Tailor-made memory: natural differences in associative olfactory learning in two closely related wasp species

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Quem é homem de bem não trai
O amor que lhe quer seu bem
Quem diz muito que vai, não vai
Assim como não vai, não vem
Quem de dentro de si não sai
Vai morrer sem amar ninguém
O dinheiro de quem não dá
É o trabalho de quem não tem

Capoeira que é bom não cai
Mas se um dia ele cai, cai bem
Capoeira me mandou dizer que já chegou
Chegou para lutar
Berimbau me confirmou vai ter briga de amor
Tristeza, camará

Se não tivesse o amor
Se não tivesse essa dor
E se não tivesse o sofrer
E se não tivesse o chorar
Melhor era tudo se acabar

Eu amei, amei demais
O que eu sofri por causa de amor ninguém sofreu
Eu chorei, perdi a paz
Mas o que eu sei é que ninguém nunca teve mais, mais do que eu

Capoeira me mandou dizer que já chegou
Chegou para lutar
Berimbau me confirmou vai ter briga de amor
Tristeza camará

Dit proefschrift draag ik op aan pa (13/06/1950 - 16/08/1991)

ABSTRACT

Learning and memory formation are often seen as traits that are purely beneficial, but nevertheless, they are associated with metabolic costs. Since costs and gains of learning and memory are expected to vary between species, the ease and speed with which stable (consolidated) long-term memory (LTM) is formed, is expected to differ between species. For animals that occupy different ecological niches, 'slow' learning may be as adaptive as 'fast' learning. If an animal encounters a relatively predictable environment during its lifetime, fast learning is a good strategy. If the environment is relatively unpredictable, however, an animal may need more time and experiences to evaluate information before storing it as long-lasting memories. This concept is known as tailor-made memories: a species learns in the way that is most favourable, given the circumstances. In order to assess how such tailor-made memories evolve, I have used a multitrophic model system. This system consisted of (1) two closely related parasitic wasps (*Cotesia glomerata* and *C. rubecula*) that show a profound difference in learning, (2) the herbivorous cabbage white butterfly larvae *Pieris brassicae* and *P. rapae*, in which the parasitic wasps lay their eggs, and (3) the host plants Brussels sprouts (*Brassica oleracea* var. Gemmifera) and nasturtium (*Tropaeolum majus*).

In my experiments, the wasps could learn to associate the odours of a plant with the presence of suitable hosts, by having one or more oviposition experiences ('conditioning trials') on that plant. Previous experiments showed that *C. glomerata* needs only one conditioning trial to form LTM, whereas *C. rubecula* needs three trials spaced in time to do so. In addition to LTM, another form of consolidated memory exists; anaesthesia-resistant memory (ARM). Both LTM and ARM are resistant to retrograde amnesia, which can be induced by cooling the wasps after conditioning. In contrast to LTM however, ARM is not protein synthesis-dependent. It can therefore be seen as a 'cheap', but less stable form of long lasting memory. Consolidated memory in *C. glomerata* is thought to consist exclusively of LTM, whereas in *C. rubecula* it appears to be a mixture of both ARM and LTM.

LTM formation requires protein synthesis, a process in which the transcription factor cAMP response element-binding protein (CREB) plays a key role. As a result of alternative splicing of the *CREB* mRNA transcript, the CREB protein occurs in different forms called isoforms. In model organisms such as the fruit fly *Drosophila melanogaster*, the mollusc *Aplysia californica*, and also in mammals such as mice and men, CREB isoforms have been shown to activate or repress transcription. Therefore, it has been hypothesized that the ratio of activator and repressor isoforms acts as a molecular switch for LTM formation. Such a switch could be responsible for species-specific differences in learning and memory.

In this study the *CREB* gene of *C. glomerata* and *C. rubecula* was cloned and sequenced, and nine isoforms were identified in the two *Cotesia* species. The abundance of two of the nine mRNA variants coding for these isoforms differs significantly between *C. glomerata* and *C. rubecula*; the other variants are expressed similarly in both species. A

conditioning trial, however, seems to induce changes in the expression of some of the major isoforms, indicating that the learning process itself may establish a ratio between activators and repressors that determines whether LTM is consolidated or not.

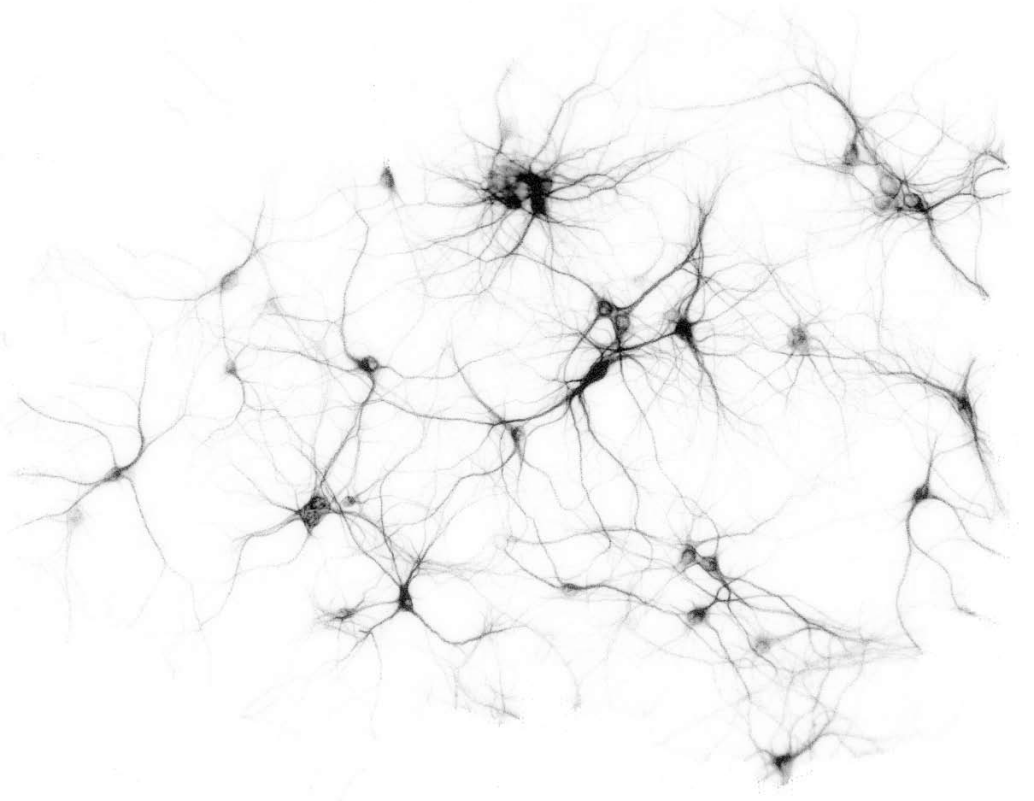
Although such molecular mechanisms can potentially act very quickly, it may sometimes take up to days or weeks before information is stored in long-lasting memories. To explain how and why such differences in memory dynamics occur, we need insight in what happens when selection acts on natural variation in learning rate. In order to investigate this, I applied a bidirectional selection regime and reared two lines of *C. glomerata* wasps that differed significantly in learning rate (the decreased-learning line (DLL) and the increased-learning line (ILL)).

By applying the protein synthesis inhibitor anisomycin before conditioning and measuring memory retention after conditioning, I showed that the memory consolidation dynamics of the selection lines differed. The DLL did not consolidate LTM anymore, whereas the ILL still did. By combining this study with experiments in which I induced retrograde amnesia by cooling at certain time intervals after conditioning, I demonstrated that in *C. glomerata*, anaesthesia-sensitive short-term memory directly consolidates into LTM, without an intermediate ARM phase. ARM represents a low-cost form of long-lasting memory (since it is not protein synthesis-dependent) and its presence is assumed to be favourable in animals that need more time to evaluate information, before storing it in the form of consolidated memories (e.g., in *C. rubecula*). The inability of *C. glomerata* to form ARM is costly because it may lead to an expenditure of energy (i.e., protein synthesis) on the 'premature' storage of unreliable information. Comparison of my selection lines showed that a high learning rate has costs. Longevity appeared to be significantly higher in wasps from the DLL than in those from the ILL. Moreover, females of the ILL have significantly larger brains than females from the DLL, while retaining a similar body size. These exciting results show that trade-offs occur (i.e., brain size vs. longevity) as a result of the bidirectional selection pressure that we applied. Moreover, the costs associated with a high learning rate seem to be of a constitutive nature. This means that animals that are able to quickly form consolidated memory pay for it by maintaining a large, costly brain and having a decreased lifespan, even when they do not actually use their learning abilities.

The results of my work show that comparative research involving a model system consisting of two closely related animals with a natural difference in learning rate yields unique information, and is preferred over the use of 'traditional' model organisms. It enables testing of various hypotheses with an ecologically relevant learning paradigm. Neuroscience (and biology in general) would benefit greatly from an increase in the use of model systems that consist of closely related species that show differences in the trait of interest. The work described in this thesis shows how fruitful such a comparative approach can be.

CONTENTS

General introduction



Michaël van den Berg

Introduction

The research presented in this thesis deals with olfactory learning and memory formation in two species of closely related parasitic wasps that differ profoundly in learning rate and memory dynamics. We are interested in how these differences have arisen in two animals that are otherwise very similar in terms of genetic makeup and physiology. We have asked questions ranging from molecular biology ('how'?) to evolutionary ecology ('why'?). The broad scope of this subject necessitates a comprehensive introduction to what learning is, and to the biology of the animals we used for our research. This introductory chapter deals with these topics, and gives an outline of the work that is presented in this thesis. Chapter 2 gives a brief review of the olfactory system of insects and of current ideas on the mechanisms underlying learning and memory, and on how differences in learning and memory may arise.

What is learning, and what are memories?

Intuitively, the concept of learning is easy to grasp. But learning eludes a strict definition (Papaj and Lewis, 1993; Papaj and Prokopy, 1989). For example, if learning is regarded as "a modification of behaviour resulting from experience", we include phenomena that do not involve learning: when an animal starts to eat after a period of starvation, this is not considered to be learning (Mery, 2009). The tendency of female parasitic *Leptopilina heterotoma* wasps to avoid laying eggs in parasitised hosts was shown to depend on previous egg-laying experiences, but not on whether those eggs had been laid in parasitised or unparasitised hosts (Henneman et al., 1995). In such a case, it is unclear whether behavioural changes reflect responses to either changes in the wasp's internal state (e.g., changes in egg load) or changes in the wasp's neural representation of the external environment (which are presumed to occur during learning). Learning requires neuronal plasticity and modification of neuronal organisation by the integration of information (Mery, 2009), so it seems reasonable to define learning as "the acquisition of neuronal representations of new information" (Dukas, 2008). Discussions of learning implicitly assume the existence of memory, which is the capacity to retain the newly acquired information for at least a short period (short-term memory) but often over long periods (long-term memory) (Dukas, 2008). The different memory phases can be distinguished on a temporal scale, but also on the basis of the genes, molecular cascades, and neural structures involved (chapter 2). Measuring learning or memory directly is impossible; they can be only inferred from behaviour, and behaviour is easily affected by factors such as physiological state, motivation, stress, circadian rhythms, and age (Cahill et al., 2001). It must be noted that even when an animal does not show conditioning in the behaviour that is measured, it may still have learned relationships among stimuli (Smith, 1993).

Learning is not a single-faceted phenomenon. A tentative classification of different

forms of learning includes sensitisation, habituation, associative learning, spatial learning, and social learning (Menzel et al., 2006; Mery, 2009). Discussion of the latter two falls outside the scope of this thesis. Habituation and sensitisation are considered the most simple forms of learning (Dukas, 2008). Habituation is a reduction of responsiveness after repeated application of the same stimulus; sensitisation is the increase of responsiveness after the experience of a strong stimulus (Menzel et al., 2006). Whether habituation and sensitisation can be considered to be true learning in terms of "the acquisition of neuronal representations of new information" (see above) is, perhaps, debatable. Both phenomena do have a neurobiological basis, however: in the sea hare *Aplysia californica*, habituation involves a reduction in the amount of glutamate released from the presynaptic sensory neuron (Kandel, 2001a). Sensitisation on the other hand, leads to an increase in the excitability of the sensory presynaptic neurons, which involves serotonin (Kandel, 2001a). As it is not my intention to focus on exact definitions of learning in this thesis, I will consider the definition of Dukas (2008) to be applicable to habituation and sensitisation as well.

Associative learning

Associative learning allows the extraction of the logical structure of the world by establishing predictive relationships between contingent events in the environment. Two major forms of associative learning are recognized. In classical (Pavlovian) conditioning, animals learn to associate an originally neutral stimulus (the conditioned stimulus or CS) with a biologically relevant stimulus (the unconditioned stimulus or US, which acts as a reward) (Dudai, 2002). In operant conditioning, animals learn the consequences of their own behaviour, by associating their behaviour with a reinforcer (Dudai, 2002; Giurfa, 2007; Menzel et al., 2006; Mery, 2009). Operant conditioning does not strictly need a CS: Classical conditioning depends on US-CS contingency, whereas operant conditioning depends on behaviour-reinforcement contingency (Dudai, 2002). Both forms of associative learning rely on the establishment of elemental links connecting two specific and unambiguous events in the animal's world. Learning through classical conditioning is said to have occurred when presentation of the CS alone elicits a response (Rescorla, 1988).

Typically, classical conditioning paradigms use single-trial conditioning or multiple trials, either with intercalated rest intervals (spaced conditioning), or with no rest intervals (massed conditioning), where spaced conditioning trials typically produce stronger, longer-lasting memory than single or massed conditioning (Carew et al., 1972; Dudai, 2002; Hammer and Menzel, 1995; Menzel, 1999; Menzel et al., 2001; Tully et al., 1994). Exceptions do exist, however, as shown in appetitive conditioning of the pond snail *Lymnaea stagnalis* (Fulton et al., 2005) and the fruit fly *Drosophila melanogaster* (Krashes and Waddell, 2008), fear conditioning in mice (Bourtchuladze et al., 1994), and oviposition learning in parasitic wasps (Collatz et al., 2006; Smid et al., 2007).

The basis of associations underlying associative learning can be traced to the level of neural circuits and single neurons. Because these forms of learning rely on a given CS and a given US, it is possible to study where and how these stimuli are encoded by the nervous system, where and how the responsible neural pathways interact during association, and how experience modifies these pathways and their activity (Giurfa, 2007).

Invertebrates have been deployed very successfully in experimental studies of the nervous system. Their advantages as model organisms include the large diameter of neurons in some invertebrates, simple nervous systems with few neurons, enhancing the tractability of neuronal circuitry, and well-defined behaviours, which lend themselves to physiological and genetic dissection (Sattelle and Buckingham, 2006). Many aspects of learning and memory formation have, therefore, been elucidated using classical conditioning paradigms with invertebrate model organisms such as insects and molluscs, and I will briefly describe three important examples below: the honeybee *Apis mellifera*, the fruit fly *Drosophila melanogaster*, and the sea hare *Aplysia californica*.

Appetitive olfactory learning in honeybees. Honeybees can be conditioned to olfactory stimuli (Bitterman et al., 1983). In this protocol, a bee is restrained in a harness, which allows only for freely moving its antennae and mouthparts. In bees, the antennae are the main chemosensory organs. When the antennae of a hungry bee are touched with sucrose solution, the animal reflexively extends its proboscis to reach out and suck the sucrose: the proboscis extension response (PER). Neutral (odour) stimuli blown to the antennae do not release this reflex in unconditioned animals. However, if an odorant is presented in contingency with a sucrose solution, an association is formed which enables the odorant to release the PER in subsequent tests. This effect constitutes a case of classical conditioning, i.e., the odorant acts as the CS and the sucrose solution as the rewarding US. This paradigm has been used extensively: on the one hand to study the complexity of the process of conditioning itself (e.g., Bitterman et al., 1983; Deisig et al., 2007; Gerber and Ullrich, 1999; Gerber et al., 1998; Hussaini et al., 2007; Komischke et al., 2002; Komischke et al., 2003; Menzel et al., 2001; Sandoz et al., 2002; Sandoz et al., 1995); on the other hand to study which molecular and neural substrates are involved in pairing the US and the CS, and how memory is formed (e.g., Eisenhardt, 2006; Erber et al., 1993; Farooqui et al., 2003; Fiala et al., 1999; Grünbaum and Müller, 1998; Hammer, 1993; Hammer and Menzel, 1995, 1998; Mauelshagen, 1993; Menzel, 1999, 2001; Menzel and Müller, 1996; Müller, 2000; Wittstock et al., 1993; Wüstenberg et al., 1998).

Aversive olfactory learning in the fruit fly. *Drosophila* has been shown to be capable of an array of learning tasks. Often used is an assay in which the animals learn to associate an odour (CS) with an electrical shock (US) (Margulies et al., 2005). After conditioning, the flies are tested for odour avoidance (Tully and Quinn, 1985). *Drosophila* studies have led to the identification of many genes involved in learning and memory formation through genetic dissection of different memory phases: short-term memory (STM),

mid-term memory (MTM), anaesthesia-resistant memory (ARM) and long-term memory (LTM). Such a multiphase model of memory formation applies to other organisms as well, although species-specific differences occur and a different terminology is sometimes used (chapter 2). Some important *Drosophila* learning mutants are *dunce* and *rutabaga*, in which STM is affected (Byers et al., 1981; Dudai et al., 1976; Livingstone, 1985; Livingstone et al., 1984); *amnesiac*, in which MTM is affected (Quinn et al., 1979); and *radish*, in which ARM is affected (Folkers et al., 1993). The formation of LTM is affected by disruption of the *CREB* gene (Yin et al., 1995a; Yin et al., 1994). The body of knowledge on learning in *Drosophila* is extensive (for some recent reviews, see Berry et al., 2008; Fiala, 2007; McGuire et al., 2005; Roman and Davis, 2001; Waddell and Quinn, 2001).

Conditioning of the gill-withdrawal reflex in *Aplysia*. Nobel prize-winner Eric R. Kandel (Medicine, 2000) was one of the pioneers in using an invertebrate model organism for the study of learning and memory. His group used the gill-withdrawal reflex in the sea hare *Aplysia* as the behavioural element in their learning paradigms. Tactile stimulation (US) of the animal's siphon leads to a quick withdrawal of the gill, which can be associated with an electric shock (CS). Kandel et al. showed that the underlying neural circuit invariantly consisted of the same cells with the same connections, and they addressed the question of how learning could occur in a circuit with fixed neuronal elements. Donald Hebb stated in 1949 that "repeated stimulation of specific receptors will lead slowly to the formation of an 'assembly' of association-area cells which can act briefly as a closed system after stimulation has ceased; this prolongs the time during which the structural changes of learning occur" (Hebb, 1949). Kandel and colleagues expanded this idea by showing that experience alters the strength and effectiveness of the existing chemical connections, a concept which is known as synaptic plasticity (see also chapter 2). Using sensitisation and classical conditioning of the gill-withdrawal reflex, Kandel showed that the cAMP/PKA pathway is involved in memory formation in *Aplysia*, and that this pathway was activated by the neurotransmitter serotonin. Later, the transcription factor cAMP responsive element-binding protein (CREB) was shown to be essential for the formation of long-lasting memory, and both activating and suppressing *CREB* genes were found to tightly control a cascade of gene activation, leading to new synaptic connections. For an excellent and extensive review of *Aplysia* memory research, see Kandel (2001a).

Parasitic wasps and learning

Clearly, invertebrates such as insects are excellent model organisms for studying learning and memory formation (Papaj and Lewis, 1993). The ability to learn is also common among parasitic wasps (Turlings et al., 1993; Vet et al., 1990). Ecologists have since long been studying the effects of experience on the response of parasitic wasps to specific stimuli, and this work has yielded hypotheses on the adaptive value of learning in

parasitic wasps (chapter 2). Many species are known to learn to associate plant odours with the presence of suitable hosts during an oviposition experience on a plant (Lewis and Takasu, 1990; Steidle and Van Loon, 2003; Turlings et al., 1993; Vet and Dicke, 1992; Vet et al., 1995). More recently, the mechanistic aspects of learning in parasitic wasps have been the subject of study as well (Bleeker et al., 2006a; Kaiser et al., 2003; Smid and Vet, 2006; Smid et al., 2007; Takasu and Lewis, 2003). The adaptive value of learning in different species is reflected in the learning rate, which indicates how readily an animal forms long-lasting, stable memory after (associative) learning. The learning rate is thought to depend, among others, on the reliability of the information an animal encounters. Neither 'fast' nor 'slow' learning is necessarily 'better' than the other in terms of a cost-benefit context; in certain environments, an animal may need more time to evaluate information before storing it as long-lasting memory, whereas the reverse is true for animals living in a stable environment where the predictive value of information is high (the concept of *tailor-made memories* - chapter 2). When discussing the adaptive value of learning, it is important to consider the roles of generalisation and discrimination. Animals commonly respond to stimuli that were not experienced during conditioning trials, but which vary from a learned CS along a defined 'perceptual' dimension (e.g., shape) (Smith, 1993). Although such generalisation may superficially seem to be erroneous, it is thought to be adaptive since it allows an animal to recognise future sensory patterns that predict important events, even when these patterns are not exactly similar. Under natural circumstances, the exact reoccurrence of a complex sensory pattern from a CS is unlikely (Shepard, 1987). When an animal generalises too broadly, however, it will effectively not discriminate among stimuli anymore. Females of the parasitic wasp *L. heterotoma* can learn to discriminate between odours from substrates that are qualitatively different, but they do not discriminate when differences are small, unless unrewarding experiences imply the absence of hosts in the substrates (Vet et al., 1998). This suggests that non-discrimination between odour stimuli in *L. heterotoma* is a functional behaviour by the parasitoid; wasps did not immediately learn to discriminate between similar types of substrates, as the resulting increase in search costs can have great fitness penalties when time available for searching is limited (Vet et al., 1998).

Summarising, the use of parasitic wasps creates excellent opportunities to study variation in learning, and the way in which ecological factors modulate the mechanisms underlying learning.

The model system

The model system (figure 1) that I used, consisting of Brussels sprouts plants (*Brassica oleracea* var. Gemmifera), cabbage white butterflies (*Pieris brassicae* and *P. rapae*), and parasitoids (the parasitic wasps *Cotesia glomerata* and *C. rubecula*) has been used extensively for research on tritrophic interactions (Bleeker et al., 2006a; Geervliet et al., 2000; Geervliet et al., 1994, 1996; Geervliet et al., 1998b; Smid et al., 2007; Steinberg

et al., 1993; Vos et al., 2001; Vos and Hemerik, 2003; Vos et al., 1998; Vos and Vet, 2004). This system is straightforward in its use as the plants and insects are easy to rear and maintain.

Figure 1. The model system that was used for the experiments described in this thesis. Top left to right: *Cotesia glomerata* female, large cabbage white *Pieris brassicae* caterpillar, *P. brassicae* butterfly. Bottom left to right: small cabbage white *P. rapae* butterfly, *P. rapae* caterpillar, *C. rubecula* female. Centre: *Brassica oleracea* var. Gemmifera (Brussels sprouts), the host plant for the two *Pieris* species.

Furthermore, a substantial body of knowledge exists on the herbivores feeding on Brussels sprouts (and other Brassicaceous plants) (Poelman et al., 2008; Root, 1973) and their parasitoids, which are attracted to the plant volatiles that are emitted upon herbivore feeding (Geervliet et al., 1994; Mattiacci et al., 1995; Smid et al., 2002). *Cotesia* wasps are koinobiont larval endoparasitoids, which means that their eggs are deposited inside host larvae, and develop while their hosts continue to feed and grow (Harvey et al., 1999). Eventually, the parasitoid larvae egress from the host and pupate. Both *C. glomerata* and *C. rubecula* rely mainly on plant volatiles to locate their hosts from a distance (Geervliet et al., 1994). Since it had already been established that *C. glomerata* and *C. rubecula* use associative learning to optimise their foraging efficiency (Geervliet et al., 1998a; Geervliet et al., 1998b), we also used the *Cotesia-Pieris-Brassica* model system for our research on learning and memory formation. Additionally, nasturtium (*Tropaeolum majus*) was used as an alternative host plant for *P. brassicae* and *P. rapae larvae*. The odour bouquet of nasturtium functioned as the CS in our olfactory conditioning paradigm. We could therefore use nasturtium vs. Brussels sprouts in a two-choice windtunnel setup, in order to measure memory retention (defined as the fraction of wasps flying towards nasturtium at a certain time interval after conditioning).

Aim and outline of this thesis

Learning is an essential adaptive trait. It shapes the way in which an animal interacts with its environment. Underlying learning are many conserved molecular and cellular mechanisms. These mechanisms act in similar ways throughout the animal kingdom (Dubnau et al., 2003). Recently, it has been argued that the dynamics of memory formation need to be seen in the context of natural behaviour under which memory formation takes place, and that the consolidation rate of memories is adapted to the demands and constraints imposed by an animal's ecological niche (Menzel, 1999). In other words: animals evolve tailor-made memories (Vet and Smid, 2007), which are shaped by selection and the ecological niche in which an animal lives. The concept of tailor-made memories implies that there are no 'good' or 'bad' ways of learning, but simply that the way an animal learns is subjected to natural selection. Thus, I refer to 'learning rate' rather than to 'learning ability' when differences in learning are studied. This thesis aims to investigate how these tailor-made memories arise and is concerned with both proximate aspects (molecular and neural pathways of memory formation) and ultimate aspects (evolutionary ecology). **Chapter 2** gives an overview of key aspects of learning and memory and provides the theoretical basis for the formulation of our hypotheses.

It has been known since a long time that the formation of LTM depends on the synthesis of new proteins (Hernandez and Abel, 2008). A key component in the pathways leading from stimulus to LTM is the transcription factor CREB, which affects the expression of genes that are involved in the synthesis of other proteins that are required for LTM. Orthologs of this protein have been identified in many organisms. CREB typically occurs in a variety of so-called isoforms (resulting from alternative splicing of the *CREB* mRNA transcript), which may act either as activators or as inhibitors of memory formation. Some fifteen years ago it was postulated that the ratio between activating and inhibiting isoforms functions as a molecular switch, which determines the rate at which LTM is formed after learning. This model has never been verified in the context of an ecologically relevant learning paradigm, however. In **chapter 3**, I describe the process of cloning and sequencing the *CREB* gene of *C. glomerata* and *C. rubecula* and the identification of several mRNA transcripts coding for different protein isoforms, which occur in both species. I then measured the relative abundance of these different alternatively spliced mRNA transcripts in the heads of unconditioned wasps from both species, in order to see whether a certain ratio (that could reflect the putative molecular switch) was present in constitutively expressed *CREB*. In **chapter 4** I tested the hypothesis that changes in the relative abundance of certain isoforms, underlying the molecular switch, are induced by the learning experience itself. Here, I describe how the relative abundances of the transcripts coding for the major CREB isoforms change over time in both wasp species, after single and spaced conditioning trials.

From changes at the molecular level, I then moved to changes at the levels of ecology,

behaviour and evolution. The observation of variation in learning performance within a natural *C. glomerata* population led us to conduct a bidirectional selection experiment, in which we aimed to create two selection lines: a decreased-learning rate line and an increased-learning rate line. Based on the previous finding that 24 h memory in *C. glomerata* consists of LTM exclusively (Smid et al., 2007), I defined long-term memory to be present when the fraction of wasps flying towards nasturtium in our windtunnel setup 24 h after conditioning is significantly higher than it is in unconditioned control wasps. In our selection procedure, *C. glomerata* wasps that did not fly towards nasturtium 24 h after single trial-conditioning but that did show 24 h memory retention after spaced trial-conditioning (indicating that the ability to form LTM was not absent) were used for propagation of a decreased-learning line. Wasps that showed 24 h memory retention after a single trial were used for propagation of an increased-learning line. Effectively, this means that we attached either a fitness penalty (no reproduction) or reward (reproduction) to the ability to form LTM after a single conditioning trial. In **chapter 5**, I give a detailed overview of this selection regime and the effects of bidirectional selection on learning rate in the two lines.

Previously, Smid et al. (2007) showed that the acquisition and consolidation of long-term memory differs between *C. glomerata* (which has a high learning rate) and *C. rubecula* (which has a low learning rate). Having established the two selection lines, I wanted to know whether selection pressure had induced similar differences between these two lines as well. Long-term memory formation depends on protein synthesis. I therefore tested the effects of the protein synthesis inhibitor anisomycin on memory retention in both selection lines, to investigate whether LTM was formed and at which rate. Among *Drosophila* researchers it is debated whether LTM either coexists, or is mutually exclusive with another, low-cost form of consolidated memory, i.e., anaesthesia-resistant memory (ARM), which does not depend on protein synthesis. Both memory traces are thought to arise from the early, more labile anaesthesia-sensitive memory (ASM) phase, which is sensitive to retrograde amnesia induced by cooling. I used cooling treatments at different time intervals after a conditioning trial to test the duration of the ASM phase, to infer whether ARM was present or not, and whether or not ARM and LTM coexist or are mutually exclusive. I also tested for ASM and ARM in unselected *C. glomerata* wasps. The results of these experiments are described in **chapter 6**.

Selection work with, e.g., *Drosophila* has indicated that trade-offs may occur as a consequence of changes in learning rate. Learning and subsequent LTM formation is energetically costly, so we hypothesized that selection for a decrease in learning rate would be accompanied by constitutive costs (i.e., genetic costs that are paid irrespective of whether learning actually takes place). This hypothesis was investigated in **chapter 7**. I tested whether changes in learning rate were associated with constitutive costs by measuring adult body sizes, brain sizes and mushroom body calyx sizes (mushroom bodies are higher integrative centres in insect brains involved in associative learning), and longevity. **Chapter 8** consists of a summarising discussion of this thesis.

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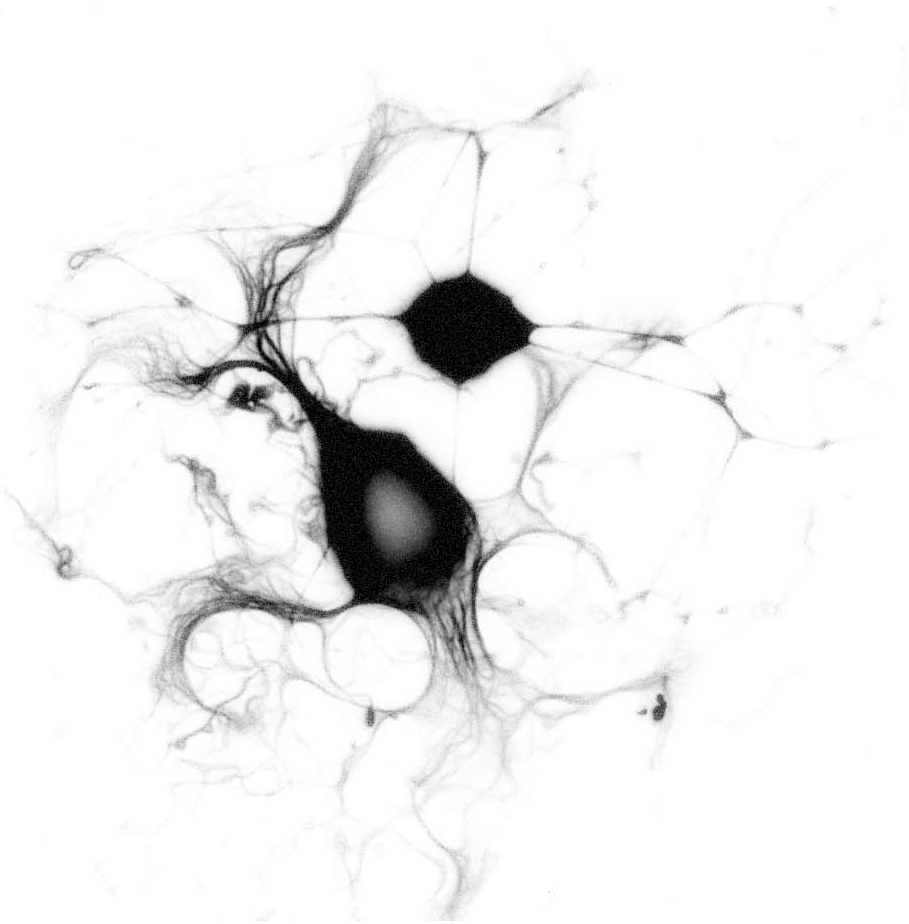
Assessing natural variation in learning and memory formation



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Abstract

One of the most remarkable aspects of animal behaviour is the ability to modify that behaviour by learning. Learning and subsequent memory formation are very similar across all animal phyla, at the molecular, cellular and behavioural level, and a limited group of model organisms has traditionally been used to elucidate biological processes underlying learning and memory. But inherent to the use of a limited number of unrelated model species is a lack of insight in interspecific natural variation in learning rate and the ultimate factors that cause this variation. Learning and memory formation are associated with constitutive and operating costs. Therefore, the ease with which consolidated, long-lasting memory is formed is expected to differ between species, depending on the demands imposed by selection and the animal's ecological niche. In this thesis I have used a model system consisting of two closely related parasitic wasps (*Cotesia glomerata* and *C. rubecula*) that show a profound difference in learning. This project was set up to assess natural variation in learning at different levels of biological integration (genes - individuals - communities). Moreover, existing variation in learning rate within our wasp model species can be used to investigate how learning rate evolves when selection acts on this variation. It also allows for testing whether changes in learning rate upon selection are associated with changes in memory dynamics and trade-offs that affect life-history traits such as longevity. Here, I briefly review several key aspects of olfactory learning and memory formation, with references to our work with *C. glomerata* and *C. rubecula*, and I present the concept of tailor-made memories, which proposes that species-specific adaptations to the environment are expected to shape the way in which memories are formed.

The shaping of memories

Learning leads to memory, and as memory develops over time, its properties change (Menzel, 1999). It has been well established that memory formation in animals and humans proceeds through a sequence of temporal stages. The cellular substrates of learning and memory are strikingly similar, both between different species (*Aplysia californica*, *Drosophila melanogaster*, mouse, man) and between different forms of memory (declarative and non-declarative, appetitive and aversive, emotional and non-emotional) (Milner et al., 1998). This similarity has led to the implicit assumption that the underlying cellular machinery is responsible for shaping memory formation. But why then, does it sometimes take hours, days, or weeks before information is stored as memory? Is the neural machinery so slow? This seems unlikely, given the speed with which neural excitation can lead to gene activation, protein synthesis and translocation, and restructuring of synaptic contacts (Frey and Frey, 2008). Menzel (1999) argued that these memory dynamics need to be seen in the context of natural behaviour under which memory formation takes place, and that the consolidation rate of memory is adapted to the demands and constraints imposed by an animal's ecological niche, e.g., slow consolidation is adaptive when the environment is unpredictable, whereas fast consolidation is adaptive when the environment is predictable (Menzel, 1999). Studies of memory phases have focused primarily on their cellular and molecular substrates, but the question of how these temporal phases are adapted to the needs of an animal behaving in a natural environment has only recently begun to receive some attention, mainly in insects such as fruit flies, bumblebees, and parasitic wasps (Bleeker et al., 2006a; Mery et al., 2007a; Raine and Chittka, 2008; Smid, 2006; Smid et al., 2007). Molecular and cellular similarities are expected to reflect general and evolutionary conserved requirements for the continuous learning, storage, and retrieval of information. Species- and task-specific adaptations, however, are likely the deciding parameters for the way in which memory proceeds through different temporal phases (Menzel, 1999).

Model organisms and natural variation in learning and memory

Ideally, investigations of the mechanisms underlying learning and memory formation require a system which allows both complex behavioural studies and a cellular analysis of the behavioural components (Mauelshagen, 1993). Mammalian brains are extremely intricate and even the simplest mammals exhibit a large variety of complex behaviours. However, learning and memory formation at the molecular, cellular and behavioural level,, are remarkably similar across all animal phyla, (Dubnau et al., 2003). This has made invertebrate animals with less complex nervous systems and behaviour like the sea hare *Aplysia* (Kandel, 2001b), the pond snail *Lymnaea stagnalis* (Lukowiak et al., 2003), the fruit fly *Drosophila* (Davis, 2005; McGuire et al., 2005), and the honeybee *Apis mellifera* (Giurfa, 2007; Hammer, 1997; Menzel, 1999, 2001; Müller, 2002) favourable subjects for such neuroethological studies. Indeed, these model organisms have been

used fruitfully in research on learning and memory formation (Castellucci, 2008). But the notion of natural variation in memory formation and the evolutionary causes and consequences of this variation are still lacking. General mechanisms of learning should be separated from species-specific adaptations, which requires a comparative approach, instead of relying on relatively few unrelated model organisms (Menzel, 2001). Clearly, insects such as *A. mellifera* are excellent model organisms for studying learning and memory formation. The ability to learn is also common among other Hymenopterans, such as parasitic wasps, and interspecific differences in parasitoid learning in an ecological context have been described (Turlings et al., 1993; Vet et al., 1995; Vet et al., 1990). In addition to empirical behavioural studies on learning with different parasitoid species these earlier papers offered a conceptual framework on why and when parasitoids are likely to learn visual, olfactory, and mechanical cues. Recent studies involving a model system consisting of two closely related (Michel-Salzat and Whitfield, 2004) parasitic wasps that differ profoundly in learning rate, *Cotesia glomerata* and *C. rubecula*, are the first to link the consequences of behavioural ecology to natural variation in learning and memory (Bleeker et al., 2006a; Smid, 2006; Smid et al., 2007).

Olfaction

Chemical sensitivity is of fundamental importance to life. In their quest for locating important resources such as mates, nutrients, oviposition, and resting sites, insects rely to a large extent on their chemical senses (De Bruyne and Baker, 2008). The predisposition of cells to be affected by chemical stimuli led to the evolution of specific receptor proteins and, ultimately, to chemosensory organs and systems such as the olfactory systems (Ache and Young, 2005). While gustatory neurons signal the quality of food or mates after contact, it is the information encoded by olfactory neurons that provides cues where to search in the first place (De Bruyne and Baker, 2008). For many insect species, the use of odour information is optimised through learning, which makes olfactory learning very suitable for insect learning paradigms in experimental setups. Odour information is processed in the olfactory pathway, which I will describe below in terms of the neural structures involved (figure 1).

The olfactory pathway

Odour processing starts with the detection of the odours by olfactory receptor neurons (ORNs) that are (in insects) predominantly located in the antennae (Rospars, 1988); they can also be found on other head appendages such as maxillary and labial palps (De Bruyne and Baker, 2008). These ORNs, together with accessory cells and the cuticular structures, in which the cells are located, form small sensory organs: the olfactory sensilla. These structures are recognized by the multiple pores in the wall of the sensillum (Steinbrecht, 1997). Several ORNs are present in each sensillum and different odorants activate different subsets of ORNs, depending on which type of olfactory receptor (OR) protein is expressed (Hallem and Carlson, 2004). The ORN axons join to form the

antennal nerve, which projects to the antennal lobe (AL) in the brain (De Bruyne and Baker, 2008; Hallem and Carlson, 2004).

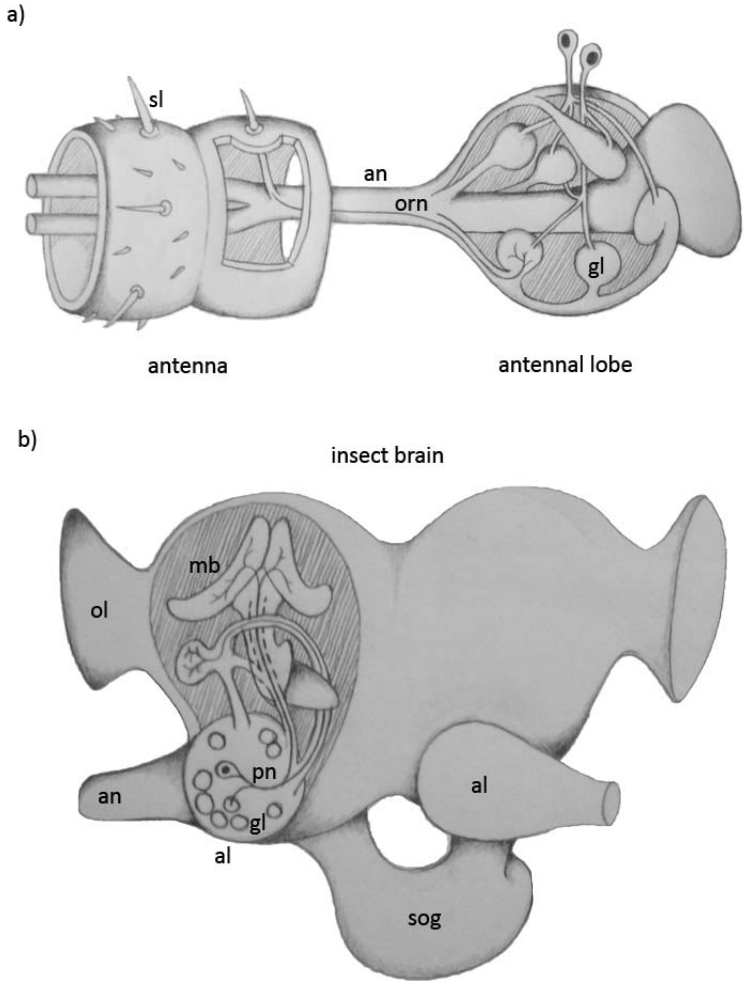

Figure 1. Schematic overview of the olfactory pathway, showing a) the antenna and the antennal lobe, and b) the brain and the major olfactory processing centres. Abbreviations: al = antennal lobe, an = antennal nerve, gl = glomerulus, mb = mushroom body, pn = projection neuron, ol = optic lobe, orn = olfactory receptor neuron, sl = sensillum, sog = suboesophageal ganglion. Drawing by MvdB, adapted from Rospars (1988).

In the AL, the first stage of processing of the olfactory information occurs before it is sent to higher brain centres. In the AL, ORNs synapse onto second order neurons called projection neurons (PNs). The AL can be subdivided into spherical units called glomeruli. Individual ORNs send axons to only one or a few glomeruli, and individual PNs typically innervate only a single glomerulus (Hallem and Carlson, 2004). Axons

of ORNs, expressing the same OR protein, project to the same glomerulus (Gao et al., 2000). Glomeruli numbers are constant in a given species and can be as high as 200, and there is also constancy in shape and position of the glomeruli between individuals of the same species (Rospars, 1988). In *Drosophila*, as in vertebrates, axons of ORNs expressing the same odorant receptor converge onto only one or a few glomeruli, which results in a highly precise spatial map of ORN projections (Hallem and Carlson, 2004). Every PN typically innervates only a single glomerulus, with its afferent axons projecting to the mushroom bodies (MBs) and the lateral horn of the brain (Hallem and Carlson, 2004). The MBs are located in the protocerebrum and are the centres of higher order processing and olfactory learning in insects (Davis, 2005; Eisenhardt, 2006; Farris, 2008). Within the MBs are so-called intrinsic neurons: the Kenyon cells (KCs), which are grouped in bundles that make up the different MB lobes (Berry et al., 2008).

The neural substrates of classical conditioning

In classical conditioning, an unconditioned stimulus (US) acts as a reward and triggers a response from the animal. Evidently, this response is initiated through the nervous system, and during classical olfactory conditioning, somehow, a link needs to be established between the neural substrates that represent the US (the reward, e.g., sucrose) and the CS (an odour) in the nervous system. A brilliant example of such a US-CS pairing was found by Hammer (1993). Neurons mediating the reinforcing property of the US in conditioning need to 1) respond to the US and 2) converge with the CS (olfactory) pathway. By measuring the response of neurons in the honeybee suboesophageal ganglion (SOG) of the brain to stimulation of the antennae and the proboscis with sucrose, Hammer found that the ventral unpaired median (VUM) neuron VUMmx1 met both criteria. It responded to sucrose stimulation with a burst of action potentials, and it innervated the AL glomeruli, the lateral protocerebrum, and the mushroom bodies, all of which are involved in olfactory processing. Proof for the idea that the VUMmx1 neuron indeed acted as the neural substrate for the US was obtained when it was shown that olfactory PER conditioning could also take place when the sucrose stimulation was replaced with an artificial depolarisation of the VUMmx1 neuron (Hammer, 1993). The VUM neuron acts through release of the neuromodulator octopamine (Hammer and Menzel, 1998). It can be expected that octopaminergic neurons in other insects may also mediate the reward in associative learning. Indeed, VUM neurons are found in many insects (Bräunig and Pflüger, 2001; Pflüger and Stevenson, 2005), including *C. glomerata* and *C. rubecula* (Bleeker et al., 2006b). The latter study is part of our ongoing research on how putative differences in the olfactory pathway in *C. glomerata* and *C. rubecula* relate to their differences in learning and memory formation. We also analysed the olfactory receptive range (Smid et al., 2002), and we made three-dimensional maps of the AL glomeruli from both species (Smid et al., 2003).

Memory dissection

A central issue in neuroscience is the role of activity-dependent synaptic plasticity in learning and memory; synaptic plasticity is a physiological phenomenon whereby specific patterns of neural activity give rise to changes in synaptic efficacy and neural excitability that long outlast the events that trigger them (Martin et al., 2000). It is generally assumed that synaptic plasticity and memory are linked as follows: activity-dependent synaptic plasticity is induced at appropriate synapses during memory formation, and is both necessary and sufficient for the information storage underlying the type of memory mediated by the brain area in which that plasticity is observed (Martin et al., 2000). Currently, memory is studied at the cellular level using well-defined models that measure changes in synaptic strength, termed long-term potentiation (LTP) and long-term depression (LTD) in vertebrates and long-term facilitation (LTF) in invertebrates (Kandel, 2001b; Malenka and Bear, 2004). Memory depends on specific sets of connected neurons, which together support the 'memory trace' (McGaugh, 1972; Thompson, 2005). These memory traces underlie the temporal changes in memory levels that are observed after learning (see chapter 1: STM, MTM, ARM, LTM) and can be defined by the brain structures and genes that are involved. In terms of molecules, the cAMP/PKA/CREB signalling pathway (figure 2) has been shown to be essential to memory formation (among others, see below). Because of space limitations, I will restrict the following discussion to the memory traces of the honeybee *A. mellifera* and the fruit fly *Drosophila*.

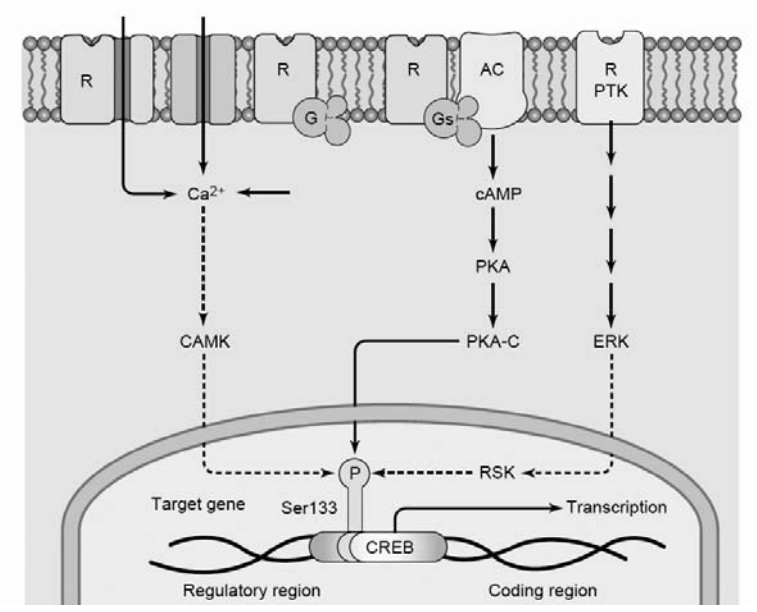

Figure 2. Schematic diagram (generic) of the mechanisms by which various second messengers induce phosphorylation of the serine 133 residue ('ser133') of the cAMP response element-binding protein (CREB). Some pathways increase Ca2+ concentrations, which then leads to activation of Ca2+ / calmodulin-de-

pendent protein kinases (CAMK), which also phosphorylate CREB on ser133. Other pathways activate extracellular signal-regulated kinase (ERK) through receptor-associated protein tyrosine kinases (R-PTK). Activation of ERK then leads to phosphorylation and activation of ribosomal S6 kinase (RSK), which also phosphorylates CREB on ser133. The most important and well studied pathway (in terms of memory formation) increases cAMP concentrations through the activation of G-protein-coupled receptors (R), adenylyl cyclase (AC), and protein kinase A (PKA), which then phosphorylates CREB on ser133. Activation of PKA leads to its dissociation and generation of the free catalytic subunit (PKA-C), which then translocates to the nucleus and phosphorylates CREB. The process of single transduction from cytoplasm to nucleus is less well established for the CaMKs and ERK, as indicated by the dashed lines. Phosphorylation of CREB on ser133 activates its transcriptional activity and leads to changes in the rate of transcription of target genes.

Short-term memory traces

Early experiments with the honeybee in which the AL was inactivated by cooling indicated that this structure was involved in the formation of short-term memories (< 3 minutes) (Erber et al., 1980). Later, it was shown that associative olfactory learning indeed transformed odour representations in the AL (Faber et al., 1999). Menzel describes an early and a late STM phase (eSTM and lSTM), in which eSTM lasts seconds and is induced by the US, whereas lSTM is induced by US-CS pairing and lasts up to minutes (Menzel, 1999, 2001). After a single conditioning trial, eSTM/lSTM is accompanied by a brief increase in PKA activity in the AL and consolidates slowly into MTM, which lasts about 1 day. After multiple spaced conditioning trials, strong PKA and nitric oxide (NO) synthase activation occurs and eSTM/lSTM consolidates into MTM and LTM (see below) (Eisenhardt, 2006; Menzel, 2001). In the context of floral foraging behaviour (where flowers occur in scattered patches), eSTM is thought to be associated with 'intrapatch' choices, where the bee is most likely to encounter similar flowers. The lSTM phase is then associated with 'interpatch' choices, where the bee probably encounters other plant species and is forced to make decisions between 'same' and 'different' (see also my discussion of 'generalisation of information' in chapter 1). Both STM phases seem to represent working memory, within one foraging bout (Menzel, 1999).

Drosophila is able to form STM as well. Much of the initial research on *Drosophila* STM was focused on the MBs, where mutations in a number of proteins highly expressed in this structure inhibited memory formation when their effects were investigated behaviourally shortly after conditioning. The affected genes were *dunce* and *rutabaga*, which code for cAMP phosphodiesterase and calcium/calmodulin-activated adenylyl cyclase, respectively (Davis, 2005). These genes are directly involved in the cAMP/PKA signalling pathway. Rescue experiments with *rutabaga* mutants showed that the *rutabaga*-dependent part of the short-term memory trace is located in the MB Kenyon cells (Zars et al., 2000). Later, anatomical data of the AL, combined with ORN mapping studies suggested that odours could be encoded by patterns of activated glomeruli (Jefferis et al., 2001; Vosshall et al., 2000). Subsequently, functional in vivo imaging of the AL neurons was used to determine how neural activity was altered by associative olfactory conditioning (Yu et al., 2004). It was believed that another part of the short-term memory trace would form at the neural intersection of the CS (odour) and the US (electric shock)

pathways. Only the PNs in the AL were found to respond to both odour and electric shock, and after training, the PNs from some of the glomeruli that were inactive in unconditioned flies were recruited into the representation of the learned odour. This cellular memory trace formed rapidly, with recruitment of new PNs observed at 3 minutes after conditioning, and it faded within 7 minutes (Yu et al., 2004).

Mid-term memory traces

After a single conditioning trial, lSTM in the honeybee slowly consolidates into an MTM phase, which lasts about 1 day (Menzel, 2001). After multiple spaced conditioning trials, a faster consolidation of eSTM/lSTM into MTM occurs, which in this case is accompanied by a rise in protein kinase C (PKC) activity during the time window of its behavioural expression in the AL (Eisenhardt, 2006; Grünbaum and Müller, 1998). This MTM is distinct from the other longer-lasting memory traces (see below) which are formed in parallel after spaced conditioning; it is sensitive to treatment with thiol-proteases, while the other memory traces are not (Eisenhardt, 2006). Under natural conditions, bees have usually returned to the hive and departed on a new foraging bout within the time-window of MTM. Upon arrival at the feeding area, memory for flower cues does not reside in the eSTM anymore, but needs to be retrieved from a more permanent store: MTM (Menzel, 1999).

Aversive olfactory memory in *Drosophila* also contains an intermediate memory phase that is often referred to as MTM. This memory trace was first deduced from experiments using flies that had a mutation in the *amnesiac* gene (Tully and Quinn, 1985). By comparing the retention curves of normal flies and *amnesiac* mutants, the latter were shown to have near-normal memory retention immediately after a single training session, and again around seven hours later. In between, memory retention in the mutants was lower than normal (Tully and Quinn, 1985). Additionally, experiments with temperature-sensitive mutants of the *DC0* gene, which encodes a subunit of PKA, showed that learning in these mutants was disrupted upon a temperature shift in a way that was indistinguishable from *amnesiac* mutants (Li et al., 1996; Skoulakis et al., 1993). *Drosophila* MTM is thus dependent on *amnesiac* and *DC0*. The amnesiac (AMN) protein was found to be preferentially expressed in the dorsal paired median (DPM) neurons. Moreover, transgenic expression of AMN in the DPM neurons rescued the memory defects of amnesiac mutants (Waddell et al., 2000). Yu et al. (2005) showed that DPM neurons respond to the US (electric shock), that they responded to a variety of odours, and that they form odour-specific memory traces (shown by increased odour-evoked calcium influx and synaptic transmission). The memory trace in the DPM neurons appears at 30 minutes after olfactory classical conditioning (Yu et al., 2005).

Long-term memory traces

Only multiple-trial conditioning is sufficient to induce LTM in honeybees, and this depends on the profound prolongation of PKA activation in the AL, mediated by the NO/cGMP pathway (Müller, 2000). Two forms of LTM occur in honeybees: translation-

dependent early LTM (eLTM, after 1-2 days), and transcription-dependent late LTM (lLTM, after >2 days) (Friedrich et al., 2004). Early LTM is formed predominantly after massed conditioning trials, while lLTM is formed after spaced conditioning (Menzel et al., 2001); in other words, these forms of LTM depend on the intertrial interval (ITI), which is short for massed conditioning and long for spaced conditioning (Menzel et al., 2001). Interestingly, PKA levels are influenced by satiation levels (i.e., whether or not the bee has fed shortly before conditioning): starved bees form stable MTM, eLTM, and lLTM after spaced conditioning, while sucrose-fed bees do not. Moreover, pharmacological rescue only restores lLTM (Friedrich et al., 2004). The biological relevance of two forms of LTM may be related to the distinction between forms of learning which lead to life-long memories (e.g., visual and olfactory cues characterizing the home colony) and those which are stable but need updating on a regular basis (e.g., visual and olfactory cues of feeding places) (Menzel, 1999).

Pharmacological treatments suggested that long-lasting, consolidated memory in *Drosophila* also consists of two mechanistically separate entities: anaesthesia-resistant memory (ARM) and long-term memory (LTM). Inhibition of protein synthesis has no effect on ARM under conditions that block LTM (Tully et al., 1994). Single and massed trainings produce ARM but do not induce LTM, while spaced training with a number of repetitions equal to a massed training induces LTM (Tully et al., 1994). The distinction between ARM and LTM is also clearly established by disruptions of the transcription factors dCREB2 (see below), Adf1, and Notch, which all block the formation of LTM without affecting ARM (Dezazzo et al., 2000; Ge et al., 2004; Presente et al., 2004; Yin et al., 1994). Conversely, *radish* mutants are deficient in ARM, but LTM remains intact (Tully et al., 1990; Yin et al., 1994). The *radish* gene encodes a protein with 23 predicted PKA phosphorylation sequences, which links it to the cAMP/PKA pathway (Folkers et al., 2006). A study by Yu et al. (2006) showed that the vertical lobes of the α/β MB neurons responded to stimulation with odours and electric shock, and only multiple spaced olfactory training cycles were capable of forming an MB cellular memory trace that appeared between 3 and 9 h after training and persisted at 24 h after training (Yu et al., 2006). These observations are consistent with an earlier experiment involving the mutant, *ala* (alpha-lobes-absent), in which proper development of the MB vertical lobe is prevented. In those flies where the mutation ablated only the vertical lobe, no LTM was observed 24 h after training, while other forms of memory remained unaffected (Pascual and Preat, 2001). The cellular localisation of ARM seems to be in the MBs, as the radish gene is highly expressed in these structures (Folkers et al., 2006).

CREB and the molecular switch

A key component mediating gene expression underlying LTM formation is the transcription factor cAMP response element-binding protein (CREB). Several signal transduction cascades (of which the cAMP/PKA pathway is the most important, figure 2)

lead to phosphorylation of the CREB protein at a specific serine residue, which is critical for transcriptional activity (reviewed by, e.g., Alberini, 2009; Carlezon et al., 2005; Lonze and Ginty, 2002; Mayr and Montminy, 2001; Shaywitz and Greenberg, 1999; Silva et al., 1998). A large body of evidence indicates that CREB-dependent transcription is essential for both long-lasting forms of synaptic plasticity and LTM, but not short-term plasticity or STM (Eisenhardt et al., 2003; Pietruck et al., 1999; Poels and Broeck, 2004; Ruppert et al., 1992). The *CREB* transcript is often alternatively spliced (Yin et al., 1995b). The resulting mRNA sequences are translated into protein isoforms, which may act either as activators or as repressors of PKA-responsive transcriptional activation (Yin et al., 1994). Previously, it was shown that overexpression of an inducible CREB repressor isoform (dCREB2b) before training abolishes LTM formation after spaced training in *Drosophila* (Yin et al., 1995a). Overexpression of an inducible CREB activator isoform (dCREB2a) before training made LTM formation possible even after a single learning trial (Yin et al., 1995a). These observations led to the hypothesis that CREB functions as part of a molecular switch that consolidates learning into LTM and that the rate of this process, and thereby the learning rate, depends on the ratio of CREB activator isoforms to CREB repressor isoforms (Tully, 1997; Yin and Tully, 1996). This idea initially led to research into the medical applications of molecules such as CREB that act as switches, specifically in the area of memory disorders (Tully et al., 2003).

In the context of our research, involving natural variation in learning and memory, we assumed *CREB* to be a suitable candidate gene that could be responsible for the observed differences between species. It is easy to see how such a molecular switch could be affected by ecological constraints and selection pressure: there only needs to occur a shift in the balance between activator and repressor isoforms, while the molecular mechanism itself does not change. In accordance with the ideas of Menzel (1999), such a molecular switch provides a good example of how a general requirement for learning and memory is reflected in a conserved molecular mechanism, while species- and task-specific adaptations are likely to modulate the way in which memory is eventually formed. With this idea in mind, I will conclude this chapter with a brief discussion of the evolutionary ecological aspects of learning and the concept of tailor-made memories, illustrated by some recent studies on natural variation in learning rate in *Drosophila* and the *C. glomerata*/*C. rubecula* model system.

Tailor-made memories

Several ultimate factors are commonly associated with variation in the learning trait (reviewed in Shettleworth, 1993; Turlings et al., 1993; Vet et al., 1995). In the light of this chapter and the idea that memory dynamics need to be seen in the context of natural behaviour under which memory formation takes place (Menzel, 1999), it is important to consider that the ecological costs of learning may differ between species (Dukas, 1998a). This concept implies that different parameters involved in learning and memory, such

as the rate of learning and the rate of memory consolidation, can differ between species according to differences in ecological needs. For different animal species, their learning rate and memory formation dynamics are thus expected to strongly depend on the balance between associated costs and benefits (Dukas, 1999; Shettleworth, 1993). Factors such as the total number of lifetime experiences, variability of the environment and reliability of information are all thought to influence the balance of costs and benefits of memory formation (Dukas, 1998b; Roitberg et al., 1993; Stephens, 1993). For example, since learning takes time, there must be a certain optimum of learning trials that an animal can afford to spend on learning. If time is costly, like it is for a short-lived insect, this optimum will be driven towards fewer learning trials. Also, if the amount of learning opportunities is limited, fewer learning trials can be used to change behaviour. An animal living in a relatively invariable environment may evolve towards a species with a high learning rate. In contrast, an animal living in a highly variable environment, where single experiences have a low predictive value, may evolve towards a species with a low learning rate (Smid, 2006). This will enable such an animal to evaluate multiple experiences and to assess the reliability of associations. Other important considerations in the light of the adaptivity of learning are generalisation and discrimination (chapter 1).

Low learning rates are costly because of suboptimal performance during learning, as shown e.g. with flower-handling skills in generalist bumblebees compared to closely related specialists (Laverty and Plowright, 1988). High learning rates and subsequent LTM formation are costly when information is unreliable, but also in terms of metabolism, e.g., because of a trade-off between protein synthesis in LTM formation and general maintenance processes in the animal. In this context, it is useful to distinguish between constitutive and operating costs of learning. Constitutive costs have a genetic nature and are paid by individuals that show high learning rates irrespective of whether learning actually takes place. These costs presumably result from developing and maintaining a sensory system and a nervous system which allows for the maintenance and repair of memories (Dukas, 1999). Operating costs are paid only when the sensory and nervous systems are effectively used to learn, and these costs presumably reflect the reallocation of metabolic resources to processes involved in memory formation (Burger et al., 2008). Costs associated with a high learning rate manifest themselves in many ways, and operating costs of learning are relatively well-studied, e.g., there is evidence for a reduced immunity in mice (Barnard et al., 2006), reduced egg productivity in *Drosophila* (Mery and Kawecki, 2004) and reduced starvation and desiccation resistance in *Drosophila* (Mery and Kawecki, 2005). Experimental evidence for a genetically based, constitutive cost of learning is beginning to accumulate as well. Examples are reduced larval competitive ability in *Drosophila* (Mery and Kawecki, 2003) and shorter lifespans of *Drosophila* flies selected for improved learning ability (Burger et al., 2008).

Because of the costs associated with memory formation it is likely that when innate behaviour can suffice, learning does not occur. Stephens (1993) suggests that learning is likely to be favoured when the predictability of the environment is low between genera-

tions, but high within generations. A completely unpredictable environment does not favour learning, as learning is based on predictability. On the other hand, a completely predictable environment is more likely to lead to innate behaviour than to learning. For example, in the context of odour learning by carnivores (e.g., parasitic wasps), the signal-to-noise ratio (SNR, i.e., plant volatiles specifically related to the presence of suitable herbivores vs. other plant volatiles) is thought to be important for determining whether or not learning is necessary (Vet, 1999). A low SNR necessitates learning of, e.g., quantitative differences in plant volatiles, in order to distinguish between signal and noise, whereas a high SNR favours the innate preference for specific plant-host combinations (Vet, 1999). Takabayashi et al. (2006) recently reviewed evidence showing that natural enemies of herbivores respond to combinations of herbivore-induced plant volatiles (HIPV) and herbivore species, either innately or after learning (Takabayashi et al., 2006). Ecologically speaking, both fast and slow learning are thought to be adaptations to the environment and therefore, both fast and slow learners are 'smart in their own way'. This idea lies at the heart of the concept of tailor-made memories: memories with different underlying consolidation dynamics, depending on the constraints imposed by a species' life history parameters and its ecological niche.

Evidently, such tailor-made memories need to evolve. For natural selection to act, heritability and variability of learning ability and memory are required (Mery, 2009). Most of the evidence for the presence of this variation was found in rats and humans (Dukas, 2004) and through selection experiments with insects (Dukas, 2008). Early work with honeybees, blowflies, and *Drosophila* confirmed that genetic differences in learning performance are present within species and can be subjected to bidirectional selection ('good' vs. 'poor' learning) (Brandes et al., 1988; Lofdahl et al., 1992; McGuire and Hirsch, 1977). Moreover, it can be quantified by means of h^2 (heritability) estimations (Brandes, 1988). More recently, the presence of heritable variation in learning rate was demonstrated by selection for improved associative learning of oviposition substrate choice in *Drosophila* (Mery and Kawecki, 2002).

Comparative studies concerning intraspecific variation in learning and memory are scarce. An important example, which is one of the first to link differences in memory formation to differences in food-searching strategy, is provided by a study of a *Drosophila* gene aptly named *foraging*. This gene, which encodes protein kinase G (PKG), has two naturally occurring variants, named rover (for^R) and sitter (for^S). Rovers have higher PKG activity than sitters and move greater distances while feeding than do sitters (Osborne et al., 1997). Interestingly, Mery et al. (2007) showed that flies with the wild-type allele for^R have better short-term memory but poorer long-term memory than flies with the for^S allele. Thus, this natural polymorphism at for may mediate an evolutionary trade-off between short- and long-term memory (Mery et al., 2007a). The respective strengths of learning performance of the two genotypes seem co-adapted with their effects on foraging behaviour: for^R flies move more between food patches and so could particularly benefit from fast learning, as they are likely to encounter new environments

(Mery, 2009; Mery et al., 2007a). In contrast, *for*^{S} flies are more sedentary, which should favour good long-term memory, as they are likely to remain in the same environment (Mery, 2009; Mery et al., 2007a; Papaj and Snell-Rood, 2007).

Studies from our own group have linked interspecific variation in learning and memory between the closely related parasitic wasp species *C. glomerata* and *C. rubecula* to the ecological niche in which these animals occur (Bleeker et al., 2006a; Geervliet et al., 1998b; Smid et al., 2007). Bleeker et al. (2006a) observed that a single oviposition experience on nasturtium led to a long-lasting increase in flight response to nasturtium in *C. glomerata* but not in *C. rubecula*, although during the first day, *C. rubecula* also showed increased response levels. These waned quickly, however (Bleeker et al., 2006a). These differences in memory dynamics were further quantified by Smid et al. (2007). They showed that *C. glomerata* forms protein synthesis-dependent LTM after a single conditioning trial; *C. rubecula* needs three spaced trials to do so. Consolidation of LTM after spaced learning was complete within 4 h in *C. glomerata*, whereas this process took 2 to 3 days in *C. rubecula*. In addition, a protein synthesis-independent memory trace, presumably ARM, was found to be present up to 48 h after spaced trial-conditioning in *C. rubecula*. In contrast, the memory trace in *C. glomerata* is built up exclusively by protein synthesis-dependent memory (Smid et al., 2007). These results suggest that LTM is consolidated exclusively in *C. glomerata* but in parallel with ARM in *C. rubecula*. Apparently, both parallel ARM-LTM and exclusive LTM consolidation can result in long-lasting memory consolidation, which is interesting given the dispute among *Drosophila* researchers, where it is claimed that ARM and LTM are either mutually exclusive (Isabel et al., 2004) or exist in parallel (Margulies et al., 2005).

What are the ultimate factors that affect the learning rate and memory dynamics of these parasitic wasps? *C. glomerata* is considered a generalist but in The Netherlands, this species prefers to lay its eggs in caterpillars of the gregarious large cabbage white *Pieris brassicae* (Geervliet et al., 2000), which is a superior host for the Dutch *C. glomerata* lines (Brodeur et al., 1998; Harvey, 2000). The large cabbage white butterfly lays her eggs in clusters of up to 150 on plants which are found in dense stands; after emergence and eating all the available leaf mass, dietary specialisation forces the caterpillars to migrate to neighbouring plants of the same species (Le Masurier, 1994; Smid, 2006). The gregarious *C. glomerata* lays 20-30 eggs in a single caterpillar (Geervliet et al., 2000; Smid, 2006). *Cotesia rubecula*, on the other hand, is a specialist on the small cabbage white *P. rapae* that lays single eggs on isolated plants (Davies and Gilbert, 1985; Root and Kareiva, 1984). Moreover, *C. rubecula* lays single eggs in its host caterpillars. This means that the number of foraging decisions for both wasps species differs dramatically: *C. glomerata* can oviposit a large part of her lifetime fecundity in a single clutch of caterpillars, whereas *C. rubecula* needs to spend much more time on locating suitable hosts and laying her eggs. Because of the egg-laying behaviour of their hosts, the predictive value of a host encounter (and subsequent oviposition by the wasp) is much higher for *C. glomerata* than it is for *C. rubecula*. According to this scenario, a quick shift towards

a different host plant species is likely to be adaptive for *C. glomerata* because the chance of finding more hosts on the same plant species nearby is high. For *C. rubecula* this strategy would be maladaptive because finding a suitable host does not imply the presence of other hosts on the same plant species in the close vicinity (Bleeker et al., 2006a). The longer LTM consolidation time provides *C. rubecula* with more time to evaluate multiple experiences. Moreover, ARM can be interpreted as a form of low-cost, long lasting memory (Mery and Kawecki, 2005). This provides an additional explanation for the difference in exclusive and parallel ARM/LTM consolidation; single trial LTM consolidation would represent high-energy expenditure for *C. rubecula*, with its high number of spaced learning experiences, but not for *C. glomerata*, with its few massed learning experiences.

Conclusions

Work on the molecular and cellular aspects of learning in honeybees has been placed in a behavioural context for a long time. It is therefore not surprising that the first notion of the limitations of purely mechanistic research, with respect to the causes of natural variation in learning and memory formation, came from those involved in honeybee research (e.g., Menzel, 1999). This notion has been acknowledged by several researchers. But even today, a decade later, most students of learning and memory remain focused on a molecular and cellular approach without appreciating how ecological factors may drive and sustain variations in the way conserved genes, molecules, and cells act in the formation of memories. In this review I have emphasised, on the one hand, the importance of the knowledge of conserved mechanisms (e.g., gene expression and neural substrates), obtained from relatively few model organisms. On the other hand, my discussion of the evolutionary and ecological aspects of learning and memory stressed that a comparative approach, involving natural variation in learning rate, illustrates how interactions between an animal and its environment can modulate the dynamics of learning and memory formation.

In this thesis, I have built on the work by Tully, Kandel, Menzel, Mery, Kawecki, and others. A leading question was how we could use our *Cotesia* model system to assess how conserved mechanisms and variation within species are linked to differences in learning rate that are driven by ecological factors. Does a molecular switch indeed underlie natural differences in learning and memory formation? Can CREB act as this putative switch, and if so, is this reflected in the way *CREB* is expressed in unconditioned and conditioned animals? In other words, can it be shown that conserved molecular mechanisms underlying memory formation are modulated by an animal's ecology? Can such variation in learning rate within a species be used as a tool to model how differential selection pressure acts on memory consolidation and trade-offs associated with learning? I have aimed to answer these questions and more, using our *Cotesia* model system. Future research will benefit from such a comparative, interdisciplinary approach, and this

favours the use of additional model systems, which show natural variation in learning and memory formation.

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CREB expression in the brains of two closely related parasitic wasp species that differ in long-term memory formation

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Abstract

Long-term memory (LTM) formation requires protein synthesis, a process in which the cAMP/PKA signalling pathway and the transcription factor cAMP response element-binding protein (CREB) play key roles. Previously it was shown that overexpression of either a CREB activator or a repressor isoform respectively enhances or abolishes LTM formation in *Drosophila*. Similar experiments with mice and rats confirmed that changes in *CREB* expression can affect both the number and the type of learning trials that are necessary for LTM formation. Therefore, it has been hypothesized that CREB acts as a molecular switch for LTM formation, depending on the ratio of activator and repressor isoforms expressed in the nervous system. We used two closely related parasitic wasp species (*Cotesia glomerata* and *C. rubecula*), which were previously shown to be profoundly different in LTM formation. *Cotesia glomerata* shows single-trial-induced LTM formation, whereas *C. rubecula*, like most animal species, needs multiple spaced trials for LTM formation in a classical conditioning setup. We cloned and sequenced the *CREB* gene of the two *Cotesia* species and found that at least nine different transcripts are present in both wasp species [1]. The splicing patterns, functional domains and amino acid sequences were similar to those found in the *CREB* genes of other organisms. The predicted amino acid sequences of the CREB isoforms were identical in both wasp species.

Using real-time quantitative PCR we analysed the relative abundance of each of the transcripts in brain homogenates of unconditioned wasps of both species, in order to test whether differences in constitutive expression levels of *CREB* transcripts are correlated with the observed differences in LTM formation. We show here that two low abundant *CREB* transcripts species are differentially expressed in the two parasitic wasps, whereas the expression levels of high abundant transcripts is similar. A subset of four isoforms constitutes the majority of all *CREB* transcripts in the brains of these wasps. The results are discussed in the context of current ideas on how LTM formation in a variety of model organisms is affected by constitutive and induced *CREB* expression.

1 The nucleotide data of the splice variants and the predicted amino acid sequence data of the isoforms can be retrieved from GenBank (http://www.ncbi.nlm.nih.gov/Genbank/index.html) under the following accession numbers: FJ155907, FJ155908, FJ155909, FJ155910, FJ155911, FJ155912, FJ155913, FJ155914, FJ155915, FJ155916, FJ155917, FJ155918, FJ155919, FJ155920, FJ155921, FJ155922, FJ155923, and FJ155924.

Introduction

Learning leads to memory, but memories are not made instantaneously (Menzel, 1999). Memory formation is a dynamic biological process involving functionally distinct temporal phases (Dubnau et al., 2003; Margulies et al., 2005). Early memory is labile and sensitive to retrograde amnesia, which can be induced by applying anaesthesia shortly after the learning experience (anaesthesia-sensitive memory [ASM]). During the hours after a learning experience, memory is consolidated into forms that are anaesthesia-resistant. Two forms of consolidated memory can be distinguished: long-term memory (LTM), which is protein-synthesis dependent, and anaesthesia-resistant memory (ARM) which does not require synthesis of proteins (Margulies et al., 2005). Long-term memory is typically formed only after multiple learning trials spaced in time (Tully, 1997; Tully et al., 1994) but exceptions to this rule exist, as shown in appetitive conditioning of the pond snail *Lymnaea stagnalis* (Fulton et al., 2005) and fruit flies (Krashes and Waddell, 2008), fear conditioning in mice (Bourtchuladze et al., 1994), and oviposition learning in parasitic wasps (Collatz et al., 2006; Smid et al., 2007).

The role and mechanisms of gene expression and protein synthesis required for LTM formation have been the focus of a substantial amount of research over the past decades (Abel and Kandel, 1998; Alberini, 1999; Carlezon et al., 2005; Dubnau et al., 2003; Kandel, 2001b; Kandel and Pittenger, 1999; Margulies et al., 2005; Menzel, 1999; Silva et al., 1998). These mechanisms are remarkably similar across the animal kingdom (Dubnau et al., 2003). A key component mediating gene expression underlying LTM formation is the transcription factor cAMP response element-binding protein (CREB). This protein specifically binds to the conserved cAMP-response element (CRE) TGACGTCA (Montminy et al., 1990) that is present in the promoter and enhancer regions of many genes (Carlezon et al., 2005). The ability of CREB to affect the function of neurons and behaviour depends on its ability to alter the expression of a set of target genes, the CREB transcriptome or regulon (Carlezon et al., 2005; Impey et al., 2004), which differs from cell type to cell type (Cha-Molstad et al., 2004).

A large number of intracellular signalling pathways converge on CREB, indicating that a variety of extracellular signals is involved in the regulation of CREB-driven transcription (Carlezon et al., 2005; Deisseroth and Tsien, 2002; Johannessen et al., 2004; Mayr and Montminy, 2001; Shaywitz and Greenberg, 1999). The major messenger molecules that are involved are cAMP, Ca^{2+}, and several types of growth factors such as nerve growth factor (NGF), respectively. Each of them activates CREB via a second messenger pathway in which the CREB protein is phosphorylated at a specific serine residue, which is critical for transcriptional activity (Gonzalez and Montminy, 1989). This residue is often referred to as 'Ser133', based on its position in rat CREB.

The *CREB* transcript is often alternatively spliced (Eisenhardt et al., 2003; Pietruck

et al., 1999; Poels and Broeck, 2004; Ruppert et al., 1992). The resulting mRNA sequences are translated into protein isoforms which may act either as activators or as repressors of PKA-responsive transcriptional activation (Yin et al., 1995b). Previously, it was shown that overexpression of an inducible CREB repressor isoform (dCREB2b) before training abolishes LTM formation after spaced training in *Drosophila melanogaster* (Yin et al., 1994). Overexpression of an inducible CREB activator isoform (dCREB2a) before training made LTM formation possible even after a single learning trial (Yin et al., 1995a). In the sea hare *Aplysia californica*, ApCREB2 represses ApCREB1-mediated transcription. When anti-ApCREB2 antibodies are injected in sensory neurons, a single pulse of serotonin is able to evoke long-term synaptic facilitation instead of the expected short-term facilitation (Bartsch et al., 1995). Long-term memory formation after fear conditioning, which is typically formed after a single learning trial, was disrupted in mice with targeted disruptions of the CREBα and CREBδ isoforms (Bourtchuladze et al., 1994). This deficiency could be overcome by spaced training (Kogan et al., 1997). Local increase of CREB levels in rat brains via viral vector-mediated gene transfer facilitates LTM formation (Brightwell et al., 2007; Josselyn et al., 2001), and local expression of mutant CREB impaired LTM in rat brains (Brightwell et al., 2008).

These experiments showed that changes in *CREB* expression can affect both the number and the type of learning trials required for LTM formation. These observations led to the hypothesis that CREB functions as part of a molecular switch that consolidates learning into LTM and that this process depends on the ratio of CREB activator isoforms to CREB repressor isoforms (Tully, 1997; Yin and Tully, 1996). Later work failed to reproduce the effects of heat shock-induced expression of the activator dCREB2a on LTM formation (Perazzona et al., 2004), which led the authors to suggest an alternative model in which the LTM-suppressing functions of dCREB2b are alleviated under the appropriate training conditions. Both models, however, assume that LTM formation depends on the relative amount of activator and repressor isoforms.

We recently showed that species-specific differences in the acquisition and formation of LTM in Dutch strains of the closely related (Michel-Salzat and Whitfield, 2004) parasitic wasps *Cotesia glomerata* and *C. rubecula* (Hymenoptera: Braconidae) can be quantified with a classical conditioning setup, using single, spaced and massed learning experiences, while interfering with LTM formation through transcription or translation inhibitors (Smid et al., 2007). These wasps lay their eggs in caterpillars of cabbage white butterflies (Geervliet et al., 1998). Associative learning of plant odours occurs when a wasp has an oviposition experience on a different host plant: *C. glomerata* forms LTM after a single experience, whereas *C. rubecula* needs three experiences spaced in time to do so (Smid et al., 2007).

Because of this clear-cut difference in LTM formation, these parasitic wasps form an ideal model system in which to study the role of CREB in LTM formation. This model system allows for a comparative approach featuring a naturally occurring difference in

LTM formation, which is fundamentally different from previous research on current genetic model species. Rather than experimentally modifying *CREB* expression or activity and measuring the induced effects on memory, we want to search for naturally occurring differences in *CREB* expression or activity between our two wasp species. Using the candidate gene approach (Fitzpatrick et al., 2005) we aim to elucidate if and how CREB is involved in the observed differences in LTM formation. Presently, it is unclear whether such a difference in the role of CREB must be sought at the level of, e.g., transcription (constitutive or after conditioning), mRNA stabilization, translation, and whether these differences occur either globally or locally in brain.

In this paper we compared the structure of different CREB isoforms in both wasp species and measured transcript expression levels in heads of unconditioned wasps, to investigate the possible correlation between constitutive *CREB* expression and differences in LTM, and to provide a solid basis for further analyses of induced *CREB* expression and CREB localization. Comparisons of induced *CREB* expression after learning, and CREB isoform localization studies will be presented separately. We show here that *C. glomerata* and *C. rubecula* both have a single *CREB* gene, which we named *CgCREB* and *CrCREB*, respectively. The mRNA transcripts of these genes are alternatively spliced and yield at least nine different transcripts including one representing the complete open reading frame, which are all expressed in both wasp species. We designed a set of primers that allowed us to specifically amplify each of the nine isoforms in a real-time quantitative PCR (qPCR) assay. Using brain homogenates from unconditioned wasps of both species, we found that the relative expression levels of two low abundant transcripts were higher in *C. glomerata* than in *C. rubecula*, whereas the relative expression levels of all other transcripts were similar.

Materials and methods

Animals

Cotesia glomerata and *C. rubecula* (Hymenoptera: Braconidae) were obtained from colonies that originated from individuals collected in cabbage fields in the vicinity of Wageningen, The Netherlands, and were reared on larvae of *Pieris brassicae* L. and *P. rapae* L. (Lepidoptera: Pieridae), respectively, as described previously (Geervliet et al., 1998b). Colonies were yearly renewed to avoid unwanted artificial selection effects. *Pieris* larvae were reared on Brussels sprouts plants (*Brassica oleracea* var. Gemmifera) as described previously (Geervliet et al., 1998).

RNA isolation and cDNA synthesis

Total RNA for each cDNA template was isolated from: a) 10-20 *C. glomerata* thoraxes (CODEHOP PCR and 3'RACE PCR) and 5 *Apis mellifera* brains with retinal pigments removed (CODEHOP PCR), b) 20-30 *C. glomerata* or *C. rubecula* brains (3' and 5'RACE PCR and isoform cloning), or c) 15 *C. glomerata* or *C. rubecula* heads

(real-time qPCR). Brains or heads were dissected from young female wasps (< 7 days after emerging, without any oviposition or plant experiences) in cold Ringer's solution (Khan et al., 1982) and immediately transferred to tubes containing 1 ml of RNAwiz™ (Ambion, Austin, Texas - cat. #9736). The tissue was then homogenized by brief pulses of ultrasonic disintegration with a Sonifier B-12 (Branson Sonic Power Co., Danbury, Connecticut). Subsequent isolation of total RNA was performed according to the manufacturer's protocol; GlycoBlue (Ambion, Austin, Texas - cat. #AM9515) glycogen was added as co-precipitant. RNA concentrations were measured using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, Delaware). RNA integrity was tested by agarose gel analysis. The RNA samples were stored at -80° C until cDNA synthesis. First strand cDNA synthesis was performed according to protocol with the BD SMART™ RACE cDNA amplification kit (Clontech, Mountain View, California - cat. #634914) for CODEHOP, 3'RACE and 5'RACE PCR, and with the Verso™ cDNA synthesis kit (ABGene, Epsom, United Kingdom - cat. # AB-1453) for real-time qPCR.

PCR with CODEHOP primers

A *CREB*-homologous sequence was first obtained by using PCR with two gene-specific primers. These primers were designed using the consensus-degenerate hybrid oligonucleotide primer (CODEHOP) method (Rose et al., 2003). Briefly, CREB amino acid sequences deduced from dCREB2a (*Drosophila melanogaster*) and AmCREB5 (*A. mellifera*) proteins were aligned to identify 3 regions with conserved sequences (table 1). Amino acid sequences were then retrotranslated into nucleotide sequences, and from these sequences, primers were selected with a degenerate 3' end and a 5' consensus sequence identical to *A. mellifera*, as this species is relatively closely related to *Cotesia*. Combinations of these primers were used to amplify CREB sequence fragments using *C. glomerata* cDNA as template.

Table 1. CODEHOP primers, with corresponding *Apis mellifera* amino acid (a.a.) positions based on alignments, and degenerate cores (shaded).

primer	sequence	*Apis* a.a.	orientation
1	5'-ATGGARAGTATGGTTGARGARAAYGG-3'	1-9	sense
2	5'-GGARAAYAGTATGGTTGARGARAAYGG-3'	1-9	sense
3	5'-ACACAGGTACAATCYGTNATHCARSC-3'	62-70	sense
4	5'-GTACAATCTGTTATHCARSCNAAY-3'	64-71	sense
5	5'-ATTAGGCTGTATAACNGAYTGNAC-3'	64-71	antisense
6	5'-CGGTTTTCTAARCAYTTDATRTAYTC-3'	270-278	antisense
7	5'-CGGTTCTCCAGGCACTTGATRTAYTCYTT-3'	269-278	antisense

RACE PCR

Rapid amplification of cDNA ends (RACE) PCR was done with the BD SMART RACE cDNA Amplification Kit (BD Biosciences Clontech - cat. #634914) with 3'RACE-ready cDNA and 5'RACE-ready cDNA in order to obtain overlapping gene fragments and to identify putative *CREB* splice variants. Gene-specific primers (table 2) were designed using the partial *CREB* sequence obtained with the CODEHOP protocol. After identification of the ORF, a set of nested, gene-specific primers (table 3) flanking the ORF were used to amplify additional *CREB* splice variants. PCR reactions were performed according to the manufacturer's protocol with the Advantage 2 PCR kit (Clontech, Mountain View, California - cat. #639206).

Table 2. Nested gene-specific primers used for generating overlapping 3'-end and 5'-end *CREB* sequence fragments and splice variant cloning, with corresponding *Cotesia glomerata* amino acid (a.a.) positions.

primer	sequence	*Cotesia* a.a.	orientation
3'RACE 1	5'-CATAGCGGTGATTCGGCACCAGCAATCG-3'	20-29	sense
3'RACE 2	5'-CTCATCACCTGGTAGCCTCCACCAGC-3'	42-50	sense
5'RACE 1	5'-CTCTTTTAGTGTTTTAAGCTCATCAATGAGCG-3'	287-297	antisense

Table 3. Nested gene-specific primers used for isolating *CREB* splice variants.

primer	sequence	orientation
Forward A	5'-CGCTTGCCCACTCGTATTGC-3'	sense
Forward C	5'-TGGTGACGAGAAATACGTAACTGG-3'	sense
Reverse B	5'-CGCTGCATAAGATTGTT-3'	antisense
Reverse C	5'-CATTGGTCTGCTTAAAGACATCCG-3'	antisense

Purification of DNA fragments

PCR products were separated on a 1.2% agarose gel and stained with ethidium bromide. The bands of interest were excised from the gel under UV illumination, and DNA was purified using the QIAEX II Gel Extraction kit (Qiagen, Venlo, the Netherlands - cat. #20021) according to protocol.

Cloning, sequencing and alignments

Purified PCR product was inserted in the pGEM-T Easy Vector System I (Promega, Leiden, the Netherlands - cat. #1360) and cloned into XL2-Blue Ultracompetent cells (Stratagene, La Jolla, California - cat. #200150). Vectors containing cDNA inserts were isolated from cells using the QIAprep Spin Miniprep kit (Qiagen, Venlo, the Netherlands - cat. #27106). The fragments of interest were sequenced at MWG Biotech (Ebersberg, Germany). Alignments were made using Vector NTI software package (Invitrogen, Carlsbad, California).

Real-time quantitative PCR

In order to quantify CREB mRNA splice variant abundances, we reverse-transcribed mRNA to cDNA, and measured cDNA concentrations in all samples using real-time quantitative PCR (qPCR). All qPCR reactions were performed on a Corbett RotorGene 6000 system (Corbett Life Science, Sydney, Australia), using Absolute ™ qPCR SYBR Green Mix (ABGene, Epsom, United Kingdom - cat. #AB-1159/a) and splice variant-specific primers according to protocol (end volume of each reaction 25 µl). Primers (table 4) were designed using the Vector NTI software package (Invitrogen, Carlsbad, California).

Table 4. Splice variant-specific primers.

primer	sequence	splice variants	orientation
E1E3For	5'-CGCTACCTCGGTACAATCAGTTATCCAACC-3'	4, 8, 9	sense
E2E3For	5'-ACGTCGGGGACGACGCAGGTAC-3'	1-3, 5-7	sense
E4E6Rev	5'-GCCGGTATTACGGCTATTTCACTCGC-3'	3, 5, 8	antisense
E4E8E9Rev	5'-CCGTGGCCTGTGTAGTCGGCTATT-3'	9	antisense
E5E6Rev	5'-CCCGCCGGTATTACTGTTTGGTATTGC-3'	1, 2, 4	antisense
E5E8E9Rev	5'-CTCCGTGGCCTGTGTAGTCTGTTTGG-3'	6	antisense
E5E9Rev	5'-CAACAACGACTCCGTGGCCTGTTTG-3'	7	antisense

We used eight replicate cDNA templates from *C. glomerata* and seven cDNA templates from *C. rubecula*. Each individual cDNA template was synthesized from total RNA obtained from 10-15 female wasp heads (unconditioned wasps between 1 and 7 days old). Per template, the expression of each of the *CREB* splice variants was quantified separately.

Primers were designed to anneal to the unique exon-exon junctions that are present as a result of alternative splicing of the mRNA transcript (table 4). Seven combinations of forward and reverse primers were selected to distinguish between the different *CREB* transcripts. The splice variants coding for isoforms 1 and 3 differ from those coding for isoforms 2 and 5, respectively, only in that they contain exon 8 (a small exon of nine base pairs). It was not possible to design primers which could distinguish between splice variants 1 and 2, and between splice variants 3 and 5.

All samples were analyzed in duplicate and reactions in which C_T values of duplicates differed by > 0.5 were repeated (Nolan, 2006). We made an exception to this rule for splice variants occurring in very low copy numbers (not more than several hundred copies/µL). The C_T values for these splice variants are high and inherently show higher variability. In such cases, a ΔC_T of up to 1.5 is acceptable (dr. S.A. Bustin, personal communication).

Standard curves for the splice variants were constructed from serial dilutions of plasmid DNA containing cloned inserts, with concentrations ranging from 10^8 molecules/µl to 10^2 molecules/µl. Melting curve analyses were performed after each run to verify qPCR product specificity.

qPCR data analysis

Splice variant concentrations (molecules/µl) were calculated with the Corbett RotorGene 6000 software, based on standard curves constructed from serial dilutions. The contribution of each mRNA splice variant relative to the total amount of CREB mRNA was calculated for each cDNA template. Relative mRNA splice variant abundance was then compared between the two species. An independent samples t-test was used if the splice variant data passed Shapiro-Wilk's normality test and Levene's test for equality of variances. Mann-Whitney's rank sum test was used to compare splice variants if the data did not pass the normality and equal variances tests. All tests were performed using SigmaPlot 11.0 (Systat Software, Inc., San Jose, CA, USA).

Results

Cloning strategy

We aimed to retrieve the predicted amino acid sequences of the putative CREB isoforms from *C. glomerata* and *C. rubecula*, by (1) cloning a transcript fragment using degenerate primers derived from known CREB sequences, (2) determining the nucleotide sequences flanking the ORF of both wasp species with 3' RACE and 5'RACE and (3) amplifying and sequencing all possible splice variants using primers annealing in these ORF-flanking regions.

PCR with CODEHOP primers - obtaining a partial *CgCREB* sequence

Different combinations of CODEHOP primer pairs (table 1) were used in PCR on *C. glomerata* thoracic cDNA template, using *A. mellifera* brain tissue-derived cDNA as a positive control. The predicted length for the amplicon of CODEHOP primer combination 1+5 was 213 bp. A band of this size was obtained from both *C. glomerata* and *A. mellifera* cDNA. This band was excised from the gel and DNA was extracted, cloned and sequenced. Subsequent BLASTing of this sequence revealed 81% identity to the homologous part of the *AmCREB* gene (as present in splice variant *AmCREB5*). This partial *CgCREB* sequence was then used to design gene-specific primers for 3'RACE and 5'RACE PCR.

RACE PCR - cloning the 3' and 5' ends of the *CREB* gene

The partial *CgCREB* sequence obtained with CODEHOP PCR was used to design nested gene-specific primers (GSPs) 3'RACE 1 and 3'RACE 2 (table 2) that were used in 3'RACE PCR on thoracic cDNA. This enabled us to clone the 3'- end of the *CgCREB* and *CrCREB* genes, including the poly-A tail.

The GSP 5'RACE 1 was designed for 5'RACE PCR, performed on two templates of *C. glomerata* brains and one template of *C. rubecula* (table 2). From the *C. glomerata* templates, two bands were obtained from which the DNA was cloned and sequenced, yielding the complete *CREB* transcript form the heaviest band and several partial *CREB* sequences from the lighter band. The 5'RACE PCR with *C. rubecula* cDNA was unsuccessful. Combining the overlapping sequences from 3'RACE and 5'RACE PCR resulted in a 921 bp open reading frame (ORF) of the entire *CREB* gene. We did not find any *CREB* transcript with 5' or 3' truncation in the ORF.

Cloning of the different *CREB* splice variants

To identify as many different transcripts resulting from alternative splicing as possible, new GSPs were designed annealing to sequences flanking the ORF, in exons 1 and 10 (table 3). Agarose gel analysis of the PCR products revealed several bands ranging in size from approximately 900 bp to 1100 bp (Fig. 1). To identify both high and low abundant transcripts, each band was cut out of the gel separately and DNA was purified and used as a template for nested PCR amplification and again analyzed on agarose gel (not shown). Approximately 25 clones obtained from each band of both species were cloned, which led to the identification of nine different transcripts, including the entire ORF (table 5). The exon structures of the splice variants are outlined in figure 2. It should be noted that not all of the transcripts were initially cloned from each species (table 5), but subsequent qPCR analysis showed that they are indeed all present in both *C. glomerata* and *C. rubecula*.

Table 5. Overview of all identified splice variants, the number of clones in which they were found, and their GenBank accession numbers of the corresponding CREB protein isoforms.

Splice variants	*C. glomerata* # of clones	Accession number of protein isoform	*C. rubecula* # of clones	Accession number of protein isoform
1	19	FJ155907	13	FJ155916
2	16	FJ155908	21	FJ155917
3	9	FJ155909	2	FJ155918
4	0	FJ155910	4	FJ155919
5	6	FJ155911	3	FJ155920
6	77	FJ155912	28	FJ155921
7	0	FJ155913	1	FJ155922
8	0	FJ155914	1	FJ155923
9	0	FJ155915	2	FJ155924
total	127		75	

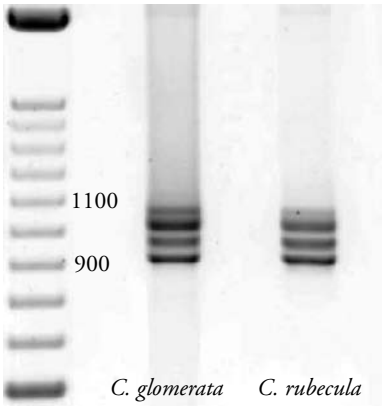


Figure 1. PCR on cDNA from both species with *CREB* gene-specific primers yielded several distinct bands in the range of 900 -1100 bp. DNA was isolated from each of these bands, inserted in a vector and cloned. Over 200 fragments were sequenced and aligned to the *CREB* open reading frame (ORF), identifying eight splice variants in addition to the complete ORF.

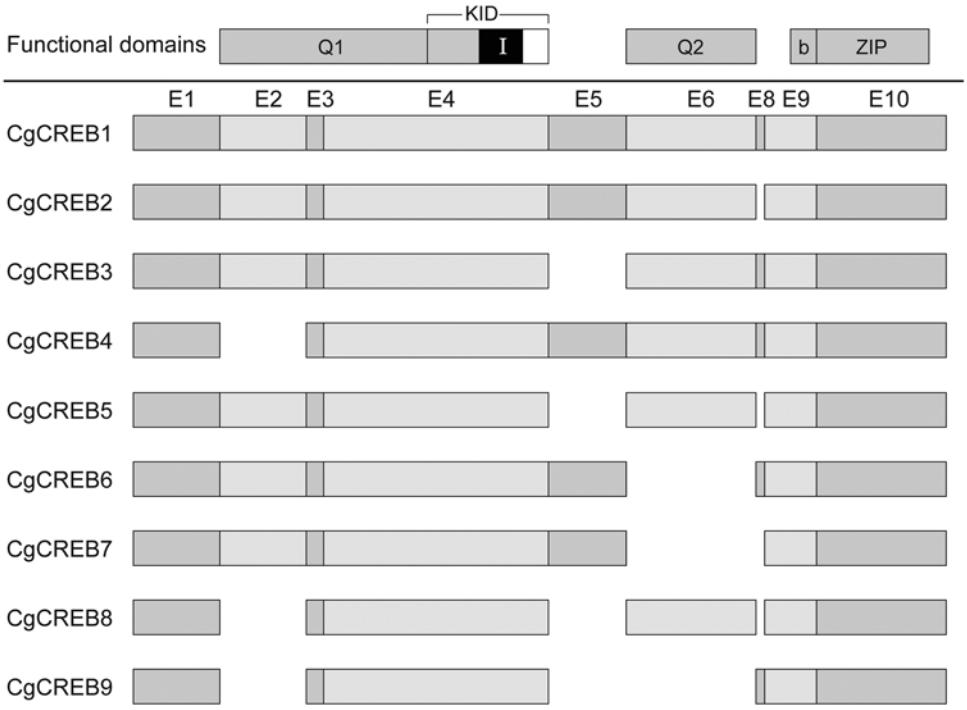


Figure 2. Schematic representation of the nine identified CREB isoforms in *C. glomerata* and *C. rubecula*. CgCREB1 represents the translation of the complete open reading frame (ORF). Note that, although the isoforms are designated CgCREB1-9 (i.e., *C. glomerata* CREB) in this figure, they are identical in *C. rubecula*. The exons are numbered E1-E10, based on Eisenhardt et al. (2003, 2006). See table 4 for the number of clones found of each of these transcripts. The homologue of *A. mellifera* exon E7 (nine amino acids) is not present in these two *Cotesia* species. Putative functional domains (see also figure 3) are mapped on top of the figure, to indicate which isoforms miss (parts of) functional domains. Q1 and Q2 are glutamine-rich domains, KID is the kinase-inducible domain, b is the basic region and ZIP is the leucine zipper region. Block I inside the KID contains the PKA-phosphorylation site with the essential serine residue.

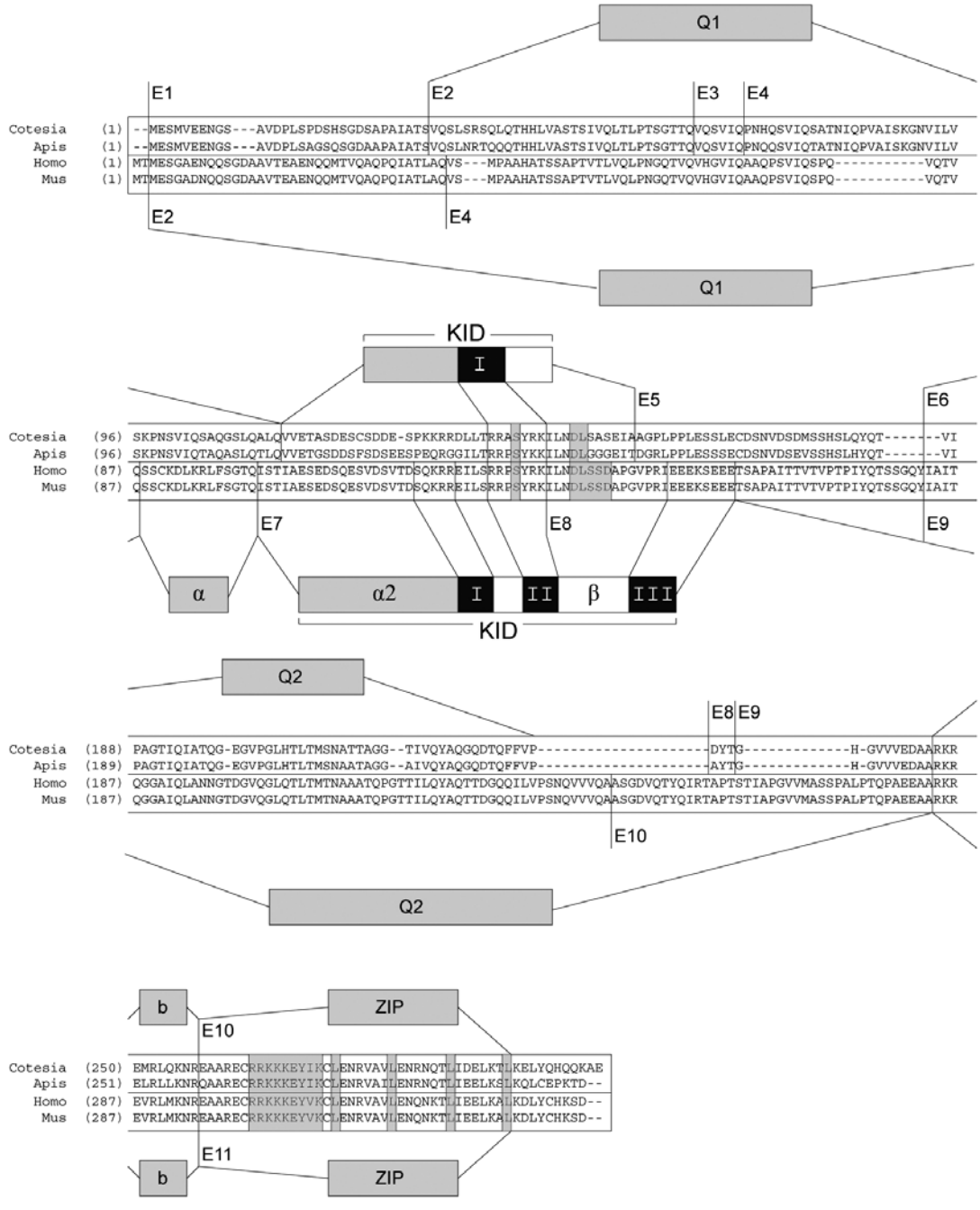
Alignments - mapping functional domains and conserved regions

The primary structures of the open reading frames of *CgCREB* and *CrCREB* differ at ten nucleotide positions, but these polymorphisms are conservative: the predicted amino acid sequences are identical. Figure 3 shows an alignment of the CgCREB/CrCREB amino acid sequence to CREB amino acid sequences from selected vertebrate and invertebrate organisms; AmCREB5 (*A. mellifera*), and CREBα (*Mus musculus* and *Homo sapiens*). The exon borders and functional domains for CgCREB are based on the AmCREB5 amino acid sequence (Eisenhardt et al., 2003; Eisenhardt et al., 2006). The *Cotesia* CREB amino acid sequences share 83% identity with AmCREB5, 28% identity with dCREB-2a, and 31% identity with mouse CREBα. Compared to *A. mellifera*, amino acid conservation within functional domains is very high (figure 3): Q1 (91%), KID (70%), Q2 (96%), basic region (82%), and ZIP (89%). Within the KID and ZIP domains, the essential amino acid residues are 100% identical. Compared to *Drosophila* (which has a very different secondary structure, notably a very long Q region preceding the KID, and no Q2 region) overall amino acid identity is very low, except in the basic region (91%) and the ZIP domain (88%). Within the *Drosophila* KID, the phosphorylation site containing the essential serine residue (RRPSYNK) shares 71% identity with the homologous site in *Cotesia* (RRASYRK).

Figure 2 shows which functional domains are possibly affected by alternative splicing. The KID and bZIP regions are present in all isoforms. Splicing mostly affects the Q1 (splice variants 4, 8, and 9 - exon 2) and Q2 (splice variants 6, 7, and 9 - exon 6) regions. Exon 5, which connects the KID and Q2 regions, is spliced out in variants 3, 5, 8, and 9. Exon 8 is spliced out in variants 2, 5, 7, and 8. Protein database searches (PROSITE) did not yield known motifs for the predicted amino acid sequence of exon 8 and its immediate surroundings.

Figure 3 (*opposite page*). Alignment of *Cotesia* CREB (Cg/CrCREB) to AmCREB5 (*Apis mellifera*) and human and mouse CREBα. The exons are labelled E1-E11. Exon borders and numbering for *Cotesia* and *A. mellifera* are according to Eisenhardt et al. (2006) except for E4, which was not split in E4 and E4_1 since we do not know if such an additional intron is present in *Cotesia*, and exon 7, of which the homologous sequence is not present in *Cotesia*. Exon borders and numbering for *Homo sapiens* and *Mus musculus* are presented according to Cole et al. (1992). Exon α is present, the rest of the alternatively spliced exons ψ, γ, and Ω are not present in this sequence. The functional domains for *Homo* and *Mus* (Brindle et al., 1993; Cole et al., 1992; Dwarki et al., 1990; Gonzalez et al., 1991; Gonzalez et al., 1989; Lee and Masson, 1993; Ruppert et al., 1992) and *Cotesia* and *A. mellifera* (Eisenhardt et al., 2003; Eisenhardt et al., 2006) are mapped to the alignment. All genes contain two glutamine-rich regions (Q1 and Q2), a kinase-inducible domain (KID) with one to three putative phosphorylation sites (I-III), a basic region (b), and a leucine zipper (ZIP). The KID region in *Homo* and *Mus* contains three phosphorylation sites (Gonzalez et al., 1989). Site I contains a protein kinase C (PKC) motif (SQKRR), site II contains a protein kinase A (PKA) motif (RRPS) and a PKC motif (SYRK), and site III contains a casein kinase II motif (EEEKSEEE). The homologous KID region in *A. mellifera* and *Cotesia* contains at least one putative phosphorylation site with a highly conserved sequence (100% identity in *A. mellifera*, a single, conservative amino acid substitution in *Cotesia*). The serine residue at position 133 (*Homo*, *Mus*), position 142 (*A. mellifera*), and position 141 (*Cotesia*) is shaded in this figure. Phosphorylation of this residue in mammals is essential for CREB activation (Gonzalez and Montminy, 1989). The phosphorylation motif compendium (Amanchy et al., 2007)

predicts other phosphorylation sites in the *Cotesia* KID region, but none of them (including Ser141) has been confirmed experimentally yet. The shaded sequence DLSSD was shown to be essential for CREB activity upon phosphorylation, with a critical role for the first residue (Gonzalez et al., 1991). This residue is conserved in *A. mellifera* and *Cotesia*. All genes have highly conserved bZIP regions, which are responsible for the DNA-binding properties of CREB. The leucine residues that occur in the repeating heptads of the leucine zipper are shaded. Also located within the bZIP region is the conserved nuclear translocation signal (NTS) RRKKKEYV (shaded) (Waeber and Habener, 1991).



Real-time qPCR - relative abundance of *CREB* splice variants

The overall splice variant expression pattern is similar in *C. glomerata* and *C. rubecula* (fig. 4). Splice variants 1+2, and variants 3+5 occur in the highest relative concentrations and account for approximately 99% of all *CREB* transcripts that are present in the brains of unconditioned wasps. Expression does not differ significantly between the two wasp species for *CREB* splice variants 1+2 ($t = -0.651$, d.f. = 13, $p = 0.53$), 3+5 ($t = 0.661$, d.f. = 13, $p = 0.52$), 6 (Mann-Whitney $U = 23$, $p = 0.61$), 7 (Mann-Whitney $U = 12$, $p = 0.07$), and 8 ($t = 1.944$, d.f. = 13, $p = 0.074$). Significant differences ($P < 0.05$) were found for splice variant 4 (Mann-Whitney $U = 6$, $p = 0.009$) and 9 ($t = 2.528$, d.f. = 13, $p = 0.025$). These differences are indicated with asterisks in figure 4.

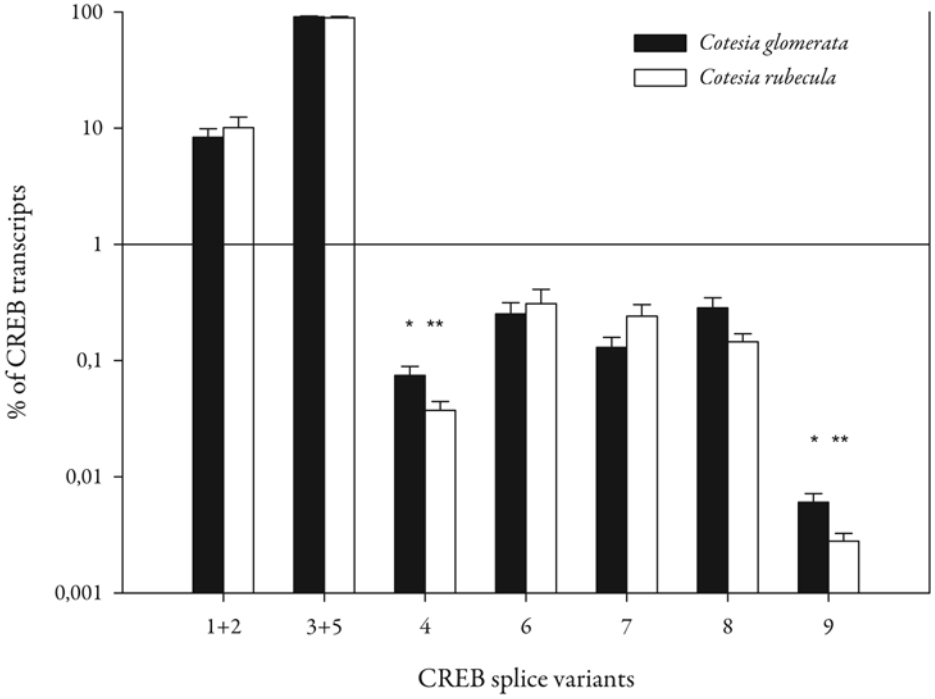


Figure 4. Real-time qPCR analysis of relative *CREB* splice variant abundance. Splice variant means (± s.e.m.) are presented as percentages of the total amount of reverse transcribed *CREB* mRNA, in unconditioned *Cotesia glomerata* (n = 8) and *C. rubecula* (n = 7) cDNA templates. Testing for significant differences in relative splice variant abundance between the two species was done using either an independent samples t-test when the assumptions of normality and equal variances were met (splice variants 1+2, 3+5, 8, and 9), or a Mann-Whitney rank sum test when these assumptions were not met (splice variants 4, 6, and 7). Expression of splice variants 4 ($p = 0.009$) and 9 ($p = 0.025$) differs significantly between *C. glomerata* and *C. rubecula*. Significant differences are indicated with asterisks. Note: values on the y axis are presented on a log scale.

Discussion

To our knowledge, this study represents the first attempt to unravel the mechanisms underlying a hypothesized molecular switch that may be responsible for differences in learning rate (Tully, 1997; Yin and Tully, 1996) by means of a specific ratio of CREB activator and inhibitor isoforms. Elucidation of *CREB* expression and its role in learning and memory formation is inherently complex. For example, in mammals three members of the *CREB* family of transcription factors have been identified: *CREB*, *CREM* and *ATF-1* (Foulkes et al., 1992; Hoeffler et al., 1988; Rehfuss et al., 1991) and alternative splicing has been shown to occur (Blendy et al., 1996; Ruppert et al., 1992). Moreover, in *CREB*-deficient mice other *CREB*-family members are upregulated to compensate for the loss of *CREB* (Hummler et al., 1994), which shows that the precise interpretation of the role of *CREB* in learning and memory in mammals is complicated when more than one *CREB* family gene is present (Barco et al., 2006). Patterns of *CREB* expression comparable to those in mammals have been found in other organisms such as the sea hare *Aplysia californica* (Bartsch et al., 1998; Bartsch et al., 1995) and the pond snail *Lymnaea stagnalis* (Sadamoto et al., 2004b), both of which have more than one *CREB* gene. In the insect model organisms *Drosophila* and *A. mellifera*, however, there is only one *CREB* family gene that plays a role in memory formation (Abel et al., 1992; Eisenhardt et al., 2003; Eisenhardt et al., 2006; Smolik et al., 1992; Usui et al., 1993; Yin et al., 1995b). The fact that in both *A. mellifera* and *Drosophila* only a single gene encodes the PKA-responsive CREB proteins reduces the complexity of gene expression analyses, which makes these insects highly attractive to investigate the role of *CREB* expression on learning rate. *Cotesia glomerata* and *C. rubecula* have the additional advantage of the naturally occurring difference in learning rate.

Our goals in this study were 1), to sequence and compare the *CREB* gene and its alternatively spliced mRNA transcripts in *C. glomerata* and *C. rubecula*, 2) to quantify the constitutive expression of the total set of *CREB* splice variants in these wasps, and 3) to see whether a specific ratio between splice variants is present in unconditioned females.

We succeeded in cloning and sequencing the *CREB* gene from the two *Cotesia* species. The *CgCREB* and *CrCREB* sequences differ at ten nucleotide positions, but these polymorphisms are conservative; the predicted amino acid sequences are identical. We found significant homology between the CgCREB/CrCREB sequences and AmCREB (85%). The overall sequence identity with dCREB-2 is low, however (20%, the majority of which is accounted for by high identity in the basic region and the ZIP domain). Compared to *A. mellifera* and *Drosophila* the majority of amino acid differences in *Cotesia* occur outside the functional domains of the CREB protein. This high degree of conservation within the domains that define the *CREB* family of genes confirms that *CgCREB* and *CrCREB* belong to this family as well.

Our cloning work showed that at least nine different *CREB* mRNA transcripts are present in the brains of unconditioned *Cotesia* females, resulting from alternative splicing of exons E2, E5, E6, and E8 (the exon numbering in *CgCREB*/*CrCREB* was adopted from *AmCREB*; see figure 3). Alignments to known sequences showed that exons E2 and E6 are part of the glutamine-rich Q1 and Q2 regions, respectively (Eisenhardt et al., 2006). The Q1 region in mammals is important for PKA-induced transcriptional activation (Gonzalez et al., 1991). The Q2 region allows basal transcriptional activity through interaction with TAFII130, a component of the transcription initiation complex (Johannessen et al., 2004; Mayr and Montminy, 2001; Mayr et al., 2005). Exon E5 is located adjacent to the KID domain but has not been implicated in the regulation of transcriptional activity. Exon E8 encodes three amino acids and its function is unknown; protein motif database searches (PROSITE) of exon 8 and surrounding sequence did not yield any results. *Cotesia* lacks the homologous counterpart of the six-amino acid exon E7, which is found in AmCREB isoform 7 but not in any of the other AmCREB isoforms. Furthermore, in the honeybee, none of the isoforms have exon E6 spliced out, whereas we found three of such isoforms (Cg/CrCREB6, 7 and 9) in Cotesia. This is an interesting difference, since E6 encodes the Q2 region. On the other hand, none of the nine isoforms in *Cotesia* has exon E3 spliced out, whereas this occurs in one of the isoforms (AmCREB6) of the honeybee.

The quantitative aspects of *CREB* splice variant abundance were analysed using qPCR. The majority of *CREB* splice variants (> 99%) in the heads of both wasps consisted of two clusters of splice variants, namely 1+2 and 3+5. It is known from previous research that the most intact CREB isoforms (i.e. the ones encoded by mRNA with the smallest number of exons spliced out) generally act as activators in CREB-dependent transcription (Bartsch et al., 1995; Poels and Broeck, 2004; Yin et al., 1995b). Intuitively, this makes sense because alternative splicing can lead to loss of functional domains and changes in protein folding (Tress et al., 2007). Moreover, our measurements showed consistently lower levels of mRNA transcripts coding for putative activator isoforms (1 and/or 2) than for repressor isoforms (3 and/or 5). This is congruent with e.g. findings in *L. stagnalis*, where mRNA coding for the activator LymCREB1 is only found in some neurons, while repressor LymCREB2 mRNA is found in larger concentrations throughout the nervous system (Sadamoto et al., 2004a; Sadamoto et al., 2004b).

We observed that the relative abundance of all nine identified splice variants in both *C. glomerata* and *C. rubecula* is highly similar. The only significant differences between the two species occurred for splice variants 4 and 9. These two splice variants are expressed in minor quantities, but they may nevertheless be important, since the spatial distribution of CREB isoforms in the nervous system is likely to play a role in LTM formation, and the molecular switch may be activated only in subsets of neurons that are specifically involved in LTM formation and consolidation. For example, the activator LymCREB1 was shown to be expressed in identifiable cells implicated in LTM of conditioned taste aversion in the pond snail *L. stagnalis* (Sadamoto et al., 2004a; Sadamoto et al., 2004b).

The *CREB*-related *CREM* gene encodes alternatively spliced activators and repressors with characteristic expression patterns throughout the rat brain (Mellström et al., 1993). In terms of LTM, this patterning implies either local *de novo* synthesis of CREB, or a molecular switch that is locally active due to spatial differences in the distribution of constitutively expressed CREB isoforms. In both scenarios, the number of CREB molecules that is actually involved in LTM formation may be obscured by the amount of CREB that is present in the brain and its surrounding tissues. Whether or not the significant differences for splice variants 4 and 9 play a role in memory formation can only be ascertained after further experimentation, e.g., *in situ* hybridization to see where the expression of these isoforms occurs in the nervous system, and RNAi to test how functional inhibition of these isoforms affects memory formation.

The fact that most of the identified splice variants in *C. glomerata* and *C. rubecula* are expressed in a similar manner in unconditioned animals of both species suggests that the molecular switch is not defined by the ratio of constitutively expressed activators and repressors. In the pond snail *L. stagnalis*, newly synthesized CREB proteins were shown to be necessary for synaptic enhancement involved in memory consolidation (Wagatsuma et al., 2006). In line with this result we believe it is possible that induced *CREB* expression may be necessary for LTM formation in *Cotesia* as well. An analysis of induced *CREB* expression at various time intervals after conditioning is currently in progress (chapter 4).

This is the first study to provide an in-depth treatment of *CREB* expression and its relation to species-specific memory dynamics, where variation in LTM acquisition is correlated to natural differences in the animal's behavioural ecology. Our parasitic wasp model system provides a unique opportunity to investigate differences in cognitive performance under natural circumstances (no transgene overexpression studies are needed) and at different biological levels of integration: from cells and organs (Bleeker et al., 2004; Bleeker et al., 2006b; Smid et al., 2003) to behavioural assays in an ecological framework (Smid et al., 2007).

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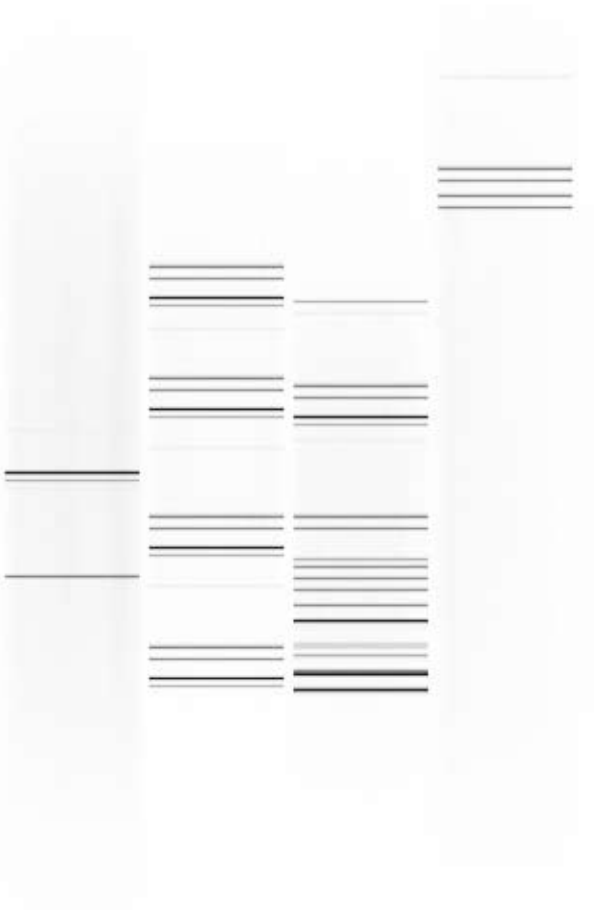
CREB expression dynamics after associative learning in the parasitic wasps *Cotesia glomerata* and *Cotesia rubecula*



Michaël van den Berg, Patrick Verbaarschot, Louise E.M. Vet, Marcel Dicke & Hans M. Smid

Abstract

The closely related parasitic wasps *Cotesia glomerata* and *C. rubecula* show a profound difference in long-term memory (LTM) formation which is related to differences in foraging behaviour and the spatial distribution of their respective hosts. This difference manifests itself in a classical conditioning setup in which the wasps learn to associate the odour of a plant species on which they had a successful oviposition in a host caterpillar.

LTM formation after conditioning requires protein synthesis and is mediated by the cAMP/PKA signalling pathway and the transcription factor CREB. It has been hypothesized that the balance between activating and inhibiting CREB isoforms acts as a molecular switch for LTM formation. It is still unclear, however, how this balance is initiated in the nervous system, and which aspect of *CREB* expression (e.g. transcription, mRNA stability, translation, localization in the brain) plays a role in the observed differences in LTM formation between *C. glomerata* and *C. rubecula*.

Previously, we cloned and sequenced the *CREB* gene and several of its splice variants in *C. glomerata* and *C. rubecula*, and we showed that constitutive *CREB* mRNA expression levels of these splice variants are similar in unconditioned females of the two wasp species. In the present study we show that, immediately after single trial conditioning, significant changes occur in the relative abundance of the major *CREB* mRNA splice variants in the brains of *C. glomerata*, but not in *C. rubecula*. We believe these changes to reflect an increase in the relative concentration of putative CREB activator isoforms, and a decrease in the relative concentration of CREB repressor isoforms. We discuss the significance of these results in the context of current ideas on how LTM formation in a variety of model organisms is affected by constitutive and induced *CREB* expression.

Introduction

The consolidation from labile short-term memory (STM) to stable long-term memory (LTM) depends on the synthesis of new proteins (Costa-Mattioli and Sonenberg, 2008). The formation of LTM typically requires multiple learning trials spaced in time (Tully, 1997; Tully et al., 1994) but exceptions to this rule exist, as shown in rats (fear conditioning), parasitic wasps (oviposition learning), the pond snail *Lymnaea stagnalis* (appetitive conditioning) and the fly *Drosophila melanogaster* (appetitive olfactory learning) (Collatz et al., 2006; Fulton et al., 2005; Igaz et al., 2002; Krashes and Waddell, 2008). Protein synthesis results from gene transcription and mRNA translation. A key component mediating the expression of genes playing a role in LTM formation is the transcription factor cAMP-response element binding protein (CREB).

Previously, it was shown that overexpression of an inducible CREB repressor isoform (dCREB2b) before training abolishes LTM formation after spaced training in *Drosophila melanogaster* (Yin et al., 1994). Overexpression of the dCREB2a activator isoform before training made LTM formation possible even after a single learning trial (Yin et al., 1995a). Later experiments with the sea hare *Aplysia californica*, mice, and rats showed that changes in *CREB* expression can affect both the number and the type of learning trials that are necessary for LTM formation (Bartsch et al., 1995; Bourtchuladze et al., 1994; Brightwell et al., 2008; Josselyn et al., 2001; Kogan et al., 1997). These observations culminated in the hypothesis that CREB functions as part of a molecular switch that consolidates learning into LTM. This process is thought to depend on the ratio of CREB activator isoforms to CREB repressor isoforms (Josselyn et al., 2001; Poels and Broeck, 2004; Tully, 1997; Yin and Tully, 1996). However, this theory was based on studies where CREB levels were artificially altered through overexpression of CREB isoforms. At present, it is unknown whether natural variation in CREB isoform levels correlates with differences in learning rate.

Already in the first paper in which the molecular switch hypothesis was postulated it is stated that "in its simplest form, this model supposes that associative learning (training) functionally induces both CREB activator and repressor isoforms" (Yin et al., 1995). Studies regarding CREB levels in the context of learning and memory are predominantly concerned with either changes in the concentration of phosphorylated CREB (pCREB) (Ahmed and Frey, 2005; Moncada and Viola, 2006; Yuan et al., 2003), or with artificially induced changes in CREB levels (overexpression) (Brightwell et al., 2007; Josselyn et al., 2001; Yin et al., 1995a). These studies show that increased levels of CREB and pCREB facilitate memory formation. It is not clear, however, whether functional induction of CREB activator and repressor isoforms under natural circumstances acts through *de novo* CREB synthesis, or whether it acts through the phosphorylation of CREB that is already present. A recent study demonstrated that in the pond snail Lymnaea stagnalis, newly synthesized activator CREB is required for synaptic enhance-

ment involved in memory consolidation (Wagatsuma et al., 2006). This suggests that under natural circumstances, the increasing CREB levels that are responsible for LTM consolidation may result from *de novo* CREB synthesis.

We recently cloned and sequenced the *CREB* gene and several of its splice variants in the parasitic wasps *Cotesia glomerata* and *C. rubecula* (chapter 3). These closely related animals show profound differences in the acquisition and consolidation of LTM upon associative olfactory learning (Smid et al., 2007). The wasps lay their eggs in caterpillars of cabbage white butterflies (*Pieris* spp.) (Geervliet et al., 1998b). *Cotesia glomerata* is a generalist but in The Netherlands it strongly prefers the large cabbage white *Pieris brassicae* as a host (Geervliet et al., 2000). It lays several eggs in each host caterpillar. *Cotesia rubecula* is a specialist and prefers to lay its eggs in the small cabbage white *P. rapae*. It lays a single egg in each host.

Associative learning of plant odours occurs when a parasitic wasp has an oviposition experience on a host plant. *Cotesia glomerata* typically forms LTM after a single conditioning trial, whereas *C. rubecula* needs spaced conditioning to do so (Smid et al., 2007). Because of this difference in LTM formation, these animals form an ideal model system in which to study the natural role of CREB in LTM formation. Rather than experimentally modifying *CREB* expression or activity and measuring the induced effects on memory, we search for naturally occurring differences in *CREB* expression or activity between our two wasp species. Presently, it is unclear at which level these differences can be expected (constitutive or induced transcription of *CREB*, mRNA stabilization, translation) and whether they occur globally in the brain or are restricted to a limited number of neurons. We previously quantified the relative abundance of the identified *CREB* splice variants in the brains of unconditioned female wasps and we showed that the constitutive *CREB* expression pattern is largely similar in both species (chapter 3).

In this study we show that significant changes in the relative abundance of the major *CREB* splice variants occur immediately upon a single conditioning trial in *C. glomerata*, but not in *C. rubecula*, which we believe to reflect an increase in the relative concentration of putative CREB activator isoforms, and a decrease in the relative concentration of CREB repressor isoforms.

Materials and methods

Animals

Cotesia glomerata and *C. rubecula* (Hymenoptera: Braconidae) were obtained from colonies that originated from individuals collected in cabbage fields in the vicinity of Wageningen, The Netherlands, and were reared on *Pieris brassicae* L. and *P. rapae* L. (Lepidoptera: Pieridae), respectively, as described previously (Geervliet et al., 1998b). Colonies were yearly renewed to avoid unwanted artificial selection effects. *Pieris* larvae

were reared on cabbage plants (*Brassica oleracea* var. Gemmifera) as described previously (Geervliet et al., 1998b).

Classical conditioning paradigm

For a more detailed description of the conditioning method and learning paradigm, we refer to previous work from our laboratory (Bleeker et al., 2006a; Smid et al., 2007). Nasturtium plants were infested 24-72 h in advance with first instar caterpillars, to induce feeding damage and volatile production by the plant. After infestation the caterpillars were removed and fresh first instar larvae were placed on the damaged leaf. Naïve female wasps were then brought into contact with the infested nasturtium leaf, ensuring that the antennae of the wasp contacted a caterpillar and its products (silk, faeces, etc.). This stimulation induced an immediate oviposition response in the wasps, lasting 10-20 seconds in *C. glomerata* and 1-3 seconds in *C. rubecula*.

The entire conditioning is defined as a form of classical conditioning. The wasp was brought into the odour space of the nasturtium leaf (the conditioned stimulus or CS) and was then rewarded by the contact with host-derived substances (the unconditioned stimulus or US) followed by the actual oviposition. This is not operant conditioning, because the naturally occurring flight approach to the host plant was not included. The oviposition response is a reflex to contact with the host and host by-products, not a behavioural response to the plant odour. Furthermore, oviposition is not required for conditioning. *Cotesia glomerata* wasps are able to learn to associate plant odours with suitable hosts when they encounter host by-products only (Geervliet et al., 1998b). However, contact with host by-products followed by contact of the ovipositor with host hemolymph constitutes a stronger reward than contact with host by-products alone, as shown for *Microplitis croceipes* (Takasu and Lewis, 2003). The conditioning sequence described above constitutes a single conditioning trial. We used two different training regimes:

1. Single trial learning: analogous to when a wasp oviposits in a solitary caterpillar on a plant.
2. A sequence of three trials, spaced in time by a 10-minute interval in a glass tube (three spaced trials): analogous to when a wasp oviposits in caterpillars on different plants of the same species, spaced in time. The interval of 10 minutes in between each trial was chosen based on studies of the effects of the intertrial interval on LTM formation in the honeybee (Eisenhardt, 2006; Gerber and Smith, 1998).

For the conditioning trials, all *C. glomerata* females were offered *P. brassicae* caterpillars and all *C. rubecula* females were offered *P. rapae* caterpillars.

Induced expression of CREB over time

In order to investigate the expression of *CREB* splice variants over time after single or spaced conditioning trials, we isolated total RNA from the heads of conditioned female

wasps (< 7 days after eclosion) at different time intervals after their conditioning trial. RNA was reverse-transcribed into cDNA, and cDNA concentrations were measured in all samples using real-time quantitative PCR (qPCR).

For each time interval we individually conditioned 10-15 wasps, immobilized them by sedation on ice at the appropriate time intervals after conditioning, and decapitated them immediately upon sedation. We sampled at 1 minute, 15 minutes, 30 minutes, 60 minutes, and 120 minutes after finishing either a single trial or a series of three spaced conditioning trials. For both single and spaced conditioning, the first trial is exactly the same and therefore the end of this trial serves as the actual t = 0 for the measurements in either treatment. Data from the spaced conditioning trials therefore starts at t = 21 minutes (1 minute after completion of 3rd trial), which is the interval when three spaced trials have been given; the other intervals are t = 35 minutes, t = 50 minutes, t = 80 minutes, and t = 140 minutes. Treatments consisted of all combinations of wasp species, single or spaced trial conditioning, and time interval after conditioning. Unconditioned female wasps were used as controls; data for the controls are presented at t = -1 minute (arbitrarily chosen), since the mRNA levels in these wasps represent the situation immediately before conditioning.

RNA isolation and cDNA synthesis

Total RNA for each cDNA template was isolated from 10-15 *C. glomerata* or *C. rubecula* heads using RNAwiz™ (Ambion, Austin, Texas - cat. #9736). Wasps were decapitated and the heads immediately transferred to tubes containing 0.5 ml of RNAwiz™. The tissue was then homogenized by brief pulses of ultrasonic disintegration with a Sonifier® B-12 (Branson Sonic Power Co., Danbury, Connecticut). Subsequent isolation of total RNA was performed according to the manufacturer's protocol; GlycoBlue (Ambion, Austin, Texas - cat. #AM9515) was added as co-precipitant. RNA concentrations were measured using a NanoDrop® ND-1000 spectrophotometer (Thermo Scientific, Wilmington, Delaware). RNA integrity was tested by agarose gel analysis. The RNA samples were stored at -80° C until cDNA synthesis. First strand cDNA synthesis was performed according to protocol with the Verso™ cDNA synthesis kit (ABGene, Epsom, United Kingdom - cat. # AB-1453) for real-time qPCR. We made two replicate time series for each treatment; replicates were blocked in time. This gave a total of 22 cDNA templates.

Real-time quantitative PCR

The relative abundance of the different *CREB* splice variants in both wasp species was analysed using real-time quantitative PCR (qPCR). All qPCR reactions were performed on a Corbett RotorGene 6000 system, using ABsolute™ qPCR SYBR® Green Mix (ABGene, cat. # AB-1159/a) and splice variant-specific primers according to protocol (end volume of each reaction 25 µl). Primers (table 1) were designed using the Vector NTI software package (Invitrogen, Carlsbad, California).

Table 1. Splice variant-specific primers.

primer	sequence	splice variants	orientation
E1E3For	5'-CGCTACCTCGGTACAATCAGTTATCCAACC-3'	4, 8, 9	sense
E2E3For	5'-ACGTCGGGGACGACGCAGGTAC-3'	1-3, 5-7	sense
E4E6Rev	5'-GCCGGTATTACGGCTATTTCACTCGC-3'	3, 5, 8	antisense
E4E8E9Rev	5'-CCGTGGCCTGTGTAGTCGGCTATT-3'	9	antisense
E5E6Rev	5'-CCCGCCGGTATTACTGTTTGGTATTGC-3'	1, 2, 4	antisense
E5E8E9Rev	5'-CTCCGTGGCCTGTGTAGTCTGTTTGG-3'	6	antisense
E5E9Rev	5'-CAACAACGACTCCGTGGCCTGTTTG-3'	7	antisense

These primers anneal to unique exon-exon junctions that are present as a result of alternative splicing of the mRNA transcript. Seven combinations of forward and reverse primers were initially selected to distinguish between the different *CREB* transcripts cf. Van den Berg et al. (2009) (chapter 3). Using this set of primers, we cannot differentiate between isoforms 1 and 2, and between isoforms 3 and 5; these are measured as the sum of 1+2, and the sum of 3+5 (chapter 3).

All samples were analyzed in duplicate and reactions in which C_T values of duplicates differed by > 0.5 were repeated (Nolan, 2006). Standard curves for the splice variants were constructed from serial dilutions of plasmid DNA containing cloned inserts, with concentrations ranging from 10^8 molecules/µl to 10^2 molecules/µl. Melting-curve analyses were performed after each run to verify qPCR product specificity.

qPCR data analysis

Splice variant concentrations (molecules/µl) were calculated with the Corbett RotorGene 6000 software, based on standard curves constructed from serial dilutions. The contribution of each mRNA splice variant relative to the total amount of *CREB* mRNA was calculated (%) for each cDNA template. Within species, data from each treatment were analyzed with a two-way ANOVA, both for the single and the spaced conditioning trials. *Splice variant percentages* were used as the dependent variable and *time in minutes* (six levels: -1, 1, 15, 30, 60, 120 for single trials and -1, 21, 35, 50, 80, and 140 for spaced trials) and *replicate* (two levels: 1 and 2) were used as factors. Post hoc Holm-Sidak multiple pairwise comparisons were performed to test which levels differed significantly from each other.

To determine whether significant differences exist between the two species, data were also analyzed with three-way ANOVA, for both single and spaced conditioning trials. *Splice variant percentages* were used as the dependent variable and *time in minutes* (six levels: -1, 1, 15, 30, 60, 120 for single trials and -1, 21, 35, 50, 80, and 140 for spaced trials), *replicate* (two levels: 1 and 2), and *species* (two levels: *C. glomerata* and *C. rubecula*) were used as factors. Post hoc multiple pairwise comparisons (Holm-Sidak method)

were performed to test which levels differed significantly from each other. All tests were performed and all graphs were rendered in SigmaPlot 11.0 (Systat Software Inc., San Jose, CA, USA).

Results

Initially, we quantified the amount of all nine identified *CREB* splice variants present in mRNA isolated from wasps at different time intervals after a conditioning trial (data not shown). It was apparent from these results that, upon conditioning, changes occurred in the expression of those transcripts that occur in the highest concentrations (1+2, and 3+5, which account for > 99% of all *CREB*). We therefore consider these splice variants as coding for putative CREB activator and repressor isoforms, and during the experiment described in this chapter we measured expression of these isoforms only.

Single trial conditioning - effects within each species

We observed that the relative abundance of the mRNA transcripts coding for CgCREB isoforms 1+2, and 3+5 in *C. glomerata* changed significantly over time ($F = 7.778$, d.f. = 5, $p = 0.021$). There was no significant effect of replicate ($F = 5.543$, d.f. = 1, $p = 0.065$) (supplementary table S1). Multiple pairwise comparisons showed a significant decrease of abundance of splice variants 1+2 at t = 1 minute, compared to the control group ($t = 5.641$, critical level = 0.003, $p = 0.002$). After this initial decrease, the concentrations of these splice variants slowly increased again over the course of the next 2 h (figure 1a). Data at none of the time points after t = 1 minute differed significantly from each other or from the data for the control group. The relative abundance of the splice variants coding for isoforms 3 and 5 increased and decreased in a complementary way (figure 1b). This is the case because we calculated abundances of the *CREB* splice variants relative to the total concentration of 1+2 and 3+5, which is by definition 100%.

In *C. rubecula*, the changes in relative concentration of the transcripts coding for isoforms 1 and 2, and 3 and 5 over time were not significant. There was, however, a significant effect of replicate ($F = 22.072$, d.f. = 1, $p = 0.005$) (supplementary table S1). In the second replicate, the relative abundance of the splice variants coding for CrCREB isoforms 1 and 2 is consistently lower (figure 1c), and for variants 3 and 5 it is consistently higher (figure 1d).

Spaced trial conditioning - effects within species

Although we do see a slight decrease in the relative abundance of splice variants 1+2 in *C. glomerata* after three spaced trials, the changes over time were not significant in any of the splice variants coding for CgCREB isoforms 1, 2, 3, and 5 (figure 2a and supplementary table S2). Since the first of the three spaced trials is identical to single trial conditioning, we expect a significant decrease in the relative abundance of splice variants 1 and 2 similar to the one we observed during single trial conditioning, to occur

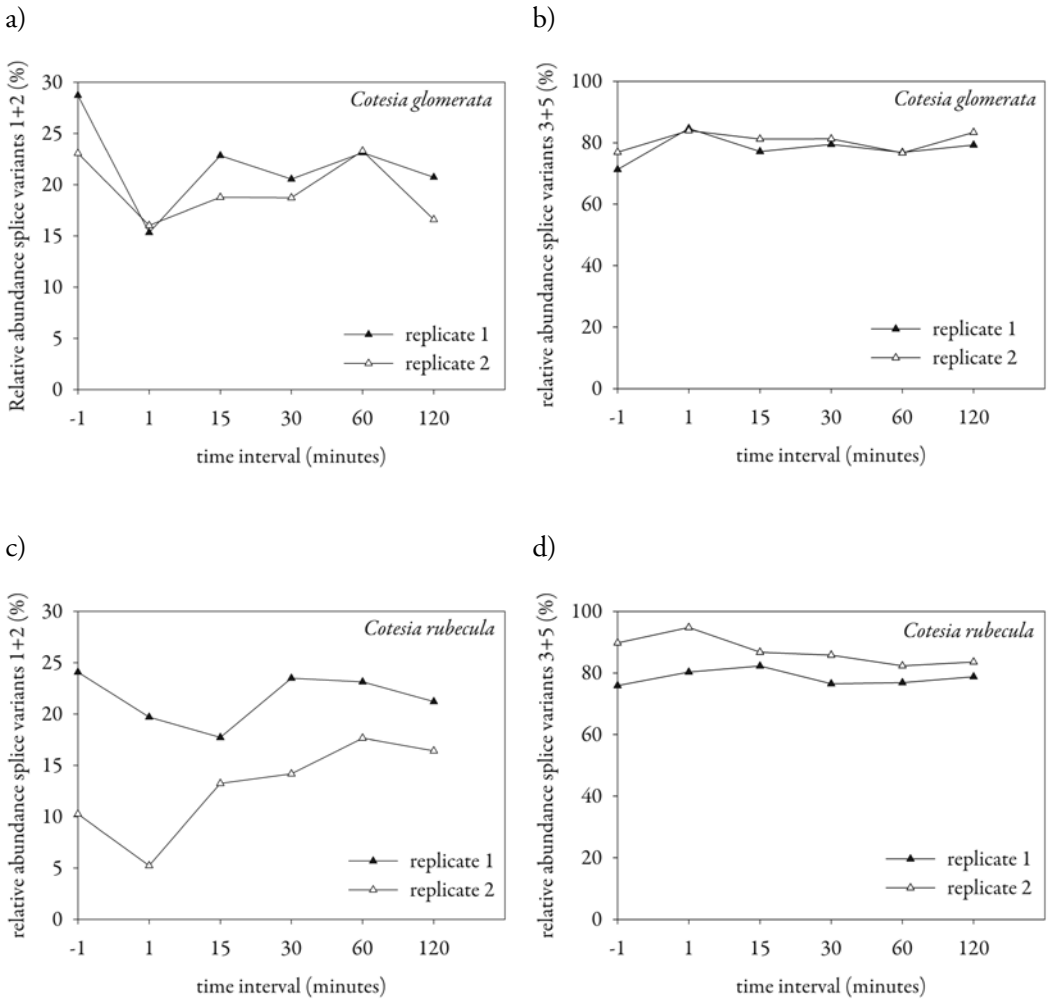


Figure 1. Relative abundance of *CREB* splice variants measured over time after a single conditioning trial. In *C. glomerata* a) the relative abundance of the splice variants coding for isoforms 1+2 decreases immediately after learning, and b) the relative abundance of the variants coding for isoforms 3+5 increases in a complementary way (because concentrations are measured relative to the sum of *CREB* variants 1, 2, 3, and 5, which by definition equals 100%). The changes over time in *C. glomerata* are significant (two-way ANOVA: $F = 7.778$, d.f. = 5, $p = 0.021$), the significant difference occurs between t = -1 minute and t = 1 minute (post hoc Holm-Sidak: $t = 5.641$, critical level = 0.003, $p = 0.002$). In *C. rubecula* c) the relative abundance of the variants coding for isoforms 1+2 does not change significantly over time, and d) consequently, neither does the abundance of the variants coding for isoforms 3+5. There is, however, a significant effect of replicate (two-way ANOVA: $F = 22.072$, d.f. = 1, $p = 0.005$). Shown are percentages relative to the total *CREB* transcript, measured over time. Time intervals are relative to the conditioning trial, and the control group is represented at the arbitrarily chosen interval t = -1 minute (i.e., representing the state just before conditioning).

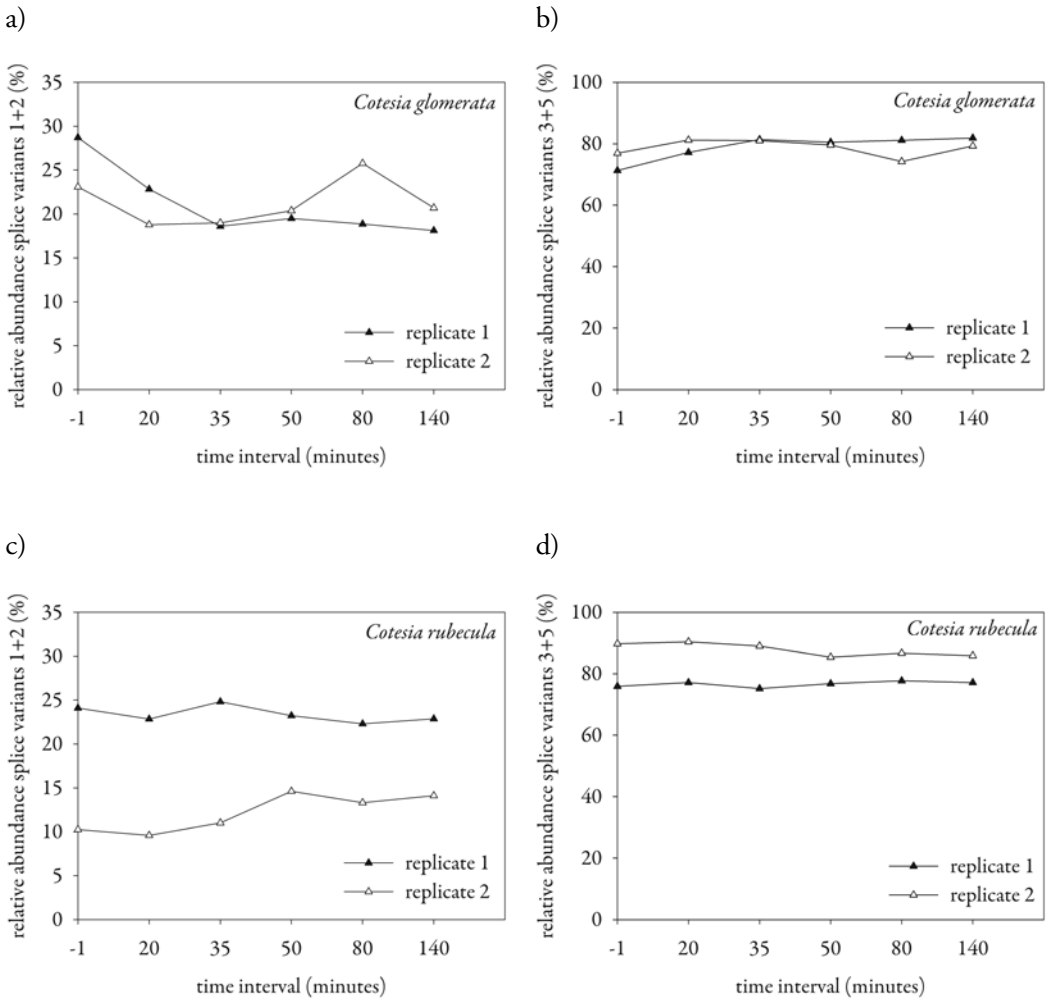


Figure 2. Relative abundance of *CREB* splice variants measured over time after three spaced conditioning trials. In *C. glomerata* a) the relative abundance of the splice variants coding for isoforms 1 and 2 does not change significantly over time, and b) neither does the relative abundance of the variants coding for isoforms 3 and 5 (these change in a complementary way because concentrations are measured relative to the sum of *CREB* variants 1, 2, 3, and 5, which by definition equals 100%). In *C. rubecula* c) the relative abundance of the variants coding for isoforms 1 and 2 does not change significantly over time, and d) consequently, neither does the abundance of the variants coding for isoforms 3 and 5. There is, however, a significant effect of replicate (two-way ANOVA: $F = 105.750$, d.f. = 1, $p < 0.001$). Shown are percentages relative to the total *CREB* transcript, measured over time. Time intervals are relative to the conditioning trial, and the control group is represented at the arbitrarily chosen interval t = -1 minute (i.e., representing the state just before conditioning).

(*continued from p. 64*) during spaced conditioning as well. This hypothesized decrease is obscured, however, because we could extract RNA only after finishing the subsequent two trials (i.e., after 20 minutes).

In *C. rubecula*, the levels of all splice variants stay more or less the same throughout the two hours after spaced conditioning; there are no significant differences in any of the splice variant abundances between the time intervals. There is, however, a significant effect of replicate (two-way ANOVA: $F = 105.750$, d.f. = 1, $p < 0.001$). (supplementary table S2). In the second replicate, the relative abundance of the splice variants coding for CrCREB isoforms 1 and 2 is consistently lower (figure 2c), and for variants 3 and 5 it is consistently higher (figure 2d).

Comparison between the two species

Single-trial conditioning. Testing for differences between the two wasp species revealed significant effects of the factors species (three-way ANOVA: $F = 9.516$, d.f. = 1, $p = 0.027$) and replicate (three-way ANOVA: $F = 24.938$, d.f. = 1, $p = 0.004$) on the observed qPCR results if they were pooled together (supplementary table S3).
Spaced-trial conditioning. Testing for differences between the two wasp species revealed significant effects of the factors species (three-way ANOVA: $F = 30.427$, d.f. = 1, $p = 0.003$) and replicate (three-way ANOVA: $F = 77.981$, d.f. = 1, $p < 0.001$) on the observed qPCR results if they were pooled together (supplementary table S3).

These tests suggest that there is not only a significant change in splice variant abundance within *C. glomerata* over time, but moreover, that overall *CREB* expression patterns differ between the two species. This may be a reflection of how natural variation in learning and memory formation is achieved through differential regulation of a shared molecular mechanism.

Discussion

This study is part of ongoing research in which we aim to unravel the mechanisms underlying a hypothesized molecular switch (Tully, 1997; Yin and Tully, 1996) that may be responsible for differences in learning rate in two species of parasitoid wasps (Bleeker et al., 2006a; Geervliet et al., 1998b; Smid et al., 2007). This switch is thought to act by means of a specific ratio of CREB activator and repressor isoforms. We employ a model system of two parasitic wasp species (*C. glomerata* and *C. rubecula*), which differ profoundly in learning rate and memory consolidation (Smid et al., 2007), for a comparative analysis of this molecular switch. Using the candidate gene approach (Fitzpatrick et al., 2005) we aim to elucidate whether and how *CREB* is involved in the observed differences in LTM formation. At present, it is unclear whether such a difference in the role of *CREB* must be sought at the level of, e.g., transcription (constitutive or after conditioning), mRNA stabilization, or translation, and whether these differences occur

either globally or locally in brain.

We started by cloning and sequencing the *CREB* gene and several of its splice variants in *C. glomerata* and *C. rubecula*, and we showed that constitutive *CREB* mRNA expression levels are similar in unconditioned females of the two wasp species (chapter 3). In the present study, we expand this work by quantifying the relative abundance of the major *CREB* splice variants over time after single and spaced conditioning trials. We show that single trial conditioning in the 'fast-learning' species *C. glomerata* is followed by a significant change in the relative abundance of the major *CREB* splice variants. In *C. rubecula*, *CREB* transcript levels roughly followed the same expression pattern after single trial conditioning as in *C. glomerata*, but the changes we observed were not significant. This is most likely due to the fact that we could only use two replicates, that, although showing the same pattern over time, differed quantitatively from each other. Spaced trial conditioning did not significantly affect relative concentrations of *CREB* transcripts in either species over time. The results from the single trial conditioning experiments suggest that the difference in LTM formation between *C. glomerata* and *C. rubecula* is at least partially regulated at the level of *CREB* mRNA. We know that *C. glomerata* typically forms LTM after a single conditioning trial, whereas *C. rubecula* needs three spaced trainings to do so (Smid et al., 2007). The fact that the abundance of the major splice variants changes transiently and significantly immediately upon conditioning in *C. glomerata* but does not change significantly in *C. rubecula*, and the significance of the factor species, seem to be congruent with these differences in LTM formation. We cannot discard the possibility, however, that the consistent and significant difference in expression levels between the two replicate time series in *C. rubecula* obscures possibly significant effects of time within this wasp species.

Both wasps form LTM after three spaced conditioning trials, but in *C. rubecula* the process of consolidation takes much longer (2-3 days as opposed to 4 h in *C. glomerata*) (Smid et al., 2007). In both species, activated CREB is expected to initiate the consolidation process, but our *CREB* mRNA level measurements after conditioning do not show significant changes over time after three spaced trials. We assume that the crucial translation events occur in the time window between the first and the third trial, which makes sense since the first 'spaced' trial is exactly the same as the single conditioning trial. The decrease in mRNA coding for isoforms 1 and 2 is therefore also expected to occur in spaced conditioning. The subsequent repetitions may enhance the effect, but we cannot quantify mRNA levels during spaced trial conditioning, as mRNA collection requires destructive sampling.

The majority of studies investigating the abundance of CREB in the nervous system are concerned with either changes in the concentration of phosphorylated CREB (pCREB) (Ahmed and Frey, 2005; Moncada and Viola, 2006; Yuan et al., 2003), or with artificially induced changes in CREB levels (overexpression) (Brightwell et al., 2007; Josselyn et al., 2001; Yin et al., 1995a). The main reason why we quantified changes in CREB

mRNA levels by performing real-time qPCR analyses of cDNA samples is because it enabled us to quantify the changes in *CREB* transcript concentrations in such a way that statistical analyses could be performed on the data (as opposed to, e.g., comparing Western blot band densities of pCREB). If the molecular switch is defined by an isoform ratio, such a quantitative approach is desirable. Moreover, this method also enabled us to analyze *CREB* expression under natural circumstances, as opposed to having to resort to overexpression studies.

The studies by Ahmed and Frey (2005), Moncada and Viola (2006), Brightwell et al. (2007), Josselyn et al. (2001) and others have shown that artificially increased levels of CREB and pCREB in the nervous system facilitate LTM formation. At present it is unclear whether this facilitation depends on phosphorylation of *de novo* synthesized CREB, or whether it acts through the phosphorylation of CREB that is already present. It has been demonstrated in the pond snail *L. stagnalis* that newly synthesized CREB is required for synaptic enhancement involved in memory consolidation (Wagatsuma et al., 2006). If *de novo* synthesis of CREB occurs, the short time window between conditioning and the transcription of downstream genes under the influence of CREB also needs to accommodate a *CREB* mRNA translation phase. Our approach of quantifying changes in *CREB* mRNA levels upon conditioning may contribute to our understanding of what happens before CREB is activated and becomes transcriptionally active.

It is known from previous research that the most intact CREB isoforms (i.e. the ones encoded by mRNA with the smallest number of exons spliced out) generally act as activators in CREB-dependent transcription (Bartsch et al., 1998; Poels and Broeck, 2004; Yin et al., 1995b). Intuitively, this makes sense because alternative splicing can lead to loss of functional domains and changes in protein folding (Tress et al., 2007). Since Cg/CrCREB isoforms 1 and/or 2 are the most intact, we hypothesised them to be the activators in *C. glomerata* and *C. rubecula*. Our results seemingly contradict this hypothesis since we observed a decrease in the amount of mRNA coding for CgCREB isoforms 1 and 2 immediately after conditioning, rather than an increase.

A possible explanation for this observation is that *CREB* mRNA (which may or may not already be present at the time of conditioning) is translated into proteins immediately upon a conditioning experience and, as a consequence, it is degraded rapidly. If the observed decrease in *CREB* mRNA indeed reflects translation and subsequent degradation of *CREB* mRNA, this means that the concentration of CREB isoform 1 and/or 2 proteins actually increases immediately after conditioning. These CREB proteins are then available for subsequent phosphorylation and activation of downstream genes involved in memory formation. The mechanisms of mRNA degradation are well understood (Garneau et al., 2007; Valencia-Sanchez et al., 2006). Indeed, the transient activity of transcription factors such as CREB is thought to be linked to mRNA stability, since rapid mRNA degradation provides an efficient mechanism for transient protein expression because it links protein synthesis directly to the gene transcription rate (Calkhoven

and Ab, 1996; Tourrière et al., 2002).

We observed an increase in the relative amount of mRNA coding for the putative repressors CgCREB isoforms 3 and 5. This seems to suggest a change in their actual concentration. However, since we measure relative concentrations in % of the total amount of CREB within a sample, the two values (activators and inhibitors) are interrelated; when one decreases, the other automatically decreases: the total remains 100%. The fact that we cannot use changes in absolute *CREB* splice variant concentrations is inherent to this experimental procedure; it is not possible to perform repeated measurements (i.e., RNA levels) on the same individual wasps over time. Using different wasps for each time interval implies that the absolute RNA concentrations in each sample are different. Therefore, changes in absolute concentrations over time are not a reliable indicator of changes in expression but rather of changes in the amount of tissue used for RNA isolation. In the light of our results, it seems plausible that the actual event we observed is *de novo* synthesis of CREB isoforms 1 and/or 2, which is reflected by a decrease in mRNA coding for isoforms 1 and/or 2: it is degraded upon translation into proteins. Alternatively, the observed changes may reflect an increase in mRNA coding for isoforms 3 and/or 5, but this requires an explanation in which, somehow, the putative repressor isoforms 3 and/or 5 initiate associative learning. This is unlikely to be the case.

Our measurements showed consistently lower levels of mRNA transcripts coding for putative activator isoforms than for repressor isoforms. This is congruent with e.g. findings in *L. stagnalis*, where mRNA coding for the activator LymCREB1 is only found in some neurons, while repressor LymCREB2 mRNA is found in larger concentrations throughout the nervous system (Sadamoto et al., 2004a; Sadamoto et al., 2004b).

We have analysed data from two replicate time series (both single- and spaced-trial conditioning) for both wasp species. In order to make definitive statements about the temporal effects of single-trial and spaced-trial conditioning on the relative abundance of *CREB* splice variants, more data are needed. Additionally, the number of CREB molecules that is actually involved in LTM formation may represent only a small fraction of the total amount of CREB that is present in the brain and its surrounding tissues (which is what we measure with qPCR). Possibly, changes in *CREB* expression that are relevant to learning and memory formation occur only locally in a subset of neurons and not throughout the whole brain. Therefore, we aim to integrate quantitative data of *CREB* expression over time with qualitative data obtained by *in situ* hybridization, which we are currently performing on wholemount *C. glomerata* and *C. rubecula* brains.

Acknowledgements

We thank Leo Koopman, André Gidding, and Frans van Aggelen for all insect rearing-related matters.

Supplementary table S1. Two-way ANOVA table for single-trial conditioning. Abbreviations: d.f. = degrees of freedom, SS = sum of squares, MS = mean squares. Significance (P < 0.05) is indicated with asterisks.

Treatment	Source of variation	d.f.	SS	MS	F	P
Cotesia glomerata single-trial conditioning isoforms 1 and 2	time	5	127.380	25.476	7.778	0.021 *
	replicate	1	18.157	18.157	5.543	0.065
	residual	5	16.377	3.275		
	total	11	161.915	14.720		
Cotesia glomerata single-trial conditioning isoforms 3 and 5	time	5	127.380	25.476	7.778	0.021 *
	replicate	1	18.157	18.157	5.543	0.065
	residual	5	16.377	3.275		
	total	11	161.915	14.720		
Cotesia rubecula single-trial conditioning isoforms 1 and 2	time	5	81.681	16.336	1.580	0.314
	replicate	1	228.266	228.266	22.072	0.005 *
	residual	5	51.710	10.342		
	total	11	361.656	32.878		
Cotesia rubecula single-trial conditioning isoforms 3 and 5	time	5	81.681	16.336	1.580	0.314
	replicate	1	228.266	228.266	22.072	0.005 *
	residual	5	51.710	10.342		
	total	11	361.656	32.878		

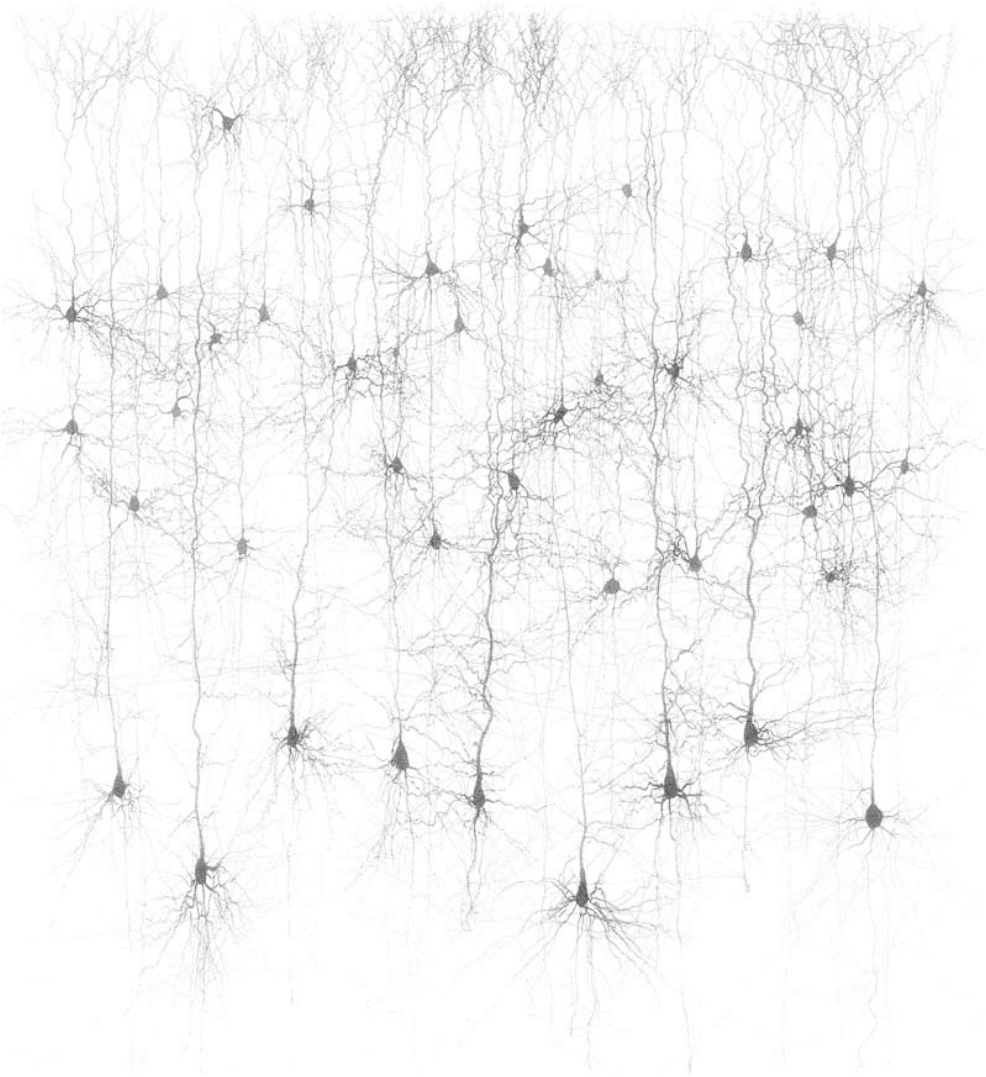
Supplementary table S2. Two-way ANOVA table for spaced-trial conditioning. Abbreviations: d.f. = degrees of freedom, SS = sum of squares, MS = mean squares. Significance (P < 0.05) is indicated with asterisks.

Treatment	Source of variation	d.f.	SS	MS	F	P
Cotesia glomerata spaced-trial conditioning isoforms 1 and 2	time	5	67.991	13.598	1.319	0.384
	replicate	1	0.0975	0.0975	0.00946	0.926
	residual	5	51.532	10.306		
	total	11	119.621	10.875		
Cotesia glomerata spaced-trial conditioning isoforms 3 and 5	time	5	67.991	13.598	1.319	0.384
	replicate	1	0.0975	0.0975	0.00946	0.926
	residual	5	51.532	10.306		
	total	11	119.621	10.875		
Cotesia rubecula spaced-trial conditioning isoforms 1 and 2	time	5	9.242	1.848	0.520	0.755
	replicate	1	376.244	376.244	105.750	< 0.001 *
	residual	5	17.789	3.558		
	total	11	403.275	36.661		
Cotesia rubecula spaced-trial conditioning isoforms 3 and 5	time	5	9.242	1.848	0.520	0.755
	replicate	1	376.244	376.244	105.750	< 0.001 *
	residual	5	17.789	3.558		
	total	11	403.275	36.661		

Supplementary table S3. Three-way ANOVA table for single-trial conditioning and spaced-trial conditioning in *C. glomerata* and *C. rubecula*. Abbreviations: d.f. = degrees of freedom, SS = sum of squares, MS = mean squares. Significance ($P < 0.05$) is indicated with asterisks.

Treatment	Source of variation	d.f.	SS	MS	F	P
single-trial conditioning isoforms 1 and 2	time	5	157.350	31.470	4.184	0.071
	species	1	71.585	71.585	9.516	0.027 *
	replicate	1	187.590	187.590	24.938	0.004 *
	residual	5	37.612	7.522		
	total	23	595.156	25.876		
single-trial conditioning isoforms 3 and 5	time	5	157.350	31.470	4.184	0.071
	species	1	71.585	71.585	9.516	0.027 *
	replicate	1	187.590	187.590	24.938	0.004 *
	residual	5	37.612	7.522		
	total	23	595.156	25.876		
spaced-trial conditioning isoforms 1 and 2	time	5	28.166	5.633	2.412	0.178
	species	1	71.058	71.058	30.427	0.003 *
	replicate	1	182.113	182.113	77.981	< 0.001 *
	residual	5	11.677	2.335		
	total	23	593.955	25.824		
spaced-trial conditioning isoforms 3 and 5	time	5	28.166	5.633	2.412	0.178
	species	1	71.058	71.058	30.427	0.003 *
	replicate	1	182.113	182.113	77.981	< 0.001 *
	residual	5	11.677	2.335		
	total	23	593.955	25.824		

Bidirectional selection on learning rate in the parasitic wasp *Cotesia glomerata*



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Abstract

The parasitic wasp species *Cotesia glomerata* has an exceptionally high learning rate. In host-plant-odour learning it forms protein synthesis-dependent long-term memory (LTM) after a single associative conditioning trial, whereas other organisms such as the closely related wasp *C. rubecula* typically need multiple spaced conditioning trials to do so. Additionally, *C. glomerata* lacks another form of consolidated memory known as anaesthesia-resistant memory (ARM), which has been shown to co-occur with LTM in other animals and is typically formed after single or massed conditioning trials. The number and type of conditioning trials required for LTM formation are expected to depend on factors such as the number of experiences and the reliability of the association that is formed, and is likely to be subject to natural selection.

Indeed, heritable variation in learning rate on which selection can act was previously shown to exist in mammals and insects. We expected similar variation to be present within *C. glomerata* as well. In order to investigate the effect of selection on learning rate and the subsequent formation of consolidated memory, we applied a bidirectional selection regime and reared a decreased-learning line and an increased-learning line of *C. glomerata* wasps. Wasps that showed 24 h memory retention after a single conditioning trial were used to propagate the increased-learning line. Wasps that did not show 24 h memory retention after a single conditioning trial, but only after three spaced conditioning trials, were used to propagate the decreased-learning line.

We show here that, over nine generations, the learning rate in the decreased-learning line became significantly lower than the learning rates of the increased-learning line. This shows that heritable variation in learning is also present in *Cotesia*. Moreover, these selection lines can be used as a tool to investigate how memory consolidation dynamics change within a species when fitness penalties are associated with learning rate. It is expected that in terms of ecology, both learning rate and memory formation dynamics strongly depend on the associated costs and benefits.

Introduction

Dutch strains of the two closely related (Michel-Salzat and Whitfield, 2004) parasitic wasps *Cotesia glomerata* and *C. rubecula* (Hymenoptera: Braconidae) exhibit species-specific differences in learning rate and the formation of a consolidated memory type known as long-term memory (LTM) (Bleeker et al., 2006a; Smid et al., 2007). This form of memory differs from another consolidated memory type known as anaesthesia-resistant memory (ARM) in that it is protein synthesis-dependent (Margulies et al., 2005). Differences in the LTM consolidation dynamics of these wasps were unravelled using single, spaced and massed learning experiences, while interfering with LTM formation through transcription or translation inhibitors (Smid et al., 2007).

It is expected that in terms of ecology, both learning rate and memory formation dynamics strongly depend on the associated costs and benefits (Dukas, 1999; Shettleworth, 1993). Low learning rates are costly because of suboptimal performance during learning, as shown, e.g., with flower-handling skills in generalist bumblebees compared to closely related specialists (Laverty and Plowright, 1988). High learning rates and subsequent LTM formation are costly in terms of metabolism, e.g. because of a trade-off between protein synthesis in LTM formation and general maintenance processes in the animal. This has been shown in *Drosophila melanogaster*, where conditioned flies that did form LTM in a spaced training protocol were significantly less resistant to starvation and desiccation than control flies that did not form LTM (Mery and Kawecki, 2005). Factors such as life span, total number of lifetime experiences, variability of the environment and reliability of information are thought to influence the balance of costs and benefits of memory formation (Dukas, 1998b; Roitberg et al., 1993; Stephens, 1993).

How does this apply to our model system? *Cotesia glomerata* is considered a generalist but in The Netherlands, this species prefers to lay its eggs in caterpillars of the gregarious large cabbage white *Pieris brassicae* (Geervliet et al., 2000), which is a superior host for the Dutch *C. glomerata* lines (Brodeur et al., 1998; Harvey, 2000). The large cabbage white butterfly lays her eggs in clusters of up to 150 on plants which are found in dense stands; after emergence and eating all the available leaf mass, dietary specialisation forces the caterpillars to migrate to neighbouring plants of the same species (Le Masurier, 1994; Smid, 2006). The gregarious *C. glomerata* lays up to 20 eggs during a single oviposition bout (Smid, 2006). *Cotesia rubecula*, on the other hand, is a specialist on the small cabbage white Pieris rapae that lays single eggs on isolated plants (Davies and Gilbert, 1985; Root and Kareiva, 1984). Moreover, *C. rubecula* lays single eggs in its host caterpillars. This means that the number of foraging decisions for both wasps species differs dramatically: *C. glomerata* can oviposit a large part of her lifetime fecundity in a single clutch of caterpillars, whereas *C. rubecula* needs to spend much more time on locating suitable hosts and laying her eggs. Because of the egg-laying behaviour of their hosts, the predictive value of a host encounter (and subsequent oviposition by the wasp)

is much higher for *C. glomerata* than it is for *C. rubecula*. According to this scenario, a quick shift towards a different host plant species is likely to be adaptive for *C. glomerata* because the chance of finding more hosts on the same plant species nearby is high. For *C. rubecula* this strategy would be maladaptive because finding a suitable host does not imply the presence of other hosts on the same plant species in the close vicinity (Bleeker et al., 2006a).

We have shown that these differences in foraging behaviour and host traits are correlated to differences in memory consolidation (Smid et al., 2007). *Cotesia glomerata* forms LTM after a single conditioning trial whereas *C. rubecula* needs three spaced trials to do so. We also showed that an additional, protein synthesis-independent ARM-like memory trace is present up to 48 h after spaced learning in *C. rubecula*, in contrast to the exclusively protein synthesis-dependent memory trace in *C. glomerata*. The idea of coexisting memory traces was inferred earlier, based on work that showed that ARM is induced in *Drosophila* by both single and massed training, whereas both LTM and ARM are induced by spaced training (Tully et al., 1994). The concept of coexisting LTM and ARM in *Drosophila* was later challenged based on the results of a study on the *Drosophila* mutant *ala*, which is incapable of LTM formation, but has fully functional ARM (Isabel et al., 2004; Pascual and Preat, 2001). The conclusion of Isabel et al. (2004) that LTM and ARM are consolidated exclusively is controversial (see Margulies et al. (2005)) but nevertheless interesting, since our results suggest that exclusive consolidation of LTM occurs in *C. glomerata* but not in *C. rubecula*, where LTM and ARM occur in parallel.

Learning and decision-making can enhance survival in new niches and adaptive zones. This suggests that cognitive traits play an important role in evolutionary change and speciation (Dukas, 2004) and that learning has positive effects on fitness (Dukas, 2005; Dukas and Bernays, 2000; Dukas and Duan, 2000; Egas and Sabelis, 2001). Natural selection can only act on naturally occurring heritable genetic variation for learning and memory. Most of the evidence for the presence of this variation was found in rats and humans (Dukas, 2004) and through selection experiments with insects (Dukas, 2008). Early work with honeybees and blowflies confirmed that genetic differences in learning behaviour are present and can be subjected to bidirectional selection ('good' vs. 'poor' learning) (Brandes et al., 1988; McGuire and Hirsch, 1977). Moreover, it can be quantified by means of h^2 (heritability) estimations (Brandes, 1988). Later, selective breeding experiments for good and poor conditioning produced two populations of *Drosophila* melanogaster which differed hugely in the percentage of animals showing good conditioning: an increase from 19% to 77% in one population, and a decrease to less than 4% in the other (Lofdahl et al., 1992) while non-associative components (i.e. sensitisation) did not differ between the populations. More recently, the presence of heritable variation in learning rate was demonstrated by selection for improved associative learning of oviposition substrate choice in *Drosophila* (Mery and Kawecki, 2002).

Variation also exists in 24 h memory retention within natural populations of *C. glom-*

erata; after a single conditioning trial on nasturtium ca. 65% of the wasps choose nasturtium instead of cabbage (Smid et al., 2007). The remaining wasps have, for whatever reason, not consolidated LTM and eventually display their innate preference for cabbage in the windtunnel (Bleeker et al., 2006a; Geervliet et al., 1998b). Natural populations of *C. glomerata* thus display variation in learning rate. This gave us the opportunity to investigate whether the observed variation in learning rate is heritable and if it can be subjected to bidirectional selection: is it possible to select for lines with increased and decreased learning rates? If so, we might speculate on how fitness constraints influence memory consolidation in a wasp species that exclusively uses protein synthesis-dependent LTM to store information. In particular, it would be interesting to see whether an ARM-like memory trace arises in a decreased-learning line, how long it lasts, and whether LTM and ARM are coexisting or mutually exclusive (chapter 6). Moreover, such differently learning lines can also be used for experiments measuring costs and benefits associated with high and low learning rates (chapter 7).

In this study, we aimed at selecting for a decreased-learning line and an increased-learning line of *C. glomerata* wasps. Since memory can only be measured indirectly, e.g., by behavioural analyses, we define memory retention at a certain time interval after conditioning as the fraction of wasps that has retained its learned preference for nasturtium. Long-term memory is assumed to be present in conditioned wasps showing significantly higher preference for nasturtium after 24 h than unconditioned wasps do. This definition is based on the results by Smid et al. (2007), who showed that 24 h memory in this wasp species consists entirely of protein synthesis-dependent LTM. Note, however, that another long-lasting form of consolidated memory exists, which is not protein synthesis-dependent (ARM) (Margulies et al., 2005; Smid et al., 2007). Decreased-learning rate is then defined as a lack of 24 h memory retention in our windtunnel bioassay after a single conditioning trial. In order to make sure that we did not select for poor learning, the wasps lacking 24 h memory retention after a single conditioning trial still needed to show 24 h memory retention after three spaced conditioning trials. In addition, we reared an increased-learning line, i.e., only wasps that did show 24 h memory retention after a single conditioning trial were allowed to reproduce. Thus, we effectively tried to diverge the variation in learning rate present in natural populations by applying bidirectional selection pressure.

We show here that, measured over nine generations, the learning rate differs significantly between the decreased-learning and the increased-learning lines. Thus, we succeeded in rearing lines of *C. glomerata*, which show learning behaviour that is adaptive in an environment in which fitness is associated with learning rate. Reproduction in the decreased-learning line is only possible when more time is spent evaluating new information, as opposed to consolidating memory immediately after a single conditioning trial, which happens in the increased-learning line. This study therefore provides new evidence for the idea that animals may have tailor-made memories: memories with different underlying consolidation dynamics, depending on the constraints imposed by the

ecological niche the animal occupies.

Materials and methods

Insects

Cotesia glomerata wasps (Hymenoptera: Braconidae) were obtained from colonies that originated from individuals collected in cabbage fields in the vicinity of Wageningen, The Netherlands (collected in summer 2007), and were reared on *Pieris brassicae* L. (Lepidoptera: Pieridae), as described previously (Geervliet et al., 1998b). *Pieris* caterpillars were reared on Brussels sprouts (*Brassica oleracea* var. Gemmifera) as described previously (Geervliet et al., 1998b). The subsequent selection regime (as described below) started in January 2008.

Upon eclosion males and females were caged together for two days to allow mating. After two days the majority of males was removed. In between experiments, wasps were kept in separate, glass cages at 20-22 °C, 50-70% relative humidity, a L16:D8 photoperiod. Water and honey were provided ad libitum. These wasps did not have any experience with hosts or plants and are referred to as 'unconditioned wasps'.

Caterpillars that were parasitised at the end of the selection cycle were transferred to cages with Brussels sprouts plants to allow feeding and development into fifth instar larvae. At this stage, developing *Cotesia* larvae egressed from the caterpillars and spun cocoons. All cocoons were collected and kept in petridishes at 20-22°C, 50-70% relative humidity, and a L16:D8 photoperiod until the wasps started to eclose, after which the wasps were transferred to cages.

Plants

Brussels sprouts plants, *B. oleracea* L. var. gemmifera cv. Cyrus, and nasturtium plants, *Tropaeolum majus* L. cv. Glorious Gleam were reared as described previously (Geervliet et al., 1998b). In all wind tunnel experiments we used nasturtium plants of six weeks old and cabbage plants of eight weeks old. To compensate for differences in size and frontal leaf density two nasturtium plants (in one pot) were tested against a single cabbage plant.

Conditioning trials and learning paradigm

Nasturtium plants were infested with 0-1 day old caterpillars which were allowed to feed for 24-48 h. Shortly before use in the conditioning trial all caterpillars were removed and substituted with new 0-1 day old caterpillars, in order to facilitate oviposition by the wasp (older caterpillars defend themselves by biting). Unconditioned female wasps were individually placed in a glass tube, which was then brought in close proximity to a caterpillar on the infested nasturtium leaf. The wasps were released onto the leaf, ensuring direct contact of their antennae with a caterpillar and its products. This stimulation

induced an immediate oviposition response, lasting approximately 10 s. After oviposition, the parasitised caterpillar was removed. After 5-10 wasps had oviposited the leaf was exchanged for a fresh one.

A conditioning trial thus consists of an oviposition experience on a different host plant species (i.e. nasturtium instead of the innately preferred cabbage). This conditioning trial is defined as a form of classical conditioning (Bleeker et al., 2006a; Smid et al., 2007). Spaced trials are repetitions of single conditioning trials with ten-minute intervals. Memory retention at a certain time interval after single or multiple conditioning trials is defined by the fraction of wasps that has retained its learned preference for nasturtium (the conditioned stimulus). We define LTM as being present in conditioned wasps when 1) 24 h memory retention is significantly higher than it is in unconditioned wasps and 2) this form of consolidated memory can be inhibited by treatment with the protein synthesis-inhibitor anisomycin (ANI). If 24 h memory retention is impaired by ANI (compared to controls that did not receive ANI) this suggests the presence of LTM. If memory retention is not impaired by ANI, this suggests the presence of another form of consolidated memory, presumably ARM. This can be tested by observing the effect of cooling-induced retrograde amnesia on memory retention.

Windtunnel

The windtunnel assay was described previously (Geervliet et al., 1994). Wind speed was set at 15-17 cm/s. Wasps were tested in a relative humidity of 40-60% and at temperatures between 23 °C and 25 ºC. In all windtunnel experiments female wasps were subjected to a two-choice test in which they could fly towards the odours of either damaged nasturtium or damaged cabbage.

Unconditioned females were tested by offering artificially damaged plants in order to prevent any contact with host-derived substances before being given a learning experience. The damage was inflicted by giving four leaves of each odour source 10 strikes with a pattern wheel, 24 h before testing. Conditioned females were tested on feeding-damaged plants infested by caterpillars.

Each wasp was individually collected from the breeding cage with a glass vial and individually transferred to the windtunnel. It was then released in the middle of an open cylinder at approximately 25 cm from the odour sources (Geervliet et al., 1994). In each test the wasps were allowed 5 minutes to show a preference by flying towards and landing on one of the plants. Landing elsewhere in the windtunnel or no flight at all counted as no-response. After testing 5 wasps, plant positions were exchanged in order to prevent bias towards one side of the windtunnel.

Selection cycle

The selection cycle consisted of several steps. Wasps to be selected for the decreased-learning line were required to show 1) an innate preference for the odours of artificially

damaged cabbage, 2) no preference for the odours of feeding-damaged nasturtium 24 h after a single conditioning trial, and 3) preference for the odours of feeding-damaged nasturtium after three spaced conditioning trials. Only wasps that fulfilled all three requirements were allowed to parasitise caterpillars and contribute to the gene pool of the next generation. Wasps to be selected for the increased-learning line were required to show 1) an innate preference for the odours of artificially damaged cabbage, and 2) preference for the odours of feeding-damaged nasturtium 24 h after a single conditioning trial.

On the day prior to the windtunnel tests the females were given either a single, or three spaced conditioning trials. They were then kept overnight in glass cages with water and honey until testing. The conditioning trials were offered when the female wasps were 2-10 days old.

The first step in the cycle ensures that all selected wasps show the 'hardwired' tendency to fly towards cabbage. Steps two and three in the decreased-learning line select for '*C. rubecula-like*' memory dynamics where, like in most other animals, a single trial-conditioning does not initiate LTM formation but spaced trial-conditioning does. Step two in the increased-learning line selects for *C. glomerata*-like memory dynamics, in which a single trial-conditioning does initiate LTM formation.

Innate plant odour preference. The flight behaviour of unconditioned wasps from the decreased- and increased-learning lines towards cabbage and nasturtium was tested with 2-7 days old females. Wasps showing an innate preference for cabbage were used for the second test in the selection cycle. All generations were tested on 5-8 different days. Wind tunnel data were only used when the percentage of responsive wasps was > 40%, except for the starting line (five days tested, but only one day with a percentage of responsive wasps > 35%.

Long-term memory formation after a single conditioning trial. Wasps that had displayed an innate preference for the odour of cabbage subsequently received a single conditioning trial on nasturtium 24 h before testing. Subsequently, flight behaviour towards cabbage and nasturtium was again recorded in the windtunnel for wasps from both lines. Wasps flying towards nasturtium were assumed to have formed LTM after a single conditioning trial. Females from the decreased-learning line that did not form LTM were subjected to the next test (spaced training). Females from the increased-learning line that did form LTM were used for further propagation of the line. All generations were tested on 2-7 different days. Wind tunnel data were only used when the percentage of responsive wasps was > 40%.

Long-term memory formation after three spaced conditioning trials. The remaining females of the decreased-learning line received three spaced conditioning trials on nasturtium. Only wasps showing LTM after three spaced conditioning trials were allowed to parasit-

ize caterpillars and propagate the decreased-learning line.

Selection was applied to each of the first six generations. From the sixth generation on we applied selection in alternate generations, in order to be able to perform experiments to assess the characteristics of the wasps in the two lines (described in chapters 6 and 7) which required large numbers of wasps.

Propagation

To maintain sufficient genetic variation in the line we ensured that each of the selected female wasps (15-20 in each generation) parasitised several *P. brassicae* caterpillars. After the parasitized caterpillars were fully grown (L5 stage, after approx. 12 days) the wasp larvae started to egress from their host and formed cocoons. These were collected and kept in petridishes. After approximately 8 days the new generation of wasps eclosed from the cocoons. These wasps were allowed to mate freely before the females were subjected to the next selection cycle.

Statistics

All data were analysed using a general linear model (GLM), using the GENMOD procedure in SAS v. 8.02 (SAS, Inc., Chicago, IL) for data with a binomial distribution of error variance and a logit-link function. When the choice distributions of the wasps were compared, the fraction of individuals landing on nasturtium in the windtunnel was used as the response variable (R), with the number of responding wasps (N) as the binomial total. Data collected on different experimental days were considered as replicates. In case of overdispersal, we allowed the variance functions of the binomial distribution to have a multiplicative overdispersion factor (DSCALE option) by dividing the square root of the deviance of the model by the degrees of freedom.

Results

Innate plant odour preference

Before applying the actual selection pressure, we assessed whether the majority of wasps we used for the procedure indeed had the innate tendency to fly towards cabbage or in other words, that they, as a group, showed a low preference for nasturtium (we used preference for nasturtium as the response variable in all analyses). We tested whether line (three levels: start line, decreased-learning line, and increased-learning line) and generation (six levels: generations 0 - 5) had an effect on choice for nasturtium (GLM: line: $\chi^2_1 = 0.44$, $p = 0.51$; generation: $\chi^2_4 = 11.06$, $p = 0.026$; generation x line: $\chi^2_4 = 1.33$, $p = 0.86$). The low levels of choice for nasturtium shown here are consistent with earlier observations (Smid et al., 2007). In several generations, the response rate was < 40%. This was the case when we used very young wasps (1-2 days old), which typically show low responsiveness in a windtunnel setup (Steinberg et al., 1992). Because of the relatively short time window available for doing all tests it was logistically impossible,

however, to use older wasps in each generation. In those cases where the flight response levels were < 40% after two days of trying, we skipped this step and moved on to the 24 h memory retention step. Therefore, we only analysed unconditioned preference data for generations 0-5. There is large variation in the flight response levels of unconditioned wasps, which is caused by large day-to-day variation (not shown).

Memory retention after a single conditioning trial

Wasps from both selection lines that flew to cabbage in the first wind tunnel test were given a single conditioning trial on a nasturtium leaf. Twenty-four hour memory retention was assessed in the windtunnel. The results are shown in figure 1. From the third generation on 24 h memory retention decreased in the decreased-learning line compared to the increased-learning line. Memory retention in the increased-learning line showed a slight but non-significant increase compared to the start and decreased-learning lines. Again, we tested the effects of line (two levels: decreased-learning line and increased-learning line) and generation (eight levels: generations 1-6, 8 and 9) (GLM, corrected for overdispersal: line: $\chi^2_1 = 18.63$, $p < 0.0001$; generation: $\chi^2_7 = 5.40$, $p = 0.61$; generation * line: $\chi^2_7 = 11.04$, $p = 0.14$; GLM, not corrected for overdispersal: line: $\chi^2_1 = 28.00$, $p < 0.0001$; generation:$\chi^2_7 = 8.11$, $p = 0.32$, generation * line: $\chi^2_7 = 16.58$, $p = 0.02$). For p-values of specific contrasts from particular comparisons, refer to figure 1. Note that we were interested in the differences between the selection lines, and not in LTM formation in the start population (which was described extensively in Smid et al., 2007) so therefore we have two levels of line instead of three. There is a strongly significant effect of line on the differences in memory retention.

Memory retention after three spaced conditioning trials in the decreased-learning line

Wasps of the decreased-learning line that did not show 24 h memory retention after a single conditioning trial were given three spaced oviposition experiences on nasturtium. Again, memory retention was measured in the windtunnel 24 h after conditioning. Only wasps that flew to nasturtium were used for parasitisation of *P. brassicae* caterpillars and thus for producing the next generation. We tested whether there was an effect of generation on memory retention in the decreased-learning line after three spaced conditioning trials (GLM: generation: $\chi^2_7 = 5.11$, $p = 0.65$). This appeared not to be the case, which implies that the ability to form LTM after three spaced conditioning trials did not change inadvertently over the course of selection.

Discussion

Our goal was to create two distinct lines of *C. glomerata* that differ in learning rate, using a bidirectional selection protocol. We succeeded in rearing and maintaining these selection lines, and we were able to change the learning rate of *C. glomerata* within relatively few generations. For propagation of the increased-learning line we used the wasps that showed 24 h memory retention after a single conditioning trial. For propagation of the

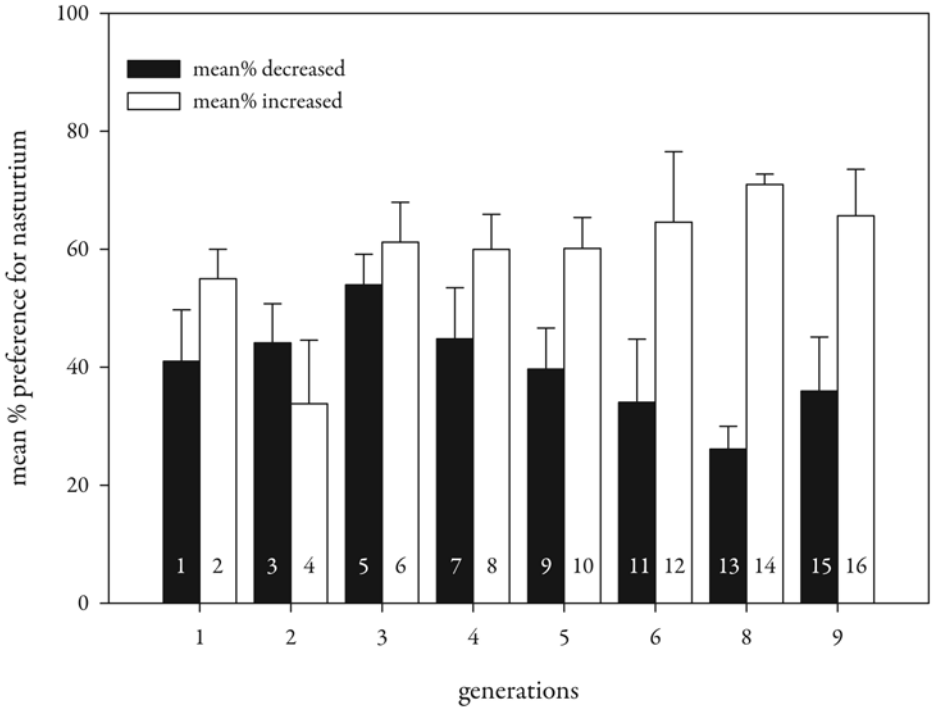

Figure 1. Mean 24 h memory retention after a single conditioning trial, expressed in the percentage of responding wasps (± s.e.m.) flying towards nasturtium in a two-choice wind tunnel test (nasturtium vs. cabbage). There is a significant difference between the decreased and increased-learning lines (GLM, corrected for overdispersal: line: $\chi^2_1 = 18.63$, $p < 0.0001$; generation: $\chi^2_7 = 5.40$, $p = 0.61$; generation x line: $\chi^2_7 = 11.04$, $p = 0.14$; GLM, not corrected for overdispersal: line: $\chi^2_1 = 28.00$, $p < 0.0001$; generation: $\chi^2_7 = 8.11$, $p = 0.32$, generation * line: $\chi^2_7 = 16.58$, $p = 0.02$).

(*continued from page 82*) decreased-learning line we used wasps that did not show 24 h memory retention after a single conditioning trial but that displayed the more common learning characteristics of 24 h memory retention after multiple spaced trials.

Our results are in line with earlier observations of heritable naturally occurring variation in learning rate (Brandes, 1988; Brandes et al., 1988; Lofdahl et al., 1992) and confirm that this variation can be subjected to selection pressure. The third generation marked the onset of the steady decrease in learning rate in the decreased-learning line compared to the increased-learning line. This is comparable to observations from the bidirectional selection experiments with honeybees, which showed that the effect of selection is already established within 1-2 parthenogenetic generations (Brandes et al., 1988). Selected lines of the fruit fly *Drosophila melanogaster* started to diverge within 5-10 generations (Lofdahl et al., 1992; Mery and Kawecki, 2002).

Memory retention after a single conditioning trial. We tested the effects of line, generation, and the interaction between line and generation. Although significant effects of line and/or generation by themselves imply that natural selection influences learning rate, a significant interaction between the two would strengthen this idea. The line-generation interaction was indeed significant, unless we corrected for overdispersal. This is most likely due to the fact that the first two generations do not conform exactly to the line that the GLM fits. The first generations were reared during winter and we have observed previously that learning rates may be compromised during this period (unpublished results), which may be an explanation for the variation in flight response (e.g., erratic behaviour and a high percentage of no-response cases) we recorded in the present study.

As selection continues, we will be able to ascertain for how long the two selection lines will continue to diverge in terms of changing learning rates. Under laboratory circumstances, we would expect the learning rate of the decreased-learning line to decrease further than what we observed so far (as was shown for *D. melanogaster* by Lofdahl et al. (1992), where it decreased to 0-4%) since there are no fitness penalties associated with the effects of a low learning rate on foraging behaviour. Foraging is expected to be influenced by changes in associative odour learning under natural conditions, but in the lab, each wasp that fulfils the selection requirements is provided with host caterpillars without having to search for them.

Memory retention after three spaced conditioning trials. There is no significant effect of generation on memory retention in the decreased-learning line after three spaced conditioning trials. This confirms, as expected, that memory consolidation still occurs. Thus, we have successfully selected *C. glomerata* to exhibit a lower learning rate, without affecting the ability to learn. Further research (chapter 6) will ensure whether 24 h memory retention represents LTM or an ARM-like memory trace.

Learning as a trait has a polygenic basis (Papaj and Snell-Rood, 2007; Tully, 1996), so selection for learning rate is likely to influence multiple genes. Underlying the observed net phenotypic effects of bidirectional selection on learning, several genetic interactions may be important, e.g., pleiotropy and epistasis, making a precise analysis difficult (Tully, 1996). One way to gain more insight into the genetic aspects of learning would be to perform quantitative trait loci (QTL) analyses, which can be used to identify regions of DNA that play a role in polygenic traits. For learning, this has been done in mice (Caldarone et al., 1997; Wehner et al., 1997) and more recently in honeybees (Chandra et al., 2001). For QTL analyses, saturated genetic maps onto which behavioural characters can be placed relative to identified regions of the genome need to be available (Chandra et al., 2001; Tanksley, 1993). Although QTL work has been done with parasitic wasps, e.g., on wing size differences between male *Nasonia vitripennis* and *N. giraulti* (Gadau et al., 2002), and on male fertility in *Leptopilina clavipes* (Pannebakker et al., 2004), such genetic maps are not yet available for *C. glomerata*. For now, as a first step towards elucidation of the genetic basis of learning and memory formation, we are investigating

whether and how the transcription factor cAMP response element-binding (CREB) protein plays a role in LTM formation in *C. glomerata* and *C. rubecula* (chapters 3 and 4) using the candidate gene approach (Fitzpatrick et al., 2005). This transcription factor is a key component in protein synthesis-dependent LTM formation (Carlezon et al., 2005; Silva et al., 1998). So far, we have cloned and sequenced the *CREB* gene and several splice variants coding for CREB isoforms in *C. glomerata* and *C. rubecula*. We have shown that constitutive *CREB* expression is largely similar in both species, and we have shown that a single conditioning trial induces a change in the ratio of the major *CREB* splice variants (chapters 3 and 4). A logical and interesting follow-up experiment would be to see whether *CREB* expression patterns have changed in our selection lines and whether the decreased-learning line still shows a learning-induced change in the relative abundance of *CREB* splice variants, or not.

The fact that we were able to obtain two lines of wasps of the same species that differ in learning rate allows us to test various hypotheses on the mechanisms and evolution of learning and memory formation. For one, it would be interesting to see whether the memory dynamics of the decreased-learning line have changed to become more like what Smid et al. (2007) observed in *C. rubecula*. *Cotesia glomerata* typically consolidates LTM after 4 h and appears not to have an anaesthesia-resistant memory (ARM) trace. *Cotesia rubecula* on the other hand, needs 2-3 days to consolidate LTM, and displays an ARM-like memory trace, which coexists with LTM during at least 48 h. In chapter 6 of this thesis, we explore LTM consolidation and the presence of ARM in our selection lines by using protein synthesis inhibitors in conjunction with single and spaced trial conditioning, and by investigating the effect of cooling-retrograde amnesia on memory retention.

From an evolutionary ecological perspective, we are interested in possible trade-offs linked to a decreased or increased learning rate. It is known from work with *Drosophila* that improved learning is associated with costs: reduced larval competitive ability (Mery and Kawecki, 2003), reduced egg-laying rate (Mery and Kawecki, 2004), reduced resistance to stress (Mery and Kawecki, 2005), and reduced longevity (Burger et al., 2008). In chapter 7 of this thesis, we investigate whether bidirectional selection for learning rate in *C. glomerata* is associated with energetic trade-offs.

Acknowledgements

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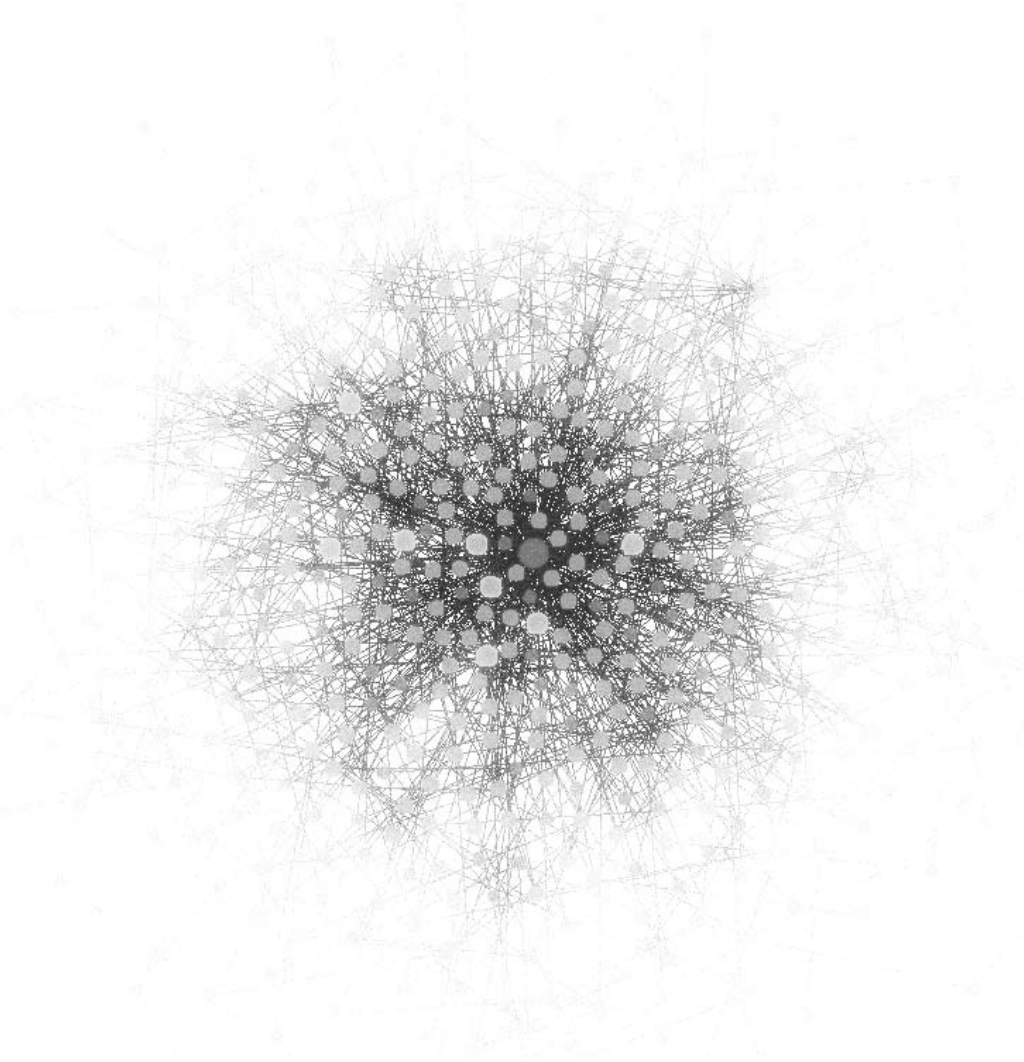
Changes in memory consolidation dynamics induced by bidirectional selection on learning rate in the parasitic wasp *Cotesia glomerata*



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Abstract

The parasitic wasp *Cotesia glomerata* and its close relative *C. rubecula* differ profoundly in learning rate and memory consolidation dynamics. One form of consolidated memory is long-term memory (LTM), which is protein synthesis-dependent. Another form of consolidated memory is anaesthesia-resistant memory (ARM), which is not protein synthesis-dependent, and which is resistant against retrograde amnesia such as amnesia induced by cooling after conditioning. *Cotesia glomerata* typically forms LTM after a single conditioning trial, whereas *C. rubecula* needs multiple conditioning trials spaced in time to do so. Moreover, consolidated memory in *C. glomerata* is thought to consist exclusively of LTM, whereas in *C. rubecula* it appears to be a mixture of both ARM and LTM.

In order to learn more about the relationship between fitness constraints, learning rate, and associated trade-offs, we performed a bidirectional selection experiment with *C. glomerata* in which we coupled a fitness penalty (i.e., no reproduction) or a fitness reward (i.e., the possibility to reproduce) to a high learning rate. This experiment resulted in a decreased-learning and an increased-learning *C. glomerata line*, which show significant differences in memory retention (chapter 5). These lines provide an excellent tool to enhance the knowledge of memory consolidation and its relation to behavioural ecology we obtained from *C. glomerata* and *C. rubecula*. To establish whether and how changes in learning rate are associated with changes in LTM consolidation dynamics, we applied the protein synthesis inhibitor anisomycin (ANI) before conditioning and measured memory retention after 1 h, 4 h, and 24 h. Furthermore, we were also interested in finding out how long the ASM phase lasts in unselected and selected *C. glomerata*, whether an ARM-like memory trace would manifest itself in the decreased-learning line, and whether or not this putative ARM trace coexists with LTM (similar to what we know from *C. rubecula*, which also has a lower learning rate). We investigated ASM and ARM by inducing retrograde amnesia through anaesthetic treatment at different time intervals after conditioning, and measuring the resulting memory retention after 4 h (at which point LTM is fully consolidated in natural *C. glomerata* populations).

Our results show that in the decreased-learning line, memory does not consolidate into protein synthesis-dependent LTM anymore. The ASM phase in the increased-learning line and the unselected *C. glomerata* wasps seems to last extremely long, compared to *Drosophila melanogaster* and *Apis mellifera*. We did not find evidence for the existence of an ARM-like memory trace in either of our selection lines or in unselected *C. glomerata*. Our results suggest that the fitness penalty associated with a lower learning rate does not translate into a more *C. rubecula*-like memory, where ARM provides a low-cost, long-lasting alternative for LTM which is advantageous considering its foraging strategy. As such, the inability of *C. glomerata* to form ARM may even represent a constitutive cost of a high learning rate.

Introduction

Memory formation is a dynamic process and memories exist in several forms, which differ in duration and stability. Many insights in the mechanisms underlying memory formation come from studies of olfactory memory formation in invertebrate model organisms such as the fruit fly *Drosophila melanogaster* and the honey bee *Apis mellifera* (Berry et al., 2008; Giurfa, 2007; Keene and Waddell, 2007; Menzel, 2001; Schwärzel and Müller, 2006). A multi-phase model describing memory dynamics was postulated by *Drosophila* researchers based on genetic and pharmacological dissection of associative olfactory conditioning, and the effects of retrograde amnesia induced by cooling (Tully et al., 1990; Tully et al., 1994; Xia et al., 1997; Xia et al., 1998a). This model broadly consists of anaesthesia-sensitive memory (ASM), anaesthesia-resistant memory (ARM) and protein synthesis-dependent long-term memory (LTM) (Margulies et al., 2005). A similar temporal division has been postulated for the honeybee (Eisenhardt, 2006; Menzel, 1999, 2001; Müller, 2002; Schwärzel and Müller, 2006).

Long-term memory is typically formed after multiple conditioning trials spaced in time, whereas a single trial or a series of trials immediately following each other (massed learning) induces ARM but is usually insufficient to induce LTM (Bailey et al., 1996; Menzel, 2001; Tully et al., 1994). In a few cases, spaced learning is not necessarily required for LTM formation, as shown by appetitive learning in the pond snail *Lymnaea stagnalis* and the fruit fly *Drosophila* (Fulton et al., 2005; Krashes and Waddell, 2008), and oviposition learning in parasitic wasps (Collatz et al., 2006; Smid et al., 2007). Furthermore, the required number of learning events (trials) for LTM acquisition can be increased or reduced, e.g., by artificial selection (Mery and Kawecki, 2002). Heritable variation in learning which could be subjected to (artificial) selection for high and low learning rates was shown to be present in the honeybee *A. mellifera*, the blowfly *Phormia regina*, and *Drosophila* (Brandes, 1988; McGuire and Hirsch, 1977; Mery and Kawecki, 2002; Mery et al., 2007b). This heritable variation allows an animal species to evolve extended time windows for evaluating new experiences, before such information is stored into fixed neural substrates (Menzel, 1999). It is expected that both learning rate and memory dynamics strongly depend on the associated costs and benefits (Dukas, 1999; Shettleworth, 1993). For example, low learning rates are costly because of suboptimal performance during learning, as shown e.g. with flower handling-skills in generalist bumblebees compared to closely related specialists (Laverty and Plowright, 1988). There is also the risk to learn wrong or unreliable information. In terms of trade-offs associated with a high learning rate, one can define either constitutive costs (genetically based, i.e., the costs are paid irrespective of whether learning actually takes place) or operating costs (which are only paid when learning, e.g., protein synthesis during LTM consolidation) (Burger et al., 2008).

As a model system for our ongoing research on learning and memory we use the two

closely related parasitic wasp species *C. glomerata* and *C. rubecula* (Michel-Salzat and Whitfield, 2004), which differ profoundly in learning and memory formation dynamics (Bleeker et al., 2006a; Geervliet et al., 1998b; Smid, 2006; Smid et al., 2007). Using the translation inhibitor anisomycin (ANI) we showed that in *C. glomerata*, LTM is formed after one conditioning trial and takes 4 h to consolidate. In contrast, *C. rubecula* needs three trials spaced in time to form LTM, with consolidation taking 2 to 3 days. Additionally, we found that a protein synthesis-independent ARM-like memory trace is present up to 48 h after spaced conditioning in *C. rubecula*, whereas the memory trace in *C. glomerata* is built up exclusively by protein synthesis-dependent memory. A situation similar to the one in *C. rubecula*, where two memory traces seem to coexist for up to 3 days, has been described for *Drosophila* (Tully et al., 1994). In this case, memory retention 24 h after 10-trial spaced conditioning consists of a protein synthesis-dependent component and a protein synthesis-independent form of consolidated memory (ARM). This concept of coexisting LTM and ARM in *Drosophila* was challenged when it was shown that the *Drosophila* mutant *ala*, which is incapable of LTM formation because of mushroom body defects (Pascual and Preat, 2001), still has fully functional ARM (Isabel et al., 2004). The *ala* mutant has normal 24 h memory retention after a single conditioning trial, but 10-trial spaced conditioning results in a complete loss of 24 h memory retention. Isabel et al. (2004) concluded from this study that LTM and ARM are consolidated exclusively. This is controversial given the results of other studies (Margulies et al., 2005) but interesting in the light of our results, which suggest that exclusive consolidation of LTM occurs in *C. glomerata* but in parallel with ARM in *C. rubecula*. Both parallel ARM-LTM and exclusive LTM formation appear to be feasible ways of long-lasting memory consolidation. Recent results in *Drosophila* show that ARM can be interpreted as a form of low-cost, long-lasting memory (Mery and Kawecki, 2005). The number and predictive value of the host finding and egg-laying experiences of *C. glomerata* (which lays multiple eggs in larvae of a gregarious host) and *C. rubecula* (which lays single eggs in larvae of a solitary host), differs enormously. This means that it is adaptive for *C. glomerata* to invest in expensive LTM, and for *C. rubecula* to rely on low-cost ARM before committing information to LTM (Smid et al., 2007).

We performed bidirectional selection on a *C. glomerata* population. By associating either a fitness penalty (i.e., no reproduction allowed) or a fitness reward (i.e., reproduction allowed) to a high learning rate, we reared a decreased-learning line and an increased-learning line (chapter 5). Measured over nine generations, the learning rate in the decreased-learning line became significantly lower than the learning rate of the increased-learning line. These selection lines provide excellent tools to answer some of the questions that arose from our earlier work with *C. glomerata* and *C. rubecula*.

Using our *C. glomerata* selection lines, we wanted to investigate whether associating a fitness penalty to a high learning rate induces a shift from (apparently) exclusive LTM consolidation to a mechanism which also includes ARM, and whether this ARM occurs in parallel or mutually exclusive with LTM. These experiments link the current dispute

on coexisting ARM and LTM in *Drosophila* with ideas on how behavioural ecology shapes memory formation. Furthermore, they add an extra dimension to our previous work (Smid et al., 2007) by testing whether selection for a low learning rate activates a 'hidden' potential in *C. glomerata* to form ARM, or whether it acts through a different mechanism, e.g., absence or slower consolidation of LTM after a single conditioning trial. In terms of ecology, we may gain new insights in trade-offs associated with changes in learning rate: ARM is a low-cost form of consolidated memory so selection might favour ARM when the fitness cost of a high learning rate increases.

We used anisomycin (ANI) to test the dynamics of the protein synthesis-dependent LTM trace in the two selection lines, and we used cooling-induced retrograde amnesia to test how long the ASM phase lasts. From the ANI and cooling data, we could then infer whether ASM directly consolidates into LTM, or whether consolidation includes an ARM phase. Additionally, we investigated the effect of cooling and ANI on memory retention in unselected wasps from our stock rearing, to ascertain whether an ARM-like memory trace is present in natural populations of *C. glomerata*. If ARM and LTM both occur, we expected the wasps to show low memory retention after cooling during the ASM phase, but above-control levels of memory retention after cooling during the ARM-phase. Similarly, above-control levels of memory retention would occur when feeding the wasps ANI before training (since the ARM trace would partially compensate for the loss of LTM). If there is no ARM, cooling treatment was expected to reveal an extended ASM phase, and ANI treatment would reduce memory retention to control levels after the initial ASM phase had passed.

In this study we show that in the decreased-learning line, memory does not consolidate into protein synthesis-dependent LTM anymore. We were able to disrupt memory retention by cooling during the ASM phase in the increased-learning line and the unselected wasps, but we could not infer evidence for the existence of an ARM-like memory trace in either of our selection lines or in unselected *C. glomerata*. Our results suggest that *C. glomerata* may pay a constitutive cost for its innate high learning rate by not being able to form ARM under conditions when this would be adaptive.

Materials and methods

Insects

1. *Cotesia glomerata* start population. The start population used for our artificially selected lines of C. glomerata wasps (Hymenoptera: Braconidae) was obtained from colonies that originated from individuals collected in cabbage fields in the vicinity of Wageningen, The Netherlands, during summer 2007. These wasps were reared on *Pieris brassicae* L. (Lepidoptera: Pieridae), as described previously (Geervliet et al., 1998). The subsequent selection regime (as described below) started in January 2008.
2. *Cotesia glomerata* selection lines. Upon egression from fifth instar *P. brassicae* cater-

pillars, all *C. glomerata* cocoons were collected and kept in Petri dishes at 20-22 °C, 50-70% relative humidity, and a L16:D8 photoperiod until the wasps started to eclose, after which emerged adults were transferred to cages and kept at 20-22 °C, 50-70% relative humidity, a L16:D8 photoperiod, and provided with water and honey. Female wasps were then subjected to a three-step selection cycle (see chapter 5 for details). This selection cycle was repeated in each subsequent generation until generation 6, after which we alternated between selection and experiments.
3. *Pieris brassicae* caterpillars used for rearing and conditioning wasps. *Pieris* larvae were reared on cabbage plants (Brassica oleracea) as described previously (Geervliet et al., 1998b). Caterpillars that were parasitized at the end of the selection cycle were transferred to cages with Brussels sprouts plants to allow feeding and development into fifth instar larvae.

For experiments I and II we used wasps from generations 8 and 9 (no selection was performed from generation 8 to generation 9). For experiment III, we used wasps from the start population.

Plants
Brussels sprouts, *B. oleracea* var. Gemmifera, and nasturtium, *Tropaeolum majus* cv. Glorious Gleam were reared as described previously (Geervliet et al., 1998b). In all wind tunnel experiments we used nasturtium plants of 5 weeks old and Brussels sprouts plants of 8 weeks old. To compensate for differences in size and frontal leaf density two nasturtium plants (in one pot) were tested against a single cabbage plant.

Conditioning trials and learning paradigm
Nasturtium plants were infested with 0-1 day old caterpillars which were allowed to feed for 24-48 h. Shortly before use in the conditioning trial all caterpillars were replaced by new 0-1 day old caterpillars, in order to facilitate oviposition by the wasp (older caterpillars defend themselves by biting). Unconditioned female wasps were individually placed in a glass tube, which was then brought in close proximity to a caterpillar on the infested nasturtium leaf. The wasps were released onto the leaf, ensuring direct contact of their antennae with a caterpillar and its products. This stimulation induced an immediate oviposition response, lasting approximately 10 s. After oviposition, the parasitised caterpillar was removed. After 5-10 wasps had oviposited, the leaf was replaced by a fresh one.

A conditioning trial thus consists of an oviposition experience on a different host plant species (i.e. nasturtium instead of the innately preferred cabbage). This conditioning trial is defined as a form of classical conditioning (Bleeker et al., 2006a; Smid et al., 2007). Spaced trials are repetitions of single conditioning trials with ten-minute intervals. Memory retention at a certain time interval after single or multiple conditioning trials is defined by the fraction of wasps that has retained its learned preference for nasturtium. We define LTM as being present in conditioned wasps when this form of consolidated memory can be inhibited by treatment with the protein synthesis-inhibitor

anisomycin (ANI). If 24 h memory retention is impaired by ANI (compared to controls that did not receive ANI) this suggests the presence of LTM. If memory retention is not impaired by ANI, this suggests the presence of another form of consolidated memory, presumably ARM. This can be tested by assessing the effect of cooling-induced retrograde amnesia on memory retention.

Windtunnel

The windtunnel assay was performed as described previously (Geervliet et al., 1994). Wind speed was set at 15-17 cm/s. Wasps were tested at a relative humidity of 40-60% and at temperatures between 23 °C and 25 °C. In all windtunnel experiments female wasps were subjected to a two-choice test in which they could fly towards either feeding-damaged nasturtium or feeding-damaged cabbage.

Each wasp was individually collected from the rearing cage with a glass vial and individually transferred to the windtunnel. It was then released in the middle of an open cylinder at approximately 25 cm from the odour sources (Geervliet et al., 1994). In each test the wasps were allowed 5 minutes to show a preference by flying towards and landing on one of the plants. Landing elsewhere in the windtunnel or no flight at all was recorded as no-response. After testing 5 wasps, plant positions were exchanged in order to prevent bias towards one side of the windtunnel.

Selection cycle

The selection cycle consisted of several steps. Wasps selected for the decreased-learning line were required to show 1) an innate preference for the odours of cabbage, 2) a preference for cabbage 24 h after a single conditioning trial, and 3) a preference for nasturtium 24 h after three spaced conditioning trials. Only wasps that fulfilled all three requirements were allowed to parasitize caterpillars and contribute to the gene pool of the next generation. Wasps from the increased-learning line were required to show 1) an innate preference for the odours of cabbage, and 2) a preference for nasturtium 24 h after a single conditioning trial. For a more detailed description, we refer to chapter 5 of this thesis.

Experiment I - LTM in selected *C. glomerata*

Memory consolidation dynamics were investigated by applying a protein synthesis inhibitor before conditioning and measuring memory retention in the windtunnel after 1 h, 4 h, or 24 h. These time intervals were chosen in order to allow a comparison of our results to those of Smid et al. (2007). To make sure that putative LTM formation was inhibited without affecting learning and protein synthesis-independent memory processes, wasps were fed the translation inhibitor anisomycin (ANI) in a sucrose solution before conditioning. Wasps serving as controls for ANI-fed animals were given the same treatment, but without ANI in the sucrose solution. The appropriate ANI concentrations have been determined previously (Smid et al., 2007).

Wasps were deprived of honey and water for 4 h, then fed 0.5 µL of a sucrose solution containing 5 mM ANI, kept in vials for 1 h or until the solution was entirely consumed, and then transferred to a glass cage with access to water and honey. Within two hours, the wasps received a conditioning experience.

Experiment II - ASM and ARM in *C. glomerata* selection lines

In order to investigate whether STM consolidates into an anaesthesia-resistant memory (ARM) trace and/or LTM during the first 4 h, we induced retrograde amnesia by applying cold-shock anaesthesia after conditioning. We cooled the wasps for 2 minutes on ice in glass vials, either 20 minutes or 3 h after conditioning, and measured memory retention in the windtunnel 4 h after conditioning. If memory was still sensitive to anaesthesia at the time of cooling, then this should have impaired 4 h memory retention. The time intervals were chosen because 1) early memory (STM and MTM) is typically anaesthesia-sensitive (Erber, 1976; Fulton et al., 2008; Quinn and Dudai, 1976; Tempel et al., 1983; Xia et al., 1998b), which is why we measured 4 h memory retention with a cooling treatment 20 minutes after conditioning, and 2) LTM was shown to have consolidated after 4 h (Smid et al., 2007), which is why we tested whether there was still ASM present just before consolidation of LTM was complete. In each case, the presence of ASM implies the absence or still incomplete consolidation of ARM/LTM. Wasps serving as controls for anaesthetised animals were given the same treatment, but without cooling.

Experiment III - ASM and ARM in unselected *C. glomerata*

We also investigated whether ARM could be present at 3 h after single trial-conditioning in unselected *C. glomerata* wasps, following the same procedures as described for experiment II. Additionally, we tested the combined effect of feeding the wasps anisomycin before conditioning and cooling 3 h after conditioning, on 4 h memory retention.

Statistics

All data were analysed using a generalized linear model (GLM), using the GENMOD procedure in SAS v. 8.02 (SAS, Inc., Chicago, IL) for data with a binomial distribution of error variance and a logit-link function. The choice that parasitoids made was coded as a binary variable (0-Brussels sprouts, 1-nasturtium). Response level of parasitoids was also binary coded (0-no-choice; 1-choice for either source). First a full model was specified with all possible interactions; non-significant interactions have been omitted from the final model. Data collected on different experimental days were considered as replicates. In case of overdispersal, we allowed the variance functions of the binomial distribution to have a multiplicative overdispersion factor (DSCALE option) by dividing the square root of the deviance of the model by the degrees of freedom.

Results

Experiment I - LTM in the *C. glomerata* selection lines

We tested the time window in which anisomycin treatment affected memory consolidation after a single conditioning trial in both selection lines. Wasps were treated individually by feeding the protein synthesis inhibitor ANI in sucrose, or sucrose alone to measure the transcription-dependent component of the observed memory levels. We investigated whether *time interval* (three levels: 1 h, 4 h, and 24 h), *treatment* (two levels: anisomycin and sucrose), and *line* (two levels: decreased-learning line and increased-learning line) had an effect on the choice for nasturtium in the windtunnel after conditioning (GLM: time interval: $\chi^2_2 = 26.37$, $p < 0.0001$; treatment: $\chi^2_1 = 5.81$, $p = 0.0159$; line: $\chi^2_1 = 16.05$, $p < 0.0001$; time interval * treatment: $\chi^2_2 = 8.19$, $p = 0.0167$). Anisomycin treatment did not affect memory retention in the decreased-learning line after any of the three time intervals: we observed memory to wane over time and memory retention did not differ significantly between the ANI-treated animals and the sucrose-fed control wasps (figure 1a). This suggests that the decreased-learning line does not consolidate protein synthesis-dependent LTM at all. Moreover, since the observed memory levels (which represent a summation of memory contributed by different memory traces) started waning immediately after conditioning, an ARM-like memory trace does not appear to exist either. In the increased-learning line, anisomycin treatment had a significant effect on 4 h memory retention and 24 h memory retention (figure 1b). This implies that after 4 h, LTM has consolidated in this line. We do not expect ARM to occur in parallel with LTM in this line, since animals treated with ANI show a control-level preference for nasturtium, as earlier observed by Smid et al. (2007). In order to test how long the ASM phase lasts and to infer whether an ARM-like memory trace is present in either or both of the selection lines, we performed experiment II.

Experiment II - ASM and ARM in the *C. glomerata* selection lines

The period of active LTM consolidation in insects can be revealed by sensitivity to cold-shock anaesthesia (Erber, 1976; Quinn and Dudai, 1976; Tully et al., 1994). During this period, both anaesthesia-sensitive STM and anaesthesia-resistant (but protein synthesis-independent) ARM can occur. In this experiment, we investigated the presence and duration of the ASM phase in the selection lines. By observing whether cooling at certain time intervals after conditioning induces retrograde amnesia or not, we can infer whether the observed levels of memory retention are the result of ASM or ARM; if memory retention is significantly higher than it is in untreated animals and not affected by cooling, this implies the presence of an ARM-like memory trace. We wanted to know whether *line* (two levels: decreased-learning line and increased-learning line), *treatment* (two levels: cooling and no cooling), and *time interval* (two levels: 20 minutes and 180 minutes) had an effect on the choice for nasturtium in the windtunnel after conditioning. Only the factor treatment had an effect (GLM: treatment: $\chi^2_1 = 6.14$, $p = 0.0132$). Since the factors line and time interval had no significant effect, we could not (*continued*

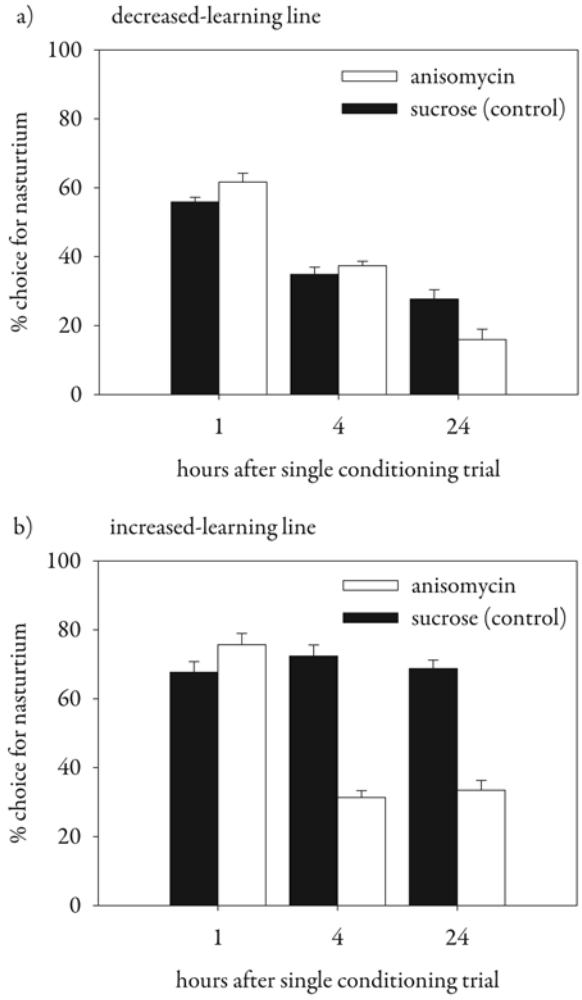


Figure 1. The effects over time of anisomycin (ANI) treatment prior to conditioning on memory consolidation after a single conditioning trial, in a) the decreased-learning line and b) the increased-learning line (mean values ± s.e.m.). Anisomycin treatment did not affect memory retention in the decreased-learning line after any of the three time intervals: we observed memory to wane over time and memory retention did not differ significantly between the ANI-treated animals and the sucrose-fed control wasps. This suggests that the decreased-learning line does not consolidate protein synthesis-dependent long-term memory at all. In the increased-learning line, anisomycin treatment had a significant effect on 4 h memory retention ($p = 0.0016$) and 24 h memory retention ($p = 0.0026$). This implies that after 4 h, LTM has been fully consolidated in this line.

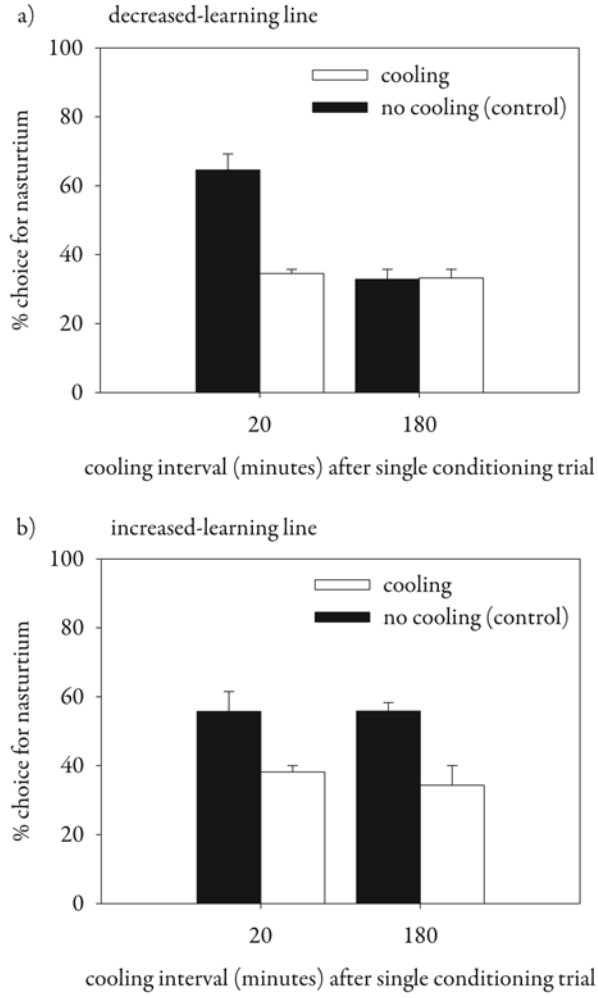


Figure 2. The effects of retrograde amnesia induced by cooling, either 20 minutes or 180 minutes after a single conditioning trial, on 4 hour memory retention in a) the decreased-learning line and b) the increased-learning line. We wanted to know whether line (two levels: decreased-learning line and increased-learning line), treatment (two levels: cooling and no cooling), and time interval (two levels: 20 minutes and 180 minutes) had an effect on the choice for nasturtium in the windtunnel after conditioning, and we found that this was the case (GLM: treatment: $\chi^2_1 = 6.14$, $p = 0.0132$). Because the factors line and time interval had no significant effect, no post hoc contrasts could be made, and hence, we could not estimate p-values for the differences between different combinations of line, treatment, and time interval. We conclude that cooling has a significant effect on memory retention in the increased-learning line.

from p. 95) make post hoc contrasts between different combinations of line, treatment, and time interval. We could, however, conclude that a cooling treatment significantly affects memory retention in the increased-learning line. When looking at figure 2a, we see that cooling after 20 minutes seems to have a large effect on 4 hour memory retention compared to the control group, whereas cooling after 180 minutes has no 'additional' effect; the observed memory levels have waned over time, which is congruent with the results of experiment I. However, 4 h memory retention in the '20 minutes control group' is higher than expected when the waning effect is taken into account. We do not have an explanation for this, although wasps do sometimes show erratic flying behaviour in the windtunnel. We conclude from experiments I and II that the decreased-learning line does not consolidate LTM, and although we can't confirm this due to the ambiguous controls of the decreased-learning line, we believe that the observed memory levels in this line represent ASM, which wanes over time. In the increased-learning line, we see an effect of cooling (figure 2b) after both 20 minutes and 180 minutes, compared to the untreated controls. This suggests that ASM is present up until at least 3 hours after conditioning, and is at that time probably consolidating into LTM, without an intermediate ARM-like trace.

Experiment III - ASM and ARM in unselected *C. glomerata* wasps

Smid et al. (2007) performed a thorough analysis of LTM consolidation in a comparative study with *C. glomerata* and its close relative *C. rubecula*. In order to gain additional insight in the memory dynamics of unselected *C. glomerata* females, we tested whether 4 h memory retention in these wasps was affected by treatment (two levels: cooling and no cooling) 3 h after a single conditioning trial (figure 3). Inducing retrograde amnesia in conditioned wasps was successful; we found a significant difference between treated and untreated wasps (GLM: treatment: $\chi^2_1 = 4.40$, $p = 0.036$). When we combined anisomycin feeding before conditioning with cooling 3 hours after conditioning, 4 hour memory retention was reduced slightly more compared to the controls (GLM: treatment: $\chi^2_1 = 4.43$, $p = 0.0353$). This means that ASM is still present 3 h after conditioning, which implies that no ARM-like memory trace has been formed. Moreover, the slight additional effect of combining cooling with anisomycin treatment suggests the immediate transition from ASM into LTM. As LTM consolidation is complete after 4 h (Smid et al., 2007), it seems unlikely that an ARM-like memory trace exists in natural populations of *C. glomerata*. With this information, we conclude from experiment II that our bidirectional selection regime has not led to changes in this respect; the increased-learning line directly consolidates LTM from ASM.

Discussion

We have shown previously that bidirectional selection for learning performance in a *C. glomerata* population yields two lines which differ significantly in 24 hour memory retention after a single conditioning trial (chapter 5). In the present study we show that

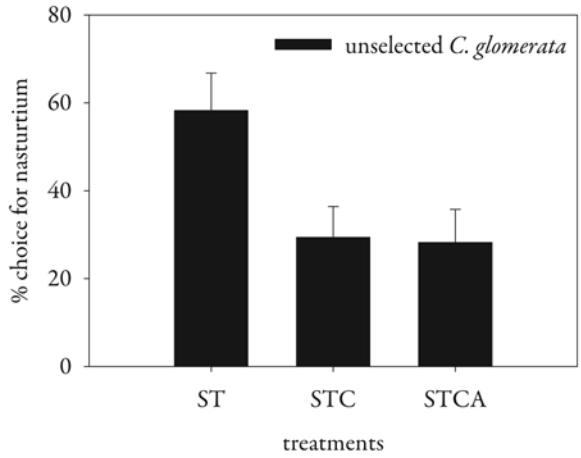


Figure 3. The effect of retrograde amnesia induced by cooling 3 h after a single conditioning trial on 4 h memory retention, either alone or in combination with anisomycin (ANI) feeding before conditioning, on unselected *C. glomerata* wasps. We wanted to know whether treatment (three levels: no cooling, cooling, and cooling + ANI) had an effect on the choice for nasturtium in the windtunnel after conditioning. Cooling 3 h after conditioning significantly reduced 4 h memory retention (GLM: $\chi^2 1 = 4.40$, $p = 0.036$), and this effect was pronounced slightly more in ANI-fed wasps (GLM: $\chi^2 1 = 4.43$, $p = 0.0353$). We therefore conclude that in unselected *C. glomerata* wasps, ASM consolidates directly into LTM, without an intermediate ARM-like memory trace. Abbreviations: ST = single-trial conditioning, STC = ST + cooling, STCA = STC + anisomycin.

the underlying memory dynamics in the decreased-learning line have changed as well. Our experiments with anisomycin revealed that after a single conditioning trial, protein synthesis-dependent LTM is no longer consolidated in the decreased-learning line. Using the same learning paradigm, the increased-learning line still consolidates LTM. Furthermore, our anaesthetic treatments indicated that the LTM phase follows directly upon the ASM phase in the increased-learning line and also in unselected wasps from the stock population. This suggests that no ARM-like memory trace is present in *C. glomerata*, be it in parallel with LTM or sequentially between ASM and LTM.

After 4 hours, LTM has not been consolidated in the decreased-learning line, whereas it has been consolidated in the increased-learning line. The latter thus exhibits the high learning rate typical for natural *C. glomerata* populations. In the control experiment with sucrose-fed wasps from the decreased-learning line, memory retention starts to wane gradually immediately after the conditioning trial and eventually returns to control levels, which implies that no LTM is formed in the decreased-learning line at all. The results from our experiment with ANI are in line with the results of Smid et al. (2007), where *C. glomerata* has consolidated LTM after 4 hours and *C. rubecula* has not. After 4 hours, memory levels have not decreased in *C. rubecula*, however, whereas they have returned to control levels in the decreased-learning *C. glomerata* line. The observed

levels of memory retention in *C. rubecula* at 4 hours after single-trial conditioning may reflect ARM, since ARM was shown to be induced after a single trial in *Drosophila* as well (Tully et al., 1994).

The ambiguous controls for cooling after 20 minutes in the decreased-learning line notwithstanding, we assume that the initially observed memory retention levels in the decreased-learning line represent ASM. These memory traces are typically short-lived, e.g., < 1 hour in *Drosophila*, (Tully, 1994; Tully et al., 1990) and indeed, after one hour, this memory starts to wane and eventually reaches control levels. This confirms the observations from the ANI experiment: if an ARM trace would be present in the decreased-learning line, we would expect above-control levels of memory retention when cooling takes place at time intervals outside the ASM phase. Cooling of wasps from the increased-learning line, either 20 minutes or 180 minutes after a single conditioning trial has an effect (figure 2b). This concurs with what we observed when cooling unselected wasps 3 hours after single-trial conditioning (experiment III). If this consolidation would occur via an intermediate ARM phase, this phase would be extremely short (< 1 hour, since 4 hour memory can be obliterated entirely by ANI and thus consists of 100% LTM), compared to ARM after single-trial conditioning in e.g. *C. rubecula*, where it lasts at least 4 hours (Smid et al., 2007) or *Drosophila*, where it lasts up to 1 day (Margulies et al., 2005; Tully et al., 1994). Additionally, the ASM phase in both the increased-learning line and in unselected wasps would be exceptionally long compared with results from *Drosophila* (Tully et al., 1994) or *A. mellifera* (Erber et al., 1980). Nevertheless, longer ASM traces (up to 90-120 minutes) have been described for *Drosophila* (Tempel et al., 1983), using a classical conditioning protocol with consecutive appetitive and electroshock reinforcement. Tempel et al. (1983) showed that the duration of the ASM phase is different, depending on the type of reinforcement that was used. In line with this, the long ASM phase that we found may be a specific feature of oviposition reward learning.

We wanted to know whether an ARM trace would arise in the decreased-learning line as a consequence of the selection regime, and whether ARM is present in the increased-learning line and the unselected wasps as well. This appeared not to be the case. We found that the ASM phase lasted comparatively long and was replaced by consolidated LTM in the increased-learning line and the unselected wasps. In the decreased-learning line, ASM gradually waned to control levels but was not replaced by LTM. A low learning rate (like in *C. rubecula*) apparently does not automatically translate into the consolidation of low-cost ARM instead of LTM. In terms of the *Drosophila* dispute (coexisting vs. mutually exclusive ARM and LTM), our current results do not favour either concept over the other. We still believe that both scenarios are feasible ways to consolidate memory, and represent adaptations to an animal's behavioural ecology (Smid et al., 2007).

The memory dynamics as inferred from the results of experiments I and II are summarised in figure 4.

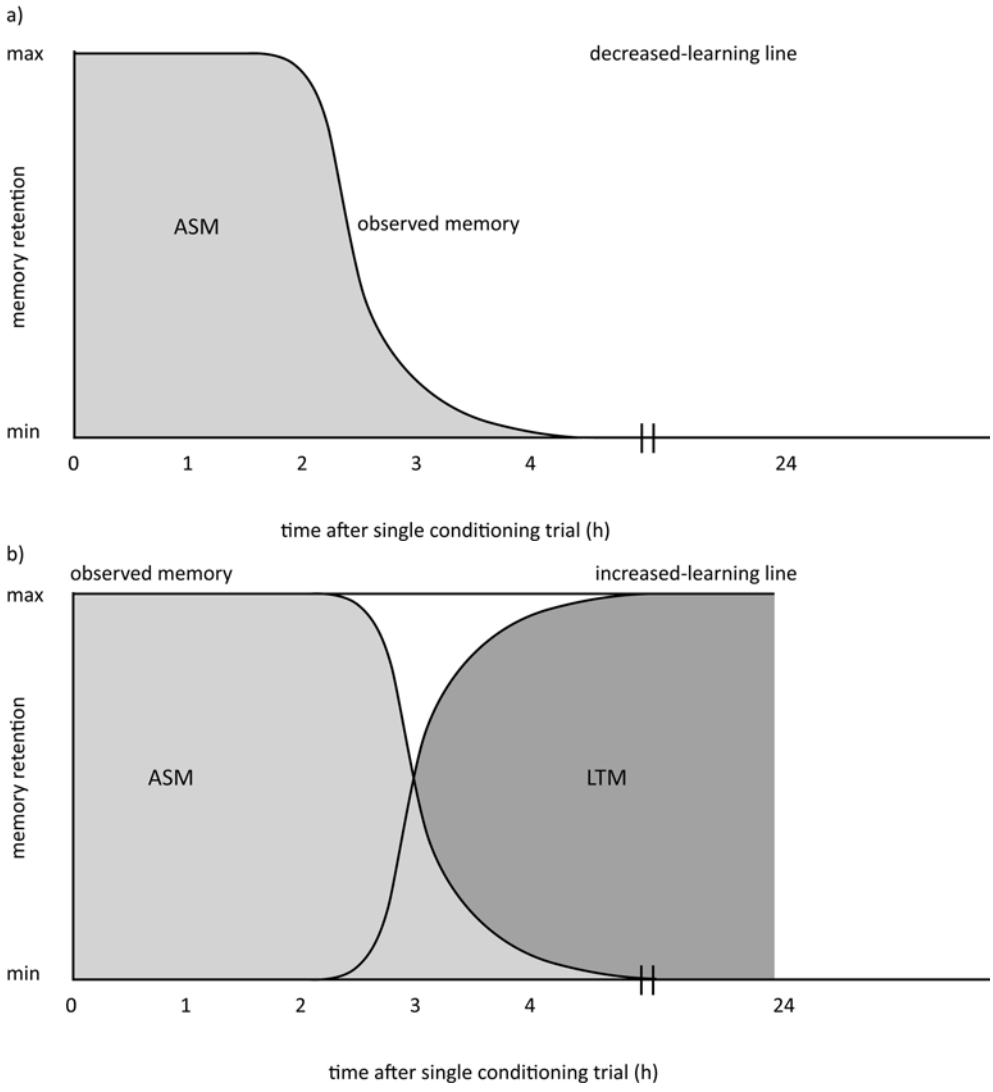


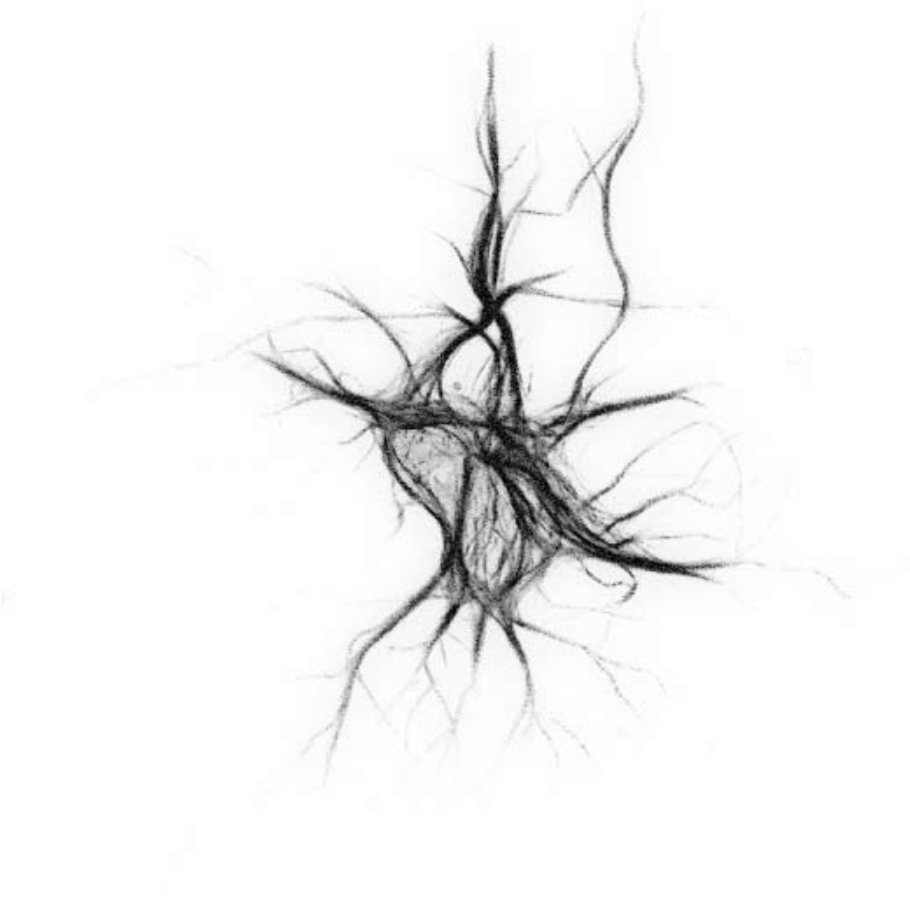
Figure 4. Memory dynamics in a) the decreased-learning and b) the increased-learning line, as inferred from the effect of anisomycin (ANI) on the consolidation of protein synthesis-dependent long-term memory (LTM) formation, and the effect of cooling on immediate memory retention (cooling induces retrograde amnesia during the anaesthesia-sensitive memory (ASM) phase). The presence of a consolidated anaesthesia-resistant memory (ARM) trace can be inferred from memory retention levels after cooling or after treatment with anisomycin, and we conclude that there is no evidence for ARM to be present in our selection lines. The presence of early ASM could not be confirmed experimentally in the decreased-learning line, but seems likely, based on literature. The different memory traces add up to the observed levels of memory retention. Minimum retention on the y-axis represents control levels of odour preference. Also note that the observed memory and the underlying dynamics are similar for wasps from the increased-learning line and the unselected *C. glomerata* population.

Previously we postulated that in the field, the number and the predictive value of the experiences encountered by *C. glomerata* allow for immediate and energetically costly investment in the formation of LTM (Smid et al., 2007). The present study builds on this concept by investigating differences in memory dynamics arising through a bidirectional selection procedure in which either a fitness penalty or a reward was attached to the learning rate. We created a situation that mimicked an environment in which the predictive value of an oviposition experience for the wasps is either very low or very high. We then asked whether (assuming differences in learning and memory are correlated with reproductive and foraging behaviour of the *Cotesia* wasps and their hosts), the association of a fitness penalty to a high learning rate may induce a shift towards *C. rubecula*-like memory dynamics. Our results confirm that in evolutionary-ecological terms, the foraging behaviour of *C. glomerata* combined with the egg-laying behaviour of its host has led to a genetically hardwired tendency to form protein synthesis-dependent LTM rather than low-cost ARM. In fact, we conclude that this feature of *C. glomerata* is a constitutive cost the animal pays for its high learning rate, as it appears that *C. glomerata* has lost its ability to form ARM under conditions where such low-cost consolidated memory would be favourable.

Acknowledgements

We thank Leo Koopman, André Gidding, and Frans van Aggelen for providing us with *C. glomerata* cocoons, and Bert Essenstam for the nasturtium plants.

Trade-offs associated with bidirectional selection on learning rate in the parasitic wasp *Cotesia glomerata*: changes in brain size and longevity



Michaël van den Berg, Louise E.M. Vet, Marcel Dicke & Hans M. Smid

Abstract

Learning and memory are ubiquitous among insects, and evidence supports the notion that individuals within an insect species exhibit genetically based variation in learning ability. In an ecological context, the learning rate and its underlying memory formation dynamics are expected to depend strongly on associated costs and benefits. Long-term memory (LTM) formation is energetically costly and it is therefore likely that investing in a high learning rate and quick, protein synthesis-dependent LTM consolidation is traded off with lower energetic expenditure on other traits.

The parasitic wasp species *Cotesia glomerata* has an exceptionally high learning rate. In host-plant-odour learning it forms LTM after a single associative conditioning trial, whereas other organisms such as the closely related wasp *C. rubecula* typically need multiple, spaced conditioning trials to do so. The number and type of conditioning trials required for LTM formation are expected to depend on factors such as the reliability of the association that is formed, and is likely to be subject to natural selection. In order to investigate the effect of selection on learning rate and the subsequent formation of consolidated memory, we applied a bidirectional selection regime and reared a decreased-learning line and an increased-learning line of *C. glomerata* wasps which differ significantly in learning rate (chapter 5) and also exhibit differences in LTM consolidation dynamics (chapter 6).

We expected bidirectional selection on learning rate to affect fitness-related parameters such as adult body size, longevity and brain size. Here, we show that longevity is significantly higher in wasps from the decreased-learning line. Moreover, females of the increased-learning line have significantly larger brains than females from the decreased-learning line, while retaining a similar body size. These results suggest that trade-offs occur (possibly brain size vs. longevity) as a result of the bidirectional selection pressure that we applied and that costs associated with a high learning rate are of a constitutive nature.

Introduction

Learning and memory are omnipresent traits among insects, and there is ample evidence that individuals within an insect species exhibit genetically based variation in learning ability (Dukas, 2008). Learning and decision-making can enhance survival in new niches and adaptive zones. This suggests that cognitive traits play an important role in evolutionary change and speciation (Dukas, 2004) and that learning affects fitness (Dukas, 2005; Dukas and Bernays, 2000; Dukas and Duan, 2000; Egas and Sabelis, 2001).

In terms of ecology, both learning rate and memory formation dynamics are expected to strongly depend on the associated costs and benefits (Dukas, 1999; Shettleworth, 1993). Factors such as the total number of lifetime experiences, variability of the environment and reliability of information are thought to influence the balance of costs and benefits of memory formation (Dukas, 1998b; Roitberg et al., 1993; Stephens, 1993). Low learning rates are costly because of suboptimal performance during learning, as shown, e.g., with flower-handling skills in generalist bumblebees compared to closely related specialists (Laverty and Plowright, 1988). High learning rates and subsequent long-term memory (LTM) formation are costly in terms of metabolism, e.g., because of a trade-off between protein synthesis in LTM formation and general maintenance processes in the animal. In this context, it is useful to distinguish between constitutive and operating costs of learning. Constitutive costs have a genetic nature and are paid by individuals that show high learning rates irrespective of whether learning actually takes place. These costs presumably result from developing and maintaining a sensory system and a nervous system which allows for maintenance and repair of memories (Dukas, 1999). Operating costs are paid only when the sensory and nervous systems are effectively used to learn, and these costs presumably reflect the reallocation of metabolic resources to processes involved in memory formation (Burger et al., 2008). Negative correlations between different forms of memory may exist as well, in addition to trade-offs between learning and other traits (Isabel et al., 2004; Mery et al., 2007a).

Costs associated with a high learning rate manifest themselves in many ways, and operating costs of learning are relatively well-studied, e.g., there is evidence for a reduced immunity in mice (Barnard et al., 2006), reduced egg productivity in *Drosophila melanogaster* (Mery and Kawecki, 2004) and reduced starvation and desiccation resistance in *D. melanogaster* (Mery and Kawecki, 2005). Experimental evidence for a genetically based, constitutive cost of learning is beginning to accumulate as well. Examples are reduced larval competitive ability in *D. melanogaster* (Mery and Kawecki, 2003) and shorter lifespans of *D. melanogaster* flies selected for improved learning ability (Burger et al., 2008).

Clearly, many traits that are affected by trade-offs associated with a high learning rate

are highly relevant to the fitness of an animal. Another trait that might be affected is the adult body size of an animal. The hypothetical positive correlation between body size and fitness has been extensively studied in insects, with an emphasis on parasitoids (Burton-Chellew et al., 2007; Edgar, 2006; Ellers and Jervis, 2003; Ellers et al., 1998; Harvey, 2005). An intriguing question is whether a high learning rate is associated with a smaller body size.

When looking at the evolutionary-ecological aspects of memory formation, an interesting angle is provided by ideas on the size of brains or brain structures and the costs of maintenance. It is thought that one way to provide high accuracy in memory storage and retrieval is redundancy, i.e., a 'surplus' presence of certain memories (Dukas, 1999; Glassman, 1987). This redundancy is associated with extra neural tissue that exerts costs because it needs to be maintained even when it is not actually used, i.e., once stored information becomes obsolete (Papaj and Snell-Rood, 2007). Brains that are comparatively large have been associated with success in terms of evolution. In birds and mammals, larger-brained, behaviourally innovative species are more likely to be successful in novel environments than are small-brained, less innovative species (Sol et al., 2008; Sol et al., 2005). Apparently, the fitness gains of large brains may outweigh the costs, although there are limits: brains appear not to scale to large sizes very well (Kaas, 2000).

Relative enlargement of brain structures, rather than enlargement of whole brains, is associated with complex behaviour and cognitive abilities as well. In the context of food acquisition, reliance upon learning and memory is associated with enlargement of the relevant brain centres in both vertebrates and invertebrates (Farris, 2008). For example, food-storing passerine birds specialised memories are reflected in a specialisation of brain anatomy: food-storers have an enlarged hippocampus relative to telencephalon volume and body size (Krebs, 1990). The insect mushroom bodies are higher-order brain centres that are of paramount importance for odour learning and relaying sensory information to other parts of the brain (Davis, 2005; Heisenberg, 2003; Strausfeld et al., 1998) and large mushroom body sizes are correlated to a generalist life history, as shown, e.g., in generalist plant-feeding scarab beetles that must assess and select among many potential host plants (Farris and Roberts, 2005). In a comparative study of European and African honeybees, it was found that brain volumes did not differ significantly, but that European honeybees had larger mushroom bodies. This difference is thought to be correlated to differences in olfactory learning (DeGrandi-Hoffman et al., 2008).

For our research on learning and memory, we use a model system which consists of Dutch strains of the two closely related (Michel-Salzat and Whitfield, 2004) parasitic wasp species, *Cotesia glomerata* and *C. rubecula* (Hymenoptera: Braconidae) which were previously shown to exhibit species-specific differences in learning rate and LTM formation (Bleeker et al., 2006a; Smid et al., 2007); *C. glomerata* forms LTM after a single conditioning trial whereas *C. rubecula* needs multiple trials spaced in time to do so (Smid et al., 2007).

In terms of evolution, natural selection on learning rate can only act on naturally occurring heritable genetic variation in learning and memory. Most of the evidence for the presence of this variation was found in rats and humans (Dukas, 2004) and through selection experiments with insects (Dukas, 2008). We expected variation in learning rate to be present in *C. glomerata* as well. This was confirmed by performing a bidirectional selection experiment with *C. glomerata* in which we succeeded to create a decreased-learning line and an increased-learning line which differ significantly in learning rate (chapter 5). Moreover, the decreased-learning line also changed with respect to memory formation dynamics; wasps from the decreased-learning line did not consolidate LTM after a single conditioning trial, in contrast to their counterparts from the increased-learning line, whereas 24 h memory retention after 3 spaced conditionings was normal (chapter 6). Thus, we succeeded in rearing lines of *C. glomerata*, which show adaptive learning behaviour in an environment where high fitness is linked to a low learning rate. For wasps from the decreased-learning line, reproduction is only possible when more time is spent on the evaluation of new information. Our work therefore provides new evidence for the idea that animals may have tailor-made memories: memories with different underlying consolidation dynamics, depending on the constraints imposed by the ecological niche the animal occupies. Moreover, the results of our selection work imply that such changes can occur within relatively few generations.

In this chapter, we show that wasps from the decreased-learning line have significantly longer lifespans and brains that are small, relative to body size, compared with wasps from the increased-learning line. This implies that bidirectional selection for learning rate in *C. glomerata* is associated with trade-offs that affect constitutive costs.

Materials and methods

Insects

We used individuals from two bidirectionally selected lines of *C. glomerata* parasitic wasps (Hymenoptera: Braconidae). Female wasps were subjected to a selection cycle in which we attached either a fitness penalty (no reproduction) or a fitness reward (reproduction) to a high learning rate, leading to a decreased-learning line and an increased-learning line (chapter 5). The base population originated from individuals collected in cabbage fields in the vicinity of Wageningen, The Netherlands, and were reared on *Pieris brassicae* L. (Lepidoptera: Pieridae), as described previously (Geervliet et al., 1998b). Pieris larvae were reared on Brussels sprouts plants (*Brassica oleracea* var. Gemmifera as described previously (Geervliet et al., 1998b).

Hind tibia length

Hind tibia length can be used as a reliable index of adult body size (Harvey et al., 1994; Soler et al., 2007). We collected wasps from generations 5 and 10 of our selection lines (♀: n = 40 and ♂: n = 40 for each group). We measured hind tibia lengths using an

ocular micrometer and a calibrated slide.

Relative mushroom body calyx size

Brains, consisting of the supra- and suboesophageal ganglia of adult, unconditioned females from generation 10 of the two selection lines were dissected from the head capsule and immediately immersed in GPA fixative (2.5 mL 25% glutaraldehyde + 7.5 mL picric acid + 100 ml 100% acetic acid), overnight at room temperature. Subsequently, the brains were washed in 70% ethanol, dehydrated to pure ethanol, followed by 20 seconds immersion in heptane in order to make the tissue more permeable for subsequent labelling procedures (Breidbach, 1990), and eventually rehydrated to PBS. The brains were then incubated for 20 min. in 1% sodium borohydride in PBS, washed, and incubated for 1 h in collagenase (Sigma-Aldrich cat. # C-5138; 0.5 mg/ml PBS). After this enzymatic treatment the brains were rinsed in three changes of PBS-T (PBS + 0.5% Triton, Sigma-Aldrich Triton® X-100, cat. # T8787), preincubated for 1 h in 1% BSA in PBS-T (PBS-T-BSA), and stained overnight in propidium iodide (Molecular Probes, cat. # P3566, diluted 1:200 in PBS-T-BSA). Thereafter the brains were washed three times in PBS-T and subsequently washed three times in PBS. In order to prepare the brains for confocal microscopy, they were then dehydrated in a graded series of ethanol, cleared in xylene, and mounted on glass microscope slides in DePeX mounting medium (Agar Scientific, cat. # R1340).

All preparations were examined with a confocal laser scanning microscope system (Zeiss LSM 510), equipped with a krypton/argon laser. The 488 nm line of this laser was used for excitation of green background fluorescence and of red propidium iodide. Fluorescent emission of green autofluorescence was captured using a band pass emission filter at 505-530 nm, propidium iodide-stained nuclei was captured using a long pass filter at 585 nm. Images were observed with a 25x oil immersion objective. Stacks of images were collected at a resolution of 1024 x 1024 pixels. Immersion oil and mounting medium both have the same refractive index (nD_{20} = 1.5); therefore no correction was made for Z-axis refractive index mismatch. Further analysis of the confocal image stacks was done with Amira 2.2 (Indeed Visual Concepts GmbH, Berlin; TGS Inc., San Diego). Image segmentation was performed by manually demarcating the brain and mushroom body (MB) calyx contours in each image, resulting in 3D arrays of voxels that were labelled as belonging to either 'brain' or 'mushroom body calyx'. Volumes (in voxels) of the complete brains and the mushroom body calyces were then calculated using the Material Statistics module in Amira, and converted to µL. A total of eight preparations were used for total brain and mushroom body volume calculations; four preparations of the decreased-learning line and four of the increased-learning line. In order to correct for absolute size differences, mushroom body calyx volumes were calculated relative to total brain volume.

Longevity

Adult wasp lifespan was measured in generation 10 of the selection lines, and in one

simultaneous generation of the base population. Cocoons were collected in a Petri dish, stored at 20°C with a L16:D8 photoperiod, and checked daily for emergence of wasps. Males generally emerge first and in quite large numbers during the first days of eclosion. Groups of 25 male wasps from the same day were collected during this time. Only a small number of females emerges during the first one or two days. These were removed, until enough females emerged on a single day to collect 25 wasps. This was done to ensure that all wasps had the same age. All six groups (n = 25 per group, males and females from the base population and the two selection lines) were kept separately in glass cages at 20°C and a photoperiod of L16:D8, and provided with water and honey. We recorded the number of dead wasps in each cage daily. Three replications were performed simultaneously for all groups.

Statistics

Hind tibia length. Hind tibia lengths were used as an index for adult body size. We used non-parametric tests to analyse these data, since they were not normally distributed. First, because parasitic wasps typically have sexually dimorphic body sizes (Hurlbutt, 1987; Mackauer, 1996) we used Mann-Whitney rank sum tests to ascertain whether or not male and female wasps from each line differed in body size (in both the 5th and the 10th generations). We then used Kruskal-Wallis rank sum tests to assess whether there were significant differences between males and between females of the two selection lines (in both generations). Finally, we used rank sum tests to see whether males and/or females from each line had changed in size over generations.

Relative mushroom body calyx size. The absolute volumes of several female wasp brains, and the absolute and relative volumes of mushroom body calyces were calculated in Amira 2.2 (Indeed Visual Concepts GmbH, Berlin; TGS Inc., San Diego). These data were normally distributed and therefore analysed with t-tests, to check for significant differences in volumes between the two selection lines.

Longevity. The adult life span of both selection lines and the base population was analysed using the Kaplan-Meier survival analysis log-rank test, with post hoc Holm-Sidak multiple comparisons to see which groups differed significantly from each other. Male and female wasps were analysed separately. Since the survival of each individual wasp was measured, no cases were censored.

All statistical tests were performed and all graphs were rendered in SigmaPlot 11.0 (Systat Software Inc., San Jose, CA, USA).

Results

Hind tibia length

We measured hind tibia lengths as an index for adult body size (table 1 and figure 1). First, because parasitic wasps typically have sexually dimorphic body sizes (Hurlbutt, 1987; Mackauer, 1996) we wanted to ascertain whether or not female and male wasps differed in body size, within both the 5th and the 10th generation (figure 1). Female wasps from both lines were significantly larger than male wasps from both lines in generation 5 (decreased-learning line: $U = 536$, $p = 0.01$; increased-learning line: $U = 547$, $p = 0.012$). Females and males from both lines did not differ significantly in body size in generation 10, although a trend of sexual dimorphism was present here as well (decreased-learning line: $U = 676$, $p = 0.233$; increased-learning line: $U = 627$, $p = 0.096$). Then we measured whether there was a difference in body size, between males and between females of the two selection lines, within both generations. In generation 5, female body sizes of the decreased-learning line were significantly smaller than those of the increased-learning line ($H = 4.103$, d.f. = 1, $p = 0.043$) whereas male body sizes did not differ significantly between the two lines ($H = 3.595$, d.f. = 1, $p = 0.058$). In generation 10, the situation was reversed: females did not differ significantly in body size ($H = 2.690$, d.f. = 1, $p = 0.101$), while males of the decreased-learning line were significantly larger than those of the increased-learning line ($H = 6.365$, d.f. = 1, $p = 0.012$). Finally, we tested whether males and/or females from each line had changed in size over generations. The body sizes of males and females from the decreased-learning line did not change significantly between generations 5 and 10 (males: $U = 659$, $p = 0.170$; females: $U = 612.5$, $p = 0.070$). The body sizes of males and females of the increased-learning line became significantly smaller between generations 5 and 10 (males: $U = 243$, $p < 0.001$; females: $U = 278.5$, $p < 0.001$).

The overall differences were larger between the two generations than between the selection lines, which suggests an effect of inbreeding.

generation	line	sex	mean HTL ± s.e.m.
5	decreased-learning	m	0.95175 ± 0.006096
5	decreased-learning	f	0.97375 ± 0.004813
5	increased-learning	m	0.96875± 0.005066
5	increased-learning	f	0.9875 ± 0.007132
10	decreased-learning	m	0.93275 ± 0.008923
10	decreased-learning	f	0.944 ± 0.010639
10	increased-learning	m	0.90975 ± 0.007368
10	increased-learning	f	0.92575 ± 0.00874

Table 1. Mean hind tibia lengths (HTL) in mm for the two selection lines and the sexes, in generations 5 and 10.

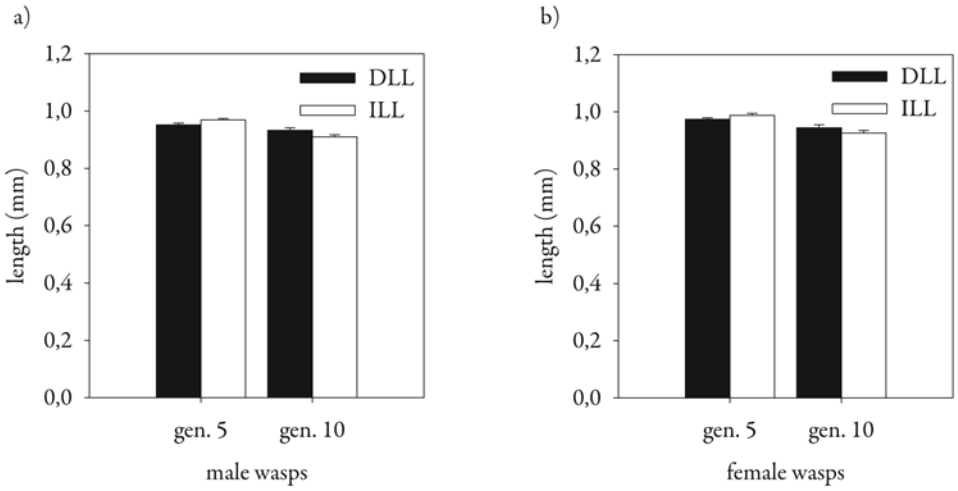


Figure 1. Mean hind tibia lengths in mm (± s.e.m.) from a) male wasps and b) female wasps. Female wasps from each line were significantly larger than male wasps from each line in generation 5 (decreased-learning line [DLL]: U = 536, p = 0.01; increased-learning line [ILL]: U = 547, p = 0.012). Females and males from both lines did not differ significantly in body size in generation 10 (decreased-learning line: U = 676, p = 0.233; increased-learning line: U = 627, p = 0.096). In generation 5, females of the decreased-learning line were significantly smaller than those of the increased-learning line (H = 4.103, d.f. = 1, p = 0.043) whereas male body sizes did not differ significantly between the two lines (H = 3.595, d.f. = 1, p = 0.058). In generation 10, the situation was reversed: females did not differ significantly in body size (H = 2.690, d.f. = 1, p = 0.101), while males of the decreased-learning line were significantly larger than those of the increased-learning line (H = 6.365, d.f. = 1, p = 0.012). Finally, males and females from the decreased-learning line did not change significantly in body size from generation 5 to 10 (males: U = 659, p = 0.170; females: U = 612.5, p = 0.070), whereas males and females from the increased-learning line did (males: U = 243, p < 0.001 ; females: U = 278.5, p < 0.001).

Longevity

We measured adult life span of wasps from the base population and the two selection lines. Male and female wasps were measured separately; all groups were measured in triplicate. The survival analysis with post hoc Holm-Sidak multiple comparisons (table 2) showed that there were significant differences in longevity between the females of the different lines (log-rank statistic = 130.971, d.f. = 2, p < 0.001) and between males of the different lines (log-rank statistic = 74.871, d.f. = 2, p < 0.001) (figure 2). All three female lines differ significantly from each other (p < 0.001 for each comparison) with wasps from the base population living longest and wasps from the increased-learning line living shortest. The mean survival was 24.556 days for females from the base population, 16.843 days for females from the decreased-learning line, and 10.529 days for females from the increased-learning line. For the male wasps, no significant differences were found between the base population and the decreased-learning line (p = 0.215), but both lived significantly longer than the increased-learning line (p < 0.001). The mean survival was 16.507 days for males from the base population, 14.125 days for

males from the decreased-learning line, and 7.313 days for males from the increased-learning line.

The results from this experiment indicate that bidirectional selection on learning rate has a strong effect on the longevity of the selection lines: wasps from the decreased-learning line have a significantly longer lifespan than wasps from the increased-learning line. Both lines have a shorter lifespan than wasps from the base population, which may be due to inbreeding (Henter, 2003) or due to pleiotropic effects of genes that are affected by our selection regime.

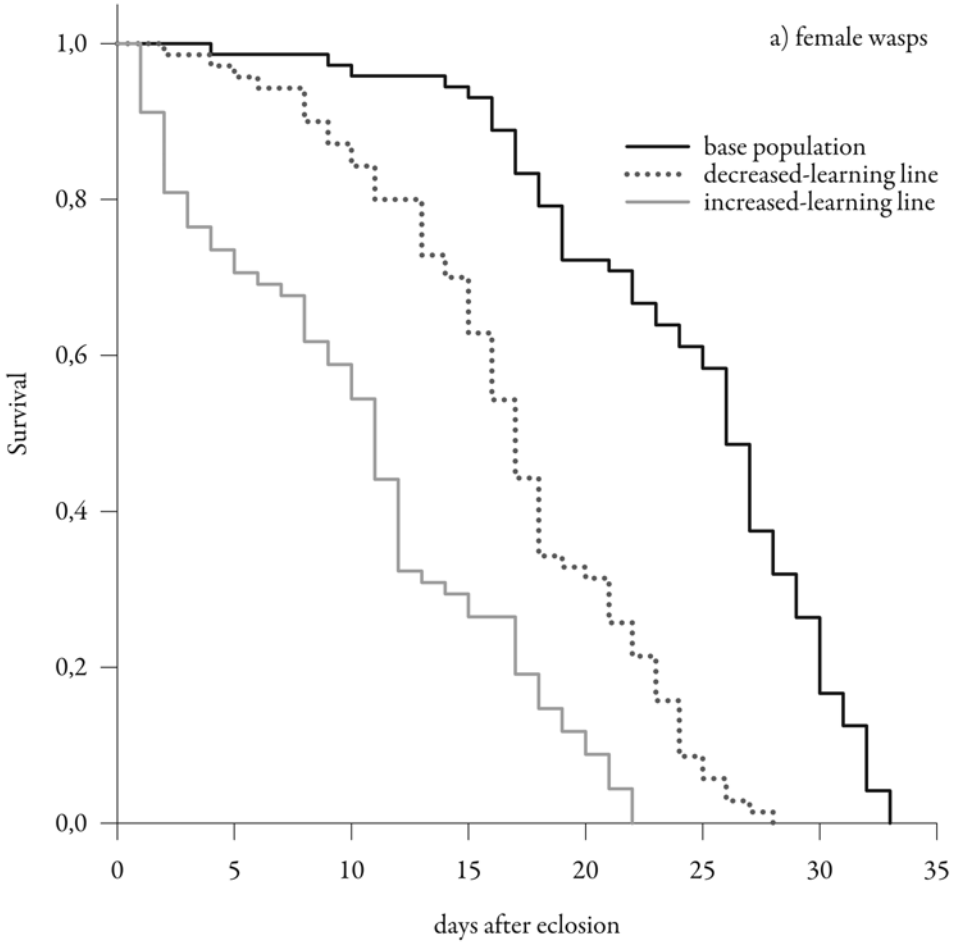


Figure 2a. Kaplan-Meier survival analysis test results. The relative survival of the wasps from the different lines is plotted against the number of days after eclosion from the cocoons. For the female wasps, longevity differs significantly (log-rank statistic = 130.971, d.f. = 2, $p < 0.001$). Post hoc Holm-Sidak multiple comparisons showed that all three lines differ significantly from each other ($p < 0.001$ for each comparison) with wasps from the base population living longest and wasps from the increased-learning line living shortest.

comparisons	sex	statistic	unadjusted p-value	critical level
lines 0-1	female	58.963	1.6×10^{-14} *	0.0253
lines 0-2	female	104.388	1.7×10^{-24} *	0.0170
lines 1-2	female	27.634	1.5×10^{-7} *	0.0500
lines 0-1	male	1.534	0.215	0.0500
lines 0-2	male	66.141	4.2×10^{-16} *	0.0170
lines 1-2	male	35.626	2.4×10^{-9} *	0.0253

Table 2. Pairwise multiple comparisons (Holm-Sidak method) for Kaplan-Meier survival analysis log-rank tests. Overall significance level = 0.05. Lines: 0 is the base population, 1 is the decreased-learning line, 2 is the increased-learning line. Asterisks indicate significant differences.

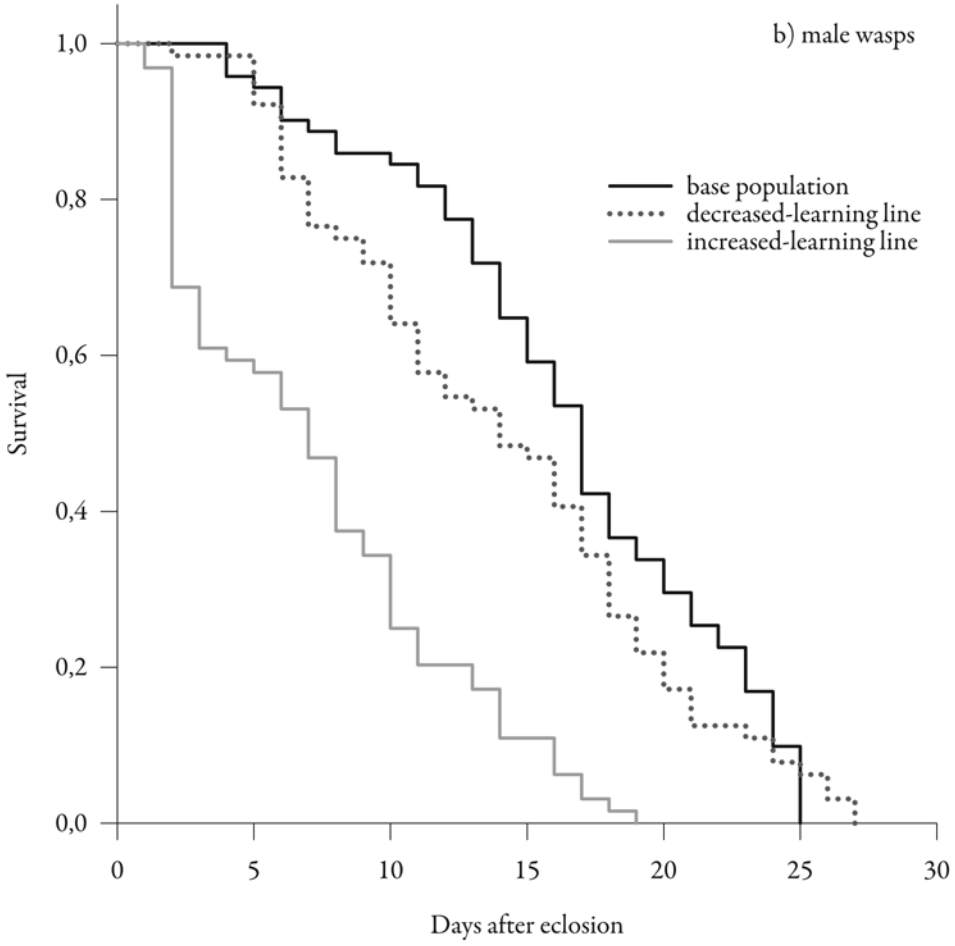


Figure 2b. Kaplan-Meier survival analysis test results. The relative survival of the wasps from the different lines is plotted against the number of days after eclosion from the cocoons. For the male wasps, longevity differs significantly (log-rank statistic = 74.871, d.f. = 2, $p < 0.001$). Post hoc Holm-Sidak multiple comparisons showed that no significant differences were found between the base population and the decreased-learning line ($p = 0.215$), but both lived significantly longer than the increased-learning line ($p < 0.001$).

Brain size and relative mushroom body calyx size

Brains and mushroom body calyces of female wasps were manually demarcated in the confocal image stacks using Amira 2.2 (Indeed Visual Concepts GmbH, Berlin; TGS Inc., San Diego). In this way, three-dimensional reconstructions could be made (figure 3). We calculated the absolute volumes of the brains and the mushroom body calyces, and the volumes of the mushroom body calyces relative to the brains. Wasps from the increased-learning line had significantly larger absolute brain volumes ($t = -5.772$, d.f. = 6, $p = 0.001$) and absolute mushroom body calyx volumes ($t = -3.596$, d.f. = 6, $p = 0.011$), as shown in figure 4. This is especially interesting given the observation that females of generation 10 (from which we analysed the brains) did not differ significantly in body size. Apparently, wasps from the decreased-learning line have smaller brains, relative to body size, than their counterparts from the increased-learning line. The relative mushroom body calyx volumes did not differ significantly between the two selection lines (figure 4c, $t = -0.448$, d.f. = 6, $p = 0.670$).

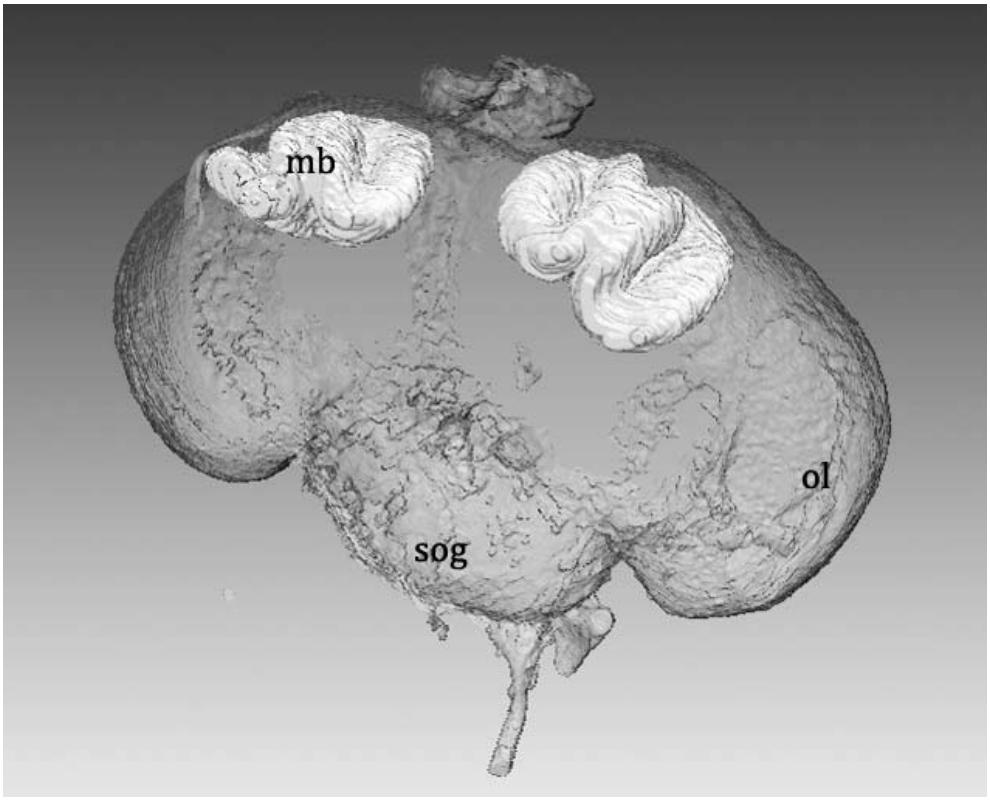


Figure 3. Three-dimensional reconstructions made in Amira 2.2, based on a confocal image stack of a female wasp brain (decreased-learning line). Transparent anterior view of a complete brain with mushroom body calyces visible inside. Abbreviations: mb = mushroom body calyx, ol = optic lobe, sog = suboesophageal ganglion.

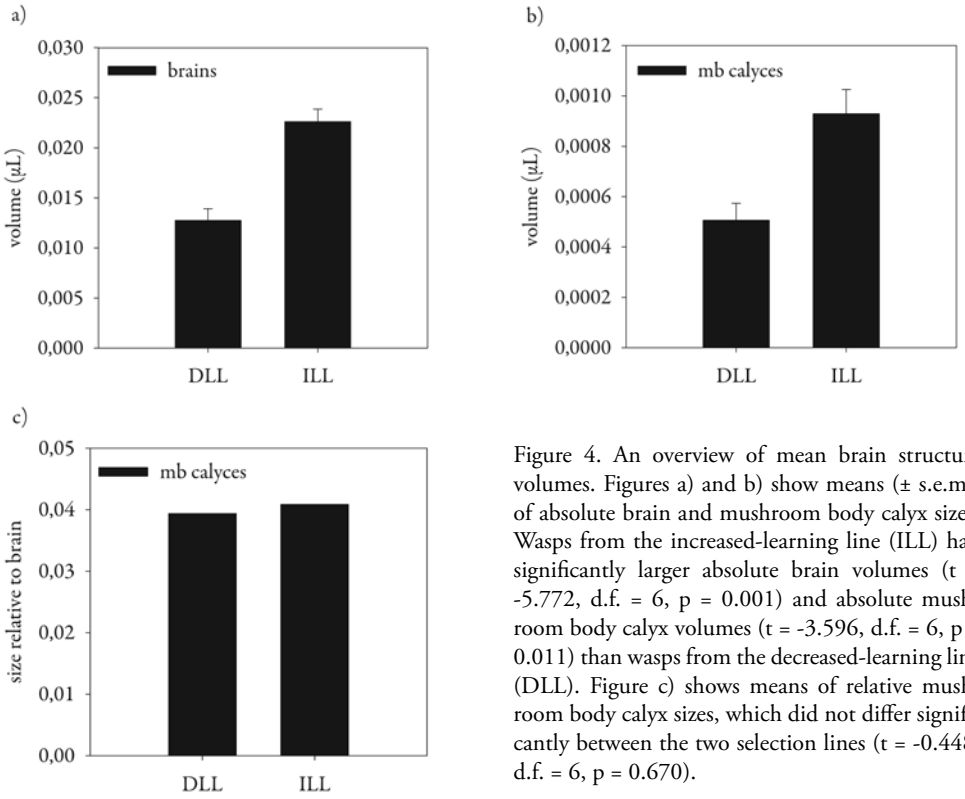


Figure 4. An overview of mean brain structure volumes. Figures a) and b) show means (± s.e.m.) of absolute brain and mushroom body calyx sizes. Wasps from the increased-learning line (ILL) had significantly larger absolute brain volumes (t = -5.772, d.f. = 6, p = 0.001) and absolute mushroom body calyx volumes (t = -3.596, d.f. = 6, p = 0.011) than wasps from the decreased-learning line (DLL). Figure c) shows means of relative mushroom body calyx sizes, which did not differ significantly between the two selection lines (t = -0.448, d.f. = 6, p = 0.670).

Discussion

Our results show that several fitness-related traits are affected by trade-offs associated with changes in learning rate: female wasps from the decreased-learning line have smaller brains, relative to body size, than their counterparts from the increased-learning line while their body sizes do not differ significantly. Furthermore, wasps from the decreased-learning line have significantly longer lifespans than wasps from the increased-learning line. These observations suggest that constitutive costs are associated with an increased learning rate. Our study is one of the first to provide evidence for such constitutive costs (see also Burger et al., 2008; Mery and Kawecki, 2003). And as far as we are aware, this is the first study in which total brain and brain structure sizes are investigated after bidirectional selection on learning rate, in the context of trade-offs associated with a high learning rate.

Adult body size is correlated with fitness in terms of the allocation of limited metabolic resources to different fitness-related physiological functions such as reproduction and survival; a large size enables individual parasitoids both to store more energy reserves (which influences longevity) and to produce more eggs (which influences reproduc-

tive success) than smaller conspecifics (Bezemer et al., 2005). We wanted to know 1) whether constitutive costs associated with a high learning rate influence body size, e.g., does a high learning rate require a larger brain and/or a relatively larger mushroom body, and 2) whether a high learning rate is traded off with a smaller body size? If this is the case, we would have expected wasps from the decreased-learning line to be larger than wasps from the increased-learning line. However, in generation 5, females of the decreased-learning line were significantly smaller than females of the increased-learning line. In generation 10, when the difference in learning rate was expected to be more pronounced than in generation 5 (see chapter 5: the learning rate of the two lines increasingly diverged through generations 3-8), females from the two lines did not differ significantly in body size anymore. There was a trend towards the opposite, however: females from the increased-learning line becoming smaller than their counterparts from the decreased-learning line, but this was not significantly different. Overall, our results show that the differences in body size between the two lines are small, and there is no correlation between learning rate and body size.

Females and males of the decreased-learning line were shown to live significantly longer than females and males of the increased-learning line. This suggests that a high learning rate is indeed associated with constitutive costs, and that a decrease in learning rate upon selection allows for a reallocation of resources to other life-history traits, i.e., a prolonged lifespan. This is in line with the results of Burger et al. (2008), who showed that *D. melanogaster* populations which were selected for improved learning lived on average 15% shorter than unselected control flies (Burger et al., 2008). A major difference between their selection regime and ours, however, is that we also selected for decreased learning, i.e., we used a bidirectional selection regime. Moreover, we used a learning paradigm which mimics the natural circumstances under which evolution of learning rate and the associated costs and benefits in *C. glomerata* is expected to take place. Both of our selection lines had shorter lifespans than wasps from the unselected base population. One obvious cause for this decreased longevity may be inbreeding. Parasitoid haplodiploidy acts as a mechanism to purge deleterious alleles because of the male's haploidy, which exposes recessive alleles to strong selection pressure (Haag-Liautard et al., 2009). Severe effects of inbreeding on longevity were indeed shown to occur in the haplodiploid wasp *Uscana semifumipennis* (Henter, 2003).

We also wanted to know whether trade-offs associated with bidirectional selection for learning rate affected the sizes of *C. glomerata* brains and brain structures that are involved in learning (i.e., the mushroom bodies). We found that, relative to body size, female wasps from the decreased-learning line have significantly smaller brains than females from the increased-learning line. This suggests that the net balance resulting from this trade-off provided wasps from the decreased-learning line with the resources necessary for the significantly longer lifespan that we observed. What is known about the relationship between brain size and cognitive performance, and about brain size in general? There is a large body of literature dealing with the relation between cognitive

performance and brain size, and although it is clearly beyond the scope of this discussion to provide an in-depth review of current ideas on brain size, some interesting points can be made. Equations representing the close correlations between body size and brain size have been established for mammals and reptiles (Martin, 1981) and a similar equation seems to apply to insects as well, as shown by studies of brain allometry in ants and bumblebees (Mares et al., 2005; Wehner et al., 2007). Interestingly, ant brains are relatively smaller (i.e., relative to body size) than brains of a hypothetical ant-sized vertebrate animal would be (Wehner et al., 2007), which may be due to differences in neural density. However, although allometry accounts for most of the variance in brain size, differences remain, which are thought to be related to cognitive advantages of larger brains (Lefebvre and Sol, 2008). This seems to be true for vertebrates, e.g., it has been shown for birds that evolution to a certain extent favours larger brains and larger relevant brain structures, e.g., the hippocampus, to increase memory performance (Sol et al., 2008; Sol et al., 2005; Sol et al., 2007). On the other hand, selection pressure causes changes to occur that depend on the costs associated with brain tissue. Indeed, there are examples of a reduction of brain size when resources are limiting and the demands on neural processing are reduced, e.g., in bats. Bats foraging in open spaces where they can fly fast and have less complex spatial information to process, have reduced brain sizes relative to the ancestral state (Niven, 2005; Safi et al., 2005). Interestingly, the study by Safi et al. (2005) also shows that bats foraging in highly structured habitats that require increased manoeuvrability have increased brain sizes, relative to the ancestral state. This study thus links brain size to the ecological niche which is occupied by the animal. Such neuro-ecological differences have been shown to occur in insects as well. But these appear to manifest themselves in increasing complexity of higher-order processing centres in the brain rather than increases in brain size, as shown by the degree of gyrification (i.e., convolutions similar to those found in vertebrate brain cortex) of the mushroom bodies (Farris, 2008; Farris and Roberts, 2005). Gyrencephalic mushroom bodies with multiple calyces are found in insects with generalist diets (e.g., *Scarites subterraneus* [Coleoptera]), opportunistic invasive lifestyles (e.g., cockroaches, Japanese beetles) or complex societies (e.g., honeybees, termites). In contrast, specialized insects such as flesh flies, ladybirds and dung beetles have mushroom bodies with single calyces (Farris, 2008; Farris and Roberts, 2005). Clearly, different scenarios can account for changes in brain size, and increased cognitive abilities are not necessarily translated into larger brains. The boundaries for the brain size of a given animal seem to be determined by a balance between increased fitness associated with a high learning rate and decreased fitness associated with metabolic expenditure on neural tissue. Allometric growth and/or increasing structural complexity of brain parts rather than whole brains may provide an alternative solution.

In this light, assuming that a high learning rate is indeed associated with a large brain, which our observations suggest, we wanted to know whether the difference in brain size between our *C. glomerata* selection lines is due to changes in overall brain size, or to allometric growth of specific brain structures, such as the mushroom bodies. We did not

find differences in the relative size of the mushroom body calyces, which suggests that bidirectional selection acted upon whole brains rather than on brain structures. Note that this does not exclude the possibility, that divergence in relative mushroom body calyx size between the two lines will occur after conditioning, which would be congruent with the observation of experience-dependent structural plasticity of honeybee mushroom bodies (Fahrbach et al., 2003; Farris et al., 2001).

Since none of the wasps that we used for this study were given conditioning trials, our results indicate that we see an effect of selection on constitutive costs of learning, i.e., on genetic costs that are paid by the individual irrespective of whether the ability to learn is actually used (Burger et al., 2008). Thus far, constitutive costs of learning have only been shown to occur in the form of reduced larval competitive ability for *Drosophila* flies with a high learning rate (Mery and Kawecki, 2003) and in the form of a symmetrical trade-off between learning rate and longevity in *Drosophila* (Burger et al., 2008). We studied trade-offs in the context of a bidirectional selection regime, however, which adds an extra dimension. We were able to investigate the cost-benefit balance from two points of view: an increased learning rate vs. a decreased learning rate.

Summarising, we conclude that:

1) Wasps from the decreased-learning line are 'relieved' from paying constitutive costs that are associated with the high learning rate typical for unselected *C. glomerata*, therefore evolve smaller brains (that are not equipped for LTM consolidation after one conditioning trial), and thus are able to invest resources in an increase of longevity.

2) Wasps from the increased-learning line are effectively selected for paying high constitutive costs (reflected, e.g., in a large brain that is able to rapidly consolidate LTM and perhaps store more memories), which may lead to a decreased lifespan.

Our results with bidirectionally selected *C. glomerata* wasps may provide new evidence to support the hypothesis that selection for changes in learning acts upon genes with antagonistic pleiotropic effects on both learning rate and lifespan. Although such genes have not been identified, various pleiotropic effects have been ascribed to alleles that affect learning ability (Dubnau and Tully, 1998; Mery et al., 2007a) and lifespan (Nuzhdin et al., 1997).

Our *C. glomerata* selection study adds an extra dimension to our ongoing research on learning and memory with *C. glomerata* and *C. rubecula*. It allows to test hypotheses on how tailor-made memories and the underlying memory dynamics evolve under selective pressure on variation in learning rate, and how animals are affected by the associated trade-offs. Some of the possible avenues for future research include: testing whether longevity and body size change directionally over multiple generations, investigating the upper and lower boundaries for brain size relative to body size, and studying the effects

of ageing and single, massed, and spaced conditioning on mushroom body size.

Acknowledgements

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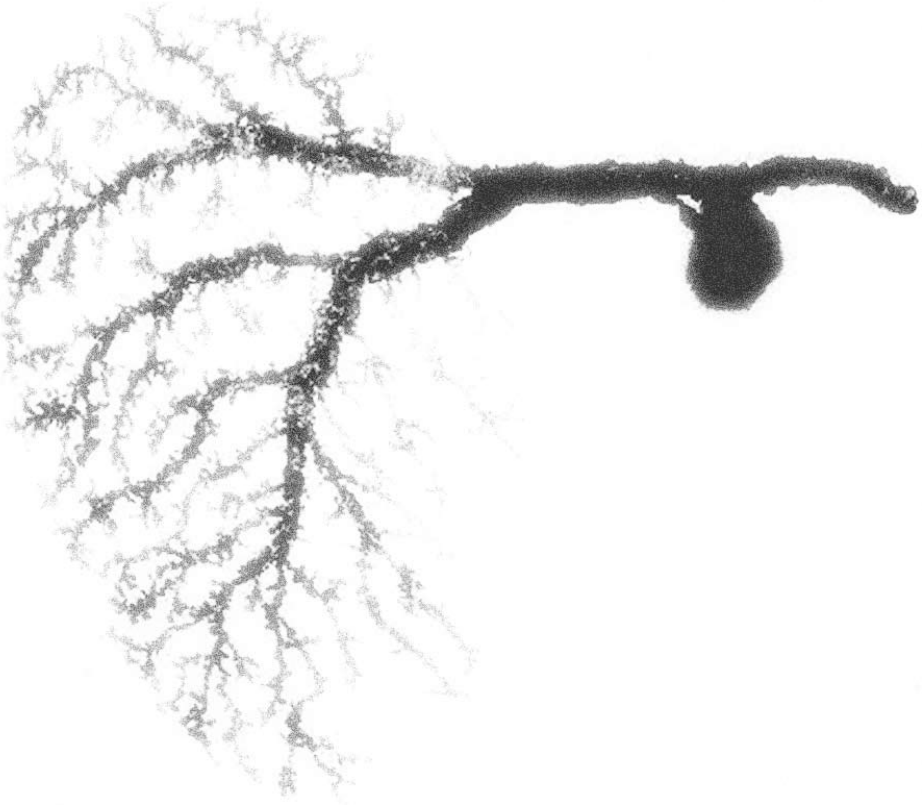
Summarising discussion



Michaël van den Berg

Introduction

It has been well established that memory formation proceeds through a sequence of temporal stages (from labile short-lasting memory to stable long-lasting memory), and that the cellular and molecular substrates underlying learning and memory are very similar in different species (Milner et al., 1998). Implicitly, this fact may lead to the assumption that the progression from short-lasting to long-lasting memory occurs in the same way in every animal. But in practice, the speed with which long-lasting memories are formed, ranges from hours or days and sometimes up to weeks (Menzel, 1999). Given the speed with which neural excitation can lead to gene activation, protein synthesis and translocation, and restructuring of synaptic contacts, all of which are necessary for long-lasting memory (Frey and Frey, 2008), there must be an alternative explanation as for why it takes varying amounts of time for long-lasting memory to consolidate (i.e., become stable). Menzel (1999) argued that the consolidation rate of memories is adapted to the demands and constraints imposed by an animal's ecological niche. This means that slow consolidation is adaptive when the environment is relatively unpredictable, whereas fast consolidation is adaptive when the environment is relatively predictable (Menzel, 1999). The question of how memory phases are adapted to the needs of an animal under natural circumstances has only recently begun to receive some attention, mainly in insects such as fruit flies, bumblebees, and parasitic wasps (Bleeker et al., 2006a; Mery et al., 2007a; Raine and Chittka, 2008; Smid, 2006; Smid et al., 2007).

In line with the ideas of Menzel, I expect molecular and cellular similarities to reflect evolutionary conserved requirements for the learning process itself, whereas species-specific adaptations are likely the deciding parameters for the way in which memory proceeds through different temporal phases (Menzel, 1999). This idea lies at the heart of the concept of tailor-made memories (chapters 1 and 2). A full understanding of learning and memory thus requires knowledge of the proximate mechanisms (i.e., genes, molecules, cells, neural networks), combined with knowledge of the ultimate factors (i.e., ecology and evolutionary history). The questions I asked in this thesis therefore range from the role of gene expression (3 and 4) to behaviour, ecology, and evolution (chapters 5, 6, and 7).

In this final chapter, I reiterate the questions and hypotheses from chapters 1 - 7, I summarise the results, and present ideas for future directions.

CREB and the molecular switch: a look into the mechanisms of learning and memory

The experiments described in chapter 3 represent the first attempt to unravel the mechanisms underlying the hypothetical molecular switch that is thought be responsible for differences in learning rate by means of a specific ratio of CREB activator and inhibitor

isoforms (Tully, 1997; Yin and Tully, 1996). I sequenced and compared the *CREB* gene and its alternatively spliced mRNA transcripts in *C. glomerata* and *C. rubecula*, to quantify the constitutive expression of the total set of *CREB* splice variants in these wasps, and to see whether a specific ratio between splice variants is present in unconditioned females. Subsequently, I studied whether conditioning influences *CREB* expression and relative isoform abundance (chapter 4).

CREB expression levels in unconditioned *C. glomerata* and *C. rubecula* are highly similar but significant differences do occur

Using the candidate gene approach (Fitzpatrick et al., 2005), the *CREB* gene from the two *Cotesia* species was successfully cloned and sequenced. Although the *CgCREB* and *CrCREB* sequences differ at ten nucleotide positions, the predicted amino acid sequences are identical. These *Cotesia* species are phylogenetically relatively closely related to the honeybee, and not surprisingly, the *CgCREB*/*CrCREB* sequences share 85% identity with the *AmCREB* sequence. The overall sequence identity with the homologous *Drosophila* gene *dCREB-2* is only 20%, and the majority of it is accounted for by high identity in the basic region and the ZIP domain of the transcription factor (*Drosophila CREB* seems to be even more different from *CgCREB* and *AmCREB* than mouse/human CREB). Compared to the honeybee and *Drosophila*, most of the amino acid differences in *Cotesia* occur outside the functional domains of the CREB protein. This high degree of conservation within the domains that define the *CREB* family of genes confirms that *CgCREB* and *CrCREB* belong to this family as well.

The next step was to identify the different CREB isoforms and to determine their abundance in the *Cotesia* brains. Nine different *CREB* mRNA transcripts were reverse-transcribed and cloned from the brain tissue of unconditioned *Cotesia* females, all of them resulting from alternative splicing of exons E2, E5, E6, or E8. Alignments to known CREB amino acid sequences showed that exons E2 and E6 are part of the glutamine-rich Q1 and Q2 regions, respectively (Eisenhardt et al., 2006). In mammals, this Q1 region is important for PKA-induced transcriptional activation (Gonzalez et al., 1991). The Q2 region allows basal transcriptional activity through interaction with the transcription initiation complex (Johannessen et al., 2004; Mayr and Montminy, 2001; Mayr et al., 2005). Exons E5 and E8 have not been implicated in the regulation of transcriptional activity, so thus far, it is not known how splicing of these exons affects CREB functionality. *Cotesia* lacks the homologous counterpart of the six-amino acid exon E7, which is found in AmCREB isoform 7 but not in any of the other AmCREB isoforms. Furthermore, in the honeybee, none of the isoforms have exon E6 spliced out, whereas I found three of such isoforms (Cg/CrCREB6, 7 and 9) in *Cotesia*. This is an interesting difference, since E6 encodes the Q2 region. On the other hand, none of the nine isoforms in Cotesia has exon E3 (part of Q1) spliced out, whereas this occurs in one of the isoforms (AmCREB6) of the honeybee. In summary, I found several splice variants that lack parts of functional domains, and it is likely that they either have impaired activating functionality, or act as repressors. More research is needed to address this (see

below: 'future perspectives').

Quantitatively, over 99% of the *CREB* splice variants in the heads of both wasps consisted of two clusters of splice variants, namely cluster 1+2, and 3+5 (which could not be measured separately due to primer design restrictions). Splice variants 1 and 2 are the ones with the least exons spliced out and thus code for the most intact proteins. Previous research shows that the most intact CREB isoforms generally act as activators in CREB-dependent transcription (Bartsch et al., 1995; Poels and Broeck, 2004; Yin et al., 1995b). Intuitively, this makes sense because alternative splicing can lead to loss of functional domains and changes in protein folding (Tress et al., 2007). Moreover, our quantification showed consistently lower levels of mRNA transcripts coding for isoforms 1 and/or 2 than coding for isoforms 3 and/or 5. I therefore postulated isoforms 1 and/or 2 to act as activators, and isoforms 3 and/or 5 (and possibly the other, less abundant isoforms as well) to act as repressors (chapter 3). This is congruent with e.g. findings in the pond snail L. stagnalis, where mRNA coding for the activator LymCREB1 is only found in some neurons, while repressor LymCREB2 mRNA is found in larger concentrations throughout the nervous system (Sadamoto et al., 2004a; Sadamoto et al., 2004b).

The relative abundance of the nine identified splice variants is highly similar in both *C. glomerata* and *C. rubecula*, with significant differences only for splice variants 4 and 9. These two splice variants are present in minor quantities, but since the spatial distribution of CREB isoforms in the nervous system is likely to play a role in LTM formation, these differences may be biologically relevant. It is very well possible that a molecular switch is activated only in subsets of neurons that are specifically involved in LTM formation and consolidation rather than in the complete brain. For example, the activator LymCREB1 was shown to be expressed in identifiable cells implicated in LTM of conditioned taste aversion in L. stagnalis (Sadamoto et al., 2004a; Sadamoto et al., 2004b). The *CREB*-related *CREM* gene encodes alternatively spliced activators and repressors with characteristic expression patterns throughout the rat brain (Mellström et al., 1993). Such patterning may imply either local *de novo* synthesis of CREB, or a molecular switch that is locally active due to spatial differences in the distribution of constitutively expressed CREB isoforms. In both scenarios, the number of CREB molecules that is actually involved in LTM formation may be obscured by the total amount of CREB that is present in the brain and its surrounding tissues.

Alternatively, the fact that most of the identified splice variants in *C. glomerata* and *C. rubecula* are expressed in a similar manner in unconditioned animals of both species suggests that the molecular switch is not defined by the ratio of constitutively expressed activators and repressors. Rather, the conditioning process itself may induce a shift in the relative abundance of specific isoforms. In the pond snail *L. stagnalis*, newly synthesized CREB proteins were shown to be necessary for synaptic enhancement involved in memory consolidation (Wagatsuma et al., 2006), so I pursued the question whether conditioning induces changes in *CREB* expression in chapter 4.

CREB expression changes after conditioning in *C. glomerata*, but not in *C. rubecula*

Single-trial-conditioning in the 'fast-learning' species *C. glomerata* is followed by a significant change in the relative abundance of the major *CREB* splice variants shortly after conditioning (chapter 4). In the 'slow-learning' species *C. rubecula*, *CREB* transcript levels roughly followed the same expression pattern after single trial conditioning as in *C. glomerata*, but the changes I observed were not significant. Spaced trial conditioning did not significantly affect the relative abundance of *CREB* transcripts in either species over time. The results from the single-trial-conditioning experiments suggest that the difference in LTM formation between *C. glomerata* and *C. rubecula* is at least partially regulated at the level of *CREB* mRNA. It is known that *C. glomerata* typically forms LTM after a single conditioning trial, whereas *C. rubecula* needs three spaced trainings to do so (Smid et al., 2007). The fact that the abundance of the major splice variants changes transiently and significantly immediately upon conditioning in *C. glomerata* but does not change significantly in *C. rubecula*, and the significance of the factor species in the statistical analysis of the data seem to be congruent with these differences in LTM formation. More experiments and sufficient replicates are needed, however, to provide conclusive evidence concerning the difference between the two species.

Both *C. glomerata* and *C. rubecula* form LTM after three spaced conditioning trials. In *C. rubecula*, however, the process of consolidation takes much longer: 2-3 days as opposed to 4 hours in *C. glomerata* (Smid et al., 2007). The consolidation process is expected to be initiated by activated CREB proteins, which are either already present, or first need to be synthesized from mRNA templates. Our *CREB* mRNA level measurements after conditioning did not show significant changes over time after three spaced trials. This implies that the crucial translation events (and the accompanying 'dip' in mRNA coding for isoforms 1 and 2) occur in the time window between the first and the third trial, which makes sense since the first 'spaced' trial is exactly the same as the single-conditioning trial. The subsequent repetitions may enhance the effect, but changes in mRNA levels in individual wasps during spaced trial conditioning cannot be quantified, as mRNA collection requires destructive sampling.

I observed a decrease in the amount of mRNA coding for CgCREB isoforms 1 and 2 immediately after conditioning, rather than an increase. This seemingly contradicts our hypothesis that isoforms 1 and/or 2 act as transcriptional activators in *C. glomerata* and *C. rubecula* (chapter 3). One explanation is that *CREB* mRNA (whether newly synthesized or already present) is translated into proteins immediately upon a conditioning experience and subsequently degrades rapidly. This would mean that, although the concentration of mRNA decreases, the concentration of CREB isoform 1 and/or 2 proteins actually increases immediately after conditioning. The mechanisms of mRNA degradation are well understood (Garneau et al., 2007; Houseley and Tollervey, 2009; Valencia-Sanchez et al., 2006) and indeed, the transient activity of transcription factors such as CREB is thought to be linked to mRNA stability. Rapid mRNA degradation

provides an efficient mechanism for transient protein expression because it links protein synthesis directly to the gene transcription rate (Calkhoven and Ab, 1996; Tourrière et al., 2002).

***CREB* expression analysis: a quantitative approach**

Commonly, research on the abundance of CREB in the nervous system is concerned with either changes in the concentration of phosphorylated CREB (pCREB) (Ahmed and Frey, 2005; Moncada and Viola, 2006; Yuan et al., 2003), or with artificially induced changes in CREB levels (Brightwell et al., 2007; Josselyn et al., 2001; Yin et al., 1995a). I have chosen to quantify changes in *CREB* mRNA levels by performing real-time qPCR analyses of cDNA samples. This gave us the opportunity to accurately quantify the changes in *CREB* transcript concentrations, rather than, e.g., comparing Western blot band densities of pCREB, which gives approximations of concentration differences at best. If the molecular switch is defined by an isoform ratio, such a quantitative approach is desirable. Moreover, this method also enabled us to analyze *CREB* expression under natural circumstances, as opposed to having to resort to complex transgene approaches in which *CREB* is artificially overexpressed.

The studies by Ahmed and Frey (2005), Moncada and Viola (2006), Brightwell et al. (2007), Josselyn et al. (2001) and others have shown that artificially increased levels of CREB and pCREB in the nervous system facilitate LTM formation. At present it is unclear whether this facilitation acts through the phosphorylation of CREB that is already present, or whether it depends on the phosphorylation of *de novo* synthesized CREB (which in turn may be translated from newly transcribed mRNA or from mRNA that is already present). It has been demonstrated in the pond snail *L. stagnalis* that newly synthesized CREB is required for synaptic enhancement involved in memory consolidation (Wagatsuma et al., 2006). If *de novo* synthesis of CREB occurs, the short time window between conditioning and the transcription of downstream genes under the influence of CREB also needs to accommodate a *CREB* mRNA translation phase. Our approach of quantifying *CREB* mRNA levels instead of CREB protein levels, has contributed to our understanding of what happens before CREB proteins are phosphorylated and become transcriptionally active.

From conserved mechanisms to modulation by ecological factors

From the molecular approach of chapters 3 and 4, I moved on to a more evolutionary-ecological way of looking at learning and memory formation. Heritable, intraspecific variation is the source for adaptive radiation upon natural selection. Variation in learning rate does not only occur between species, but also within species. As such it may provide clues as to how fitness penalties associated with environmental circumstances can give rise to tailor-made memories. This concept formed the framework for the experiments described in chapters 5, 6 and 7, that addressed whether variation in learning

rate within a *Cotesia* species could be subjected to bidirectional selection (i.e., to derive 'slow' and 'fast' learning lines), and if so, how memory consolidation dynamics would change in these lines. Moreover, I wanted to test whether a high learning rate is traded off with other traits.

Variation in learning rate within a species: what can it tell us?

Natural selection must act on naturally occurring heritable genetic variation for learning and memory. Evidence for the presence of this variation was found, among others, through bidirectional selection experiments with insects that were selected for either 'poor' or 'good' learning (Brandes et al., 1988; Lofdahl et al., 1992; McGuire and Hirsch, 1977). More recently, the presence of heritable variation in learning rate within a species was demonstrated by selection for improved associative learning of oviposition substrate choice in *Drosophila* (Mery and Kawecki, 2002). We observed the occurrence of natural variation in learning rate within populations of *C. glomerata*. Therefore, successful bidirectional selection for increased and decreased learning rates within this typically fast-learning species would 1) prove that the observed variation is indeed heritable, and 2) give us the opportunity to study whether and how these fitness constraints influence memory consolidation dynamics in this wasp species. Furthermore, I was curious to see whether memory dynamics would change upon selection, e.g., whether a low-cost consolidated memory trace (such as ARM) would arise in the decreased-learning line, and if so how long it would last, how long the ASM phase lasts, and whether LTM and ARM are coexisting or mutually exclusive (chapter 6). These results could then be compared with the previous results of Smid et al. (2007), who showed that *C. glomerata* typically consolidates LTM after 4 h and appears not to have an anaesthesia-resistant memory (ARM) trace. *Cotesia rubecula* on the other hand, needs 2-3 days to consolidate LTM, and displays an ARM-like memory trace, which coexists with LTM during at least 48 hours. Moreover, such selection lines could be used to quantify costs and benefits associated with high and low learning rates (chapter 7).

Bidirectional selection on learning rate: decreased-learning vs. increased-learning

In order to rear two distinct lines of *C. glomerata* that differ in learning rate I used a bidirectional selection protocol (chapter 5). In this protocol, I attached either a fitness penalty or a fitness reward to a high learning rate. For propagation of the decreased-learning line I used wasps that did not show 24 h memory retention after a single conditioning trial, but that did display the more common learning characteristics of 24 h memory retention after three spaced trials. For propagation of the increased-learning line I used wasps that showed 'immediate' 24 h memory retention after a single conditioning trial. Using this bidirectional selection regime, I obtained two lines that differed significantly in single-trial-induced LTM within a few generations (chapter 5). These results are in line with earlier observations of heritable naturally occurring variation in learning rate (Brandes, 1988; Brandes et al., 1988; Lofdahl et al., 1992). Divergence in learning rate in the decreased-learning line compared to the increased-learning line became apparent

from generation three onwards. This is comparable to honeybees, in which the effect of bidirectional selection is established within 1-2 parthenogenetic generations (Brandes et al., 1988). Selected *Drosophila* lines started to diverge within 5-10 generations (Lofdahl et al., 1992; Mery and Kawecki, 2002).

Changes in memory dynamics upon bidirectional selection

Bidirectional selection for learning performance in a *C. glomerata* population yielded two lines which differed significantly in 24 h memory retention after a single conditioning trial (chapter 5). I then wanted to know whether these lines had also diverged in terms of memory dynamics and asked the following questions: 1) how long does the ASM phase last, 2) how fast does LTM consolidate, and 3) is there evidence for an ARM-like memory trace? The temporal progression through the different memory phases is typically analysed using anaesthetic treatments to interfere with short-term memory (Erber, 1976; Fulton et al., 2008; Quinn and Dudai, 1976; Tempel et al., 1983; Xia et al., 1998b), and using protein synthesis inhibitors for long-term memory (Alberini, 2008, 2009; Hernandez and Abel, 2008). I used cooling-induced retrograde amnesia to gain insight in the anaesthesia-sensitive short-term memory time window, and I used treatments with the translation inhibitor anisomycin (ANI) to test the progress of protein synthesis-dependent memory consolidation.

I found that LTM had not yet consolidated after 4 h in the decreased-learning line, in contrast to LTM in the increased-learning line, which was fully consolidated at that time. The latter thus exhibits the high learning rate typical for natural *C. glomerata* populations (Smid et al., 2007). These data suggest that the wasps from the decreased-learning line were not capable of consolidating LTM anymore; in the control experiment with sucrose-fed wasps memory retention starts to wane gradually immediately after the conditioning trial and returns to control levels within a few hours. These observations are in line with the results of Smid et al. (2007), where *C. glomerata* has consolidated LTM after 4 h and the slower-learning *C. rubecula* has not. In *C. rubecula*, memory retention levels do not decrease after 4 h however, whereas by then, they have returned to control levels in the decreased-learning *C. glomerata* line. Memory retention in *C. rubecula* 4 h after single-trial conditioning presumably reflects ARM, since ARM was shown to be induced after a single trial in *Drosophila* as well (Tully et al., 1994).

Literature suggests that memory retention immediately after conditioning represents ASM. Due to ambiguous controls, we could not prove that this is the case in the decreased-learning line, but we nevertheless assume that ASM is present. Such anaesthesia-sensitive memory traces are typically short-lived, e.g., < 1 h in *Drosophila*, (Tully, 1994; Tully et al., 1990) and indeed, after one hour, this memory starts to wane in *C. glomerata* and eventually reaches control levels. The presence of an ARM trace in the decreased-learning line would be reflected in above-control levels of memory retention when cooling takes place at time intervals outside the ASM phase. The results from the cooling treatments thus concur with those of the ANI treatments. Cooling of wasps from the

increased-learning line 3 h after conditioning still has an effect, which is in line with what we observed in unselected wasps. If consolidation occurred via an intermediate ARM phase, this ARM phase would then have to exist for less than 1 hour, since 4 h memory can be obliterated entirely by ANI and thus consists of 100% LTM. This is very short, compared to ARM after single-trial conditioning in *C. rubecula*, where it lasts at least 4 h (Smid et al., 2007) or *Drosophila*, where it lasts up to 1 day (Margulies et al., 2005; Tully et al., 1994). Additionally, the ASM phase in both the increased-learning line and in unselected wasps would then be exceptionally long compared with results from *Drosophila* (Tully et al., 1994) or *A. mellifera* (Erber et al., 1980). Nevertheless, longer ASM traces (up to 90-120 minutes) have been described for *Drosophila* (Tempel et al., 1983), using a classical conditioning protocol with consecutive appetitive and electroshock reinforcement. Tempel et al. (1983) showed that the duration of the ASM phase is different, depending on the type of reinforcement that was used. In line with this, the long ASM phase that I found may be a specific feature of oviposition-reward learning.

Absence of ARM as a constitutive cost for a high learning rate

The results of Smid et al. (2007) imply the existence of an ARM-like memory trace in *C. rubecula*. In order to investigate whether the occurrence of ARM is correlated to a 'low' learning rate, I wanted to know whether an ARM trace would arise in the decreased-learning line as a consequence of the selection regime. Moreover, I wanted to ascertain whether ARM is present in the increased-learning line and the unselected wasps. This appeared not to be the case. I found that the ASM phase lasted comparatively long and was followed by consolidated LTM in the increased-learning line and the unselected wasps. In contrast, in the decreased-learning line, putative ASM gradually waned to control levels but was not followed by LTM. A low learning rate (like in *C. rubecula*) apparently does not automatically translate into the feature of consolidating low-cost ARM instead of LTM. In terms of the *Drosophila* dispute (coexisting vs. mutually exclusive ARM and LTM), our current results do not favour either concept over the other. Both scenarios represent feasible ways to consolidate memory, and as such may represent adaptations to an animal's behavioural ecology. Our results confirm that in evolutionary-ecological terms, the foraging behaviour of *C. glomerata* combined with the oviposition behaviour of its host has led to a genetically hardwired tendency to form protein synthesis-dependent LTM rather than low-cost ARM. In fact, I conclude that this feature of *C. glomerata* is a constitutive cost that the animal pays for its high learning rate, as it appears that *C. glomerata* has lost its ability to form ARM under conditions where such low-cost consolidated memory would be favourable. The latter would be the case, for example, in *C. glomerata* wasps that use *P. rapae* as their primary host rather than *P. brassicae*. Caterpillars of this species are unpredictably distributed over plant species in the field, and hence the predictive value of a single oviposition experience in *P. rapae* is lower than it is for *P. brassicae*. Japanese *C. glomerata* do not encounter *P. brassicae* in the field and seem to have specialised on *P. rapae*, as they show a preference shift towards this host after a single conditioning trial (Bleeker, 2005).

Learning rate trade-offs: smaller brains and longer lifespans

Learning as a trait is thought to be costly, and such costs may manifest themselves in different ways. Therefore, I wanted to know whether the high learning rate in *C. glomerata* is associated with other (types of) costs than the absence of ability to form ARM. Again, the availability of the two selection lines enabled us to test this. For chapter 7, I measured longevity, adult body size, brain size, and mushroom body size in both lines. Differences between the two lines could then be interpreted in terms of costs associated with building and maintaining a nervous system that is capable of quick consolidation of long-lasting memory.

Females and males of the decreased-learning line were shown to live significantly longer than their counterparts from the increased-learning line. Clearly, a high learning rate is associated with constitutive costs, and a decrease in learning rate upon selection allows for a reallocation of resources to other life-history traits, such as a prolonged lifespan. This is in line with the results of Burger et al. (2008), who showed that *Drosophila* populations that were selected for improved learning lived on average 15% shorter than unselected control flies (Burger et al., 2008). A major difference between their selection regime and ours, however, is that I also selected for decreased learning, i.e., I used a bidirectional selection regime. Moreover, I used a learning paradigm that mimics the natural circumstances under which evolution of learning rate and the associated costs and benefits in *C. glomerata* is expected to take place.

Possibly, trade-offs associated with bidirectional selection for learning rate could affect the sizes of *C. glomerata* brains and brain structures that are involved in learning (e.g., the mushroom bodies). Equations representing the close correlations between body size and brain size have been established for mammals and reptiles (Martin, 1981) and a similar equation seems to apply to insects as well, as shown by studies of brain allometry in ants and bumblebees (Mares et al., 2005; Wehner et al., 2007).Therefore, I first tested whether there were differences in body size between the two lines, and over several generations. Adult body size is correlated with fitness in terms of the allocation of limited metabolic resources to different fitness-related physiological functions such as reproduction and survival; a large size enables individual parasitoids both to store more energy reserves (which influences longevity) and to produce more eggs (which influences reproductive success) than smaller conspecifics (Bezemer et al., 2005). Our results showed that any differences in body size between the two lines and over several generations are small, and there is no correlation between learning rate and body size (chapter 7).

I did find, however, that female wasps from the decreased-learning line have significantly smaller brains (relative to body size) than females from the increased-learning line. Cognitive advantages of larger brains may account for part of the observed brain size variance (Lefebvre and Sol, 2008). This seems to be true for vertebrates, e.g., it has been

shown for birds that evolution to a certain extent favours larger brains and larger relevant brain structures (such as the hippocampus), to increase memory performance (Sol et al., 2008; Sol et al., 2005; Sol et al., 2007). On the other hand, costs are associated with larger amounts of brain tissue. Brains may get smaller when resources are limiting and the demands on neural processing are reduced. For example, bats foraging in open spaces where they can fly fast and have less complex spatial information to process, have reduced brain sizes relative to the ancestral state (Niven, 2005; Safi et al., 2005).

Interestingly, the study by Safi et al. (2005) also shows that bats foraging in highly structured habitats that require increased manoeuvrability have increased brain sizes, relative to the ancestral state. This study thus links brain size to the ecological niche that is occupied by the animal. Such neuro-ecological differences have been shown to occur in insects as well. They are represented in an increasing complexity of higher-order processing centres in the brain rather than increases in brain size, as shown by the degree of gyrification (i.e., convolutions similar to those found in vertebrate brain cortex) of the mushroom bodies (Farris, 2008; Farris and Roberts, 2005). Gyrencephalic mushroom bodies with multiple calyces are found in insects with generalist diets (e.g., *Scarites subterraneus* [Coleoptera]), opportunistic invasive lifestyles (e.g., cockroaches, Japanese beetles) or complex societies (e.g., honeybees, termites). In contrast, specialized insects such as flesh flies, ladybirds and dung beetles have mushroom bodies with single calyces (Farris, 2008; Farris and Roberts, 2005). Clearly, different scenarios can account for changes in brain size, and increased cognitive abilities are not necessarily translated into larger brains. The boundaries for the brain size of a given animal seem to be determined by a balance between increased fitness associated with a high learning rate and decreased fitness associated with metabolic expenditure on neural tissue. Allometric growth and/or increasing structural complexity of brain parts rather than whole brains may provide an alternative solution.

In this light, assuming that a high learning rate is indeed associated with a large brain, which our observations suggest, I wanted to know whether the difference in brain size between our *C. glomerata* selection lines is due to changes in overall brain size, or to allometric growth of specific brain structures, such as the mushroom bodies. I did not find differences in the relative size of the mushroom body calyces, which suggests that bidirectional selection acted upon whole brains rather than on brain structures. Note that this does not exclude the possibility that divergence in relative mushroom body calyx size between the two lines will occur after conditioning, which would be congruent with the observation of experience-dependent structural plasticity of honeybee mushroom bodies (Fahrbach et al., 2003; Farris et al., 2001).

Since none of the wasps that I used for this study were given conditioning trials, our results indicate that we see an effect of selection on constitutive costs of learning, i.e., on genetic costs that are paid by the individual irrespective of whether the ability to learn is actually used (Burger et al., 2008). Thus far, constitutive costs of learning have only

been shown to occur in the form of reduced larval competitive ability for *Drosophila* flies with a high learning rate (Mery and Kawecki, 2003) and in the form of a symmetrical trade-off between learning rate and longevity in *Drosophila* (Burger et al., 2008).

I thus conclude that wasps from the decreased-learning line are 'relieved' from paying constitutive costs that are associated with the high learning rate typical for unselected *C. glomerata*, therefore evolve smaller brains (that are not equipped for LTM consolidation after one conditioning trial), and thus are able to invest resources in an increase of longevity. Furthermore, wasps from the increased-learning line are effectively selected for paying high constitutive costs (reflected, e.g., in a large brain that is able to rapidly consolidate LTM and perhaps store more information in the form of memory), which may lead to a decreased lifespan. Ours is one of the first studies to provide evidence for constitutive costs associated with a high learning rate (see also Burger et al., 2008; Mery and Kawecki, 2003). And as far as I am aware, this is the first time that brain sizes and brain structure sizes are investigated after bidirectional selection on learning rate, in the context of trade-offs associated with a high learning rate.

Future perspectives

The role of CREB is pivotal for LTM formation. The activity of CREB as a transcription factor, however, is obviously part of a longer chain of events. There are many steps between the perception of unconditioned and conditioned stimuli and the consolidation of long-lasting memory. In order to elucidate how conserved molecular and cellular mechanisms give rise to the species-specific differences in learning and memory formation between *C. glomerata* and *C. rubecula*, a systematic 'dissection' of these mechanisms is necessary. To this end, our group has previously investigated the olfactory receptive range (Smid et al., 2002), the antennal morphology (Bleeker et al., 2004), the organisation and anatomy of the antennal lobes (Smid et al., 2003), and the presence and distribution of octopaminergic neurons (Bleeker et al., 2006b) in *C. glomerata* and *C. rubecula*. The work on CREB described in this thesis (chapters 3 and 4) contributes to this ongoing comparative analysis by providing new clues on where in the chain of events the differences in learning and memory between the two wasps arise. I have shown that learning can induce changes in the relative abundance of *CREB* splice variants, a process which could underlie the hypothetical molecular switch. Moreover, cloning and sequencing of the *CREB* gene and its alternatively spliced transcripts has provided us with tools for the investigation of differences in local expression: we can use the sequence data of the different mRNA splice variants to generate probes for in situ hybridisation. And finally, our work has shown that the candidate gene approach (Fitzpatrick et al., 2005) is a fruitful one, which should encourage the future identification of other genes involved in learning and memory.

More 'how': comparisons at the molecular and cellular level

One of the questions that remains to be addressed is whether species-specific differences in learning and memory result from modulation of *CREB* transcription and/or translation. It is well-established that translational control is important for regulating the synthesis of specific proteins in response to neuronal activity (Costa-Mattioli et al., 2009). In eukaryotes, translation initiation is a complex process catalysed by at least twelve different initiation factors (eIFs), and it has been shown that, e.g., the phosphorylation of eIF2α inhibits general translation but selectively stimulates translation of ATF4, a repressor of CREB-mediated LTM in mice (Costa-Mattioli et al., 2007; Costa-Mattioli et al., 2009). Insect homologues of eIFs have been identified, and it should be worth investigating their role in learning and memory in *Cotesia*.

Although CREB is a key player in LTM formation, it does not act in isolation. The candidate gene approach that I used for the identification of *CgCREB* and *CrCREB* could also be used for the identification of, e.g., some of the immediate early genes (IEGs) that are activated by CREB. One example of such an IEG is the *Arc* gene (activity-regulated cytoskeleton-associated protein), which has received particular attention because of its tight experience-dependent regulation in neural networks (reviewed in Miyashita et al., 2008). Although most of the knowledge of IEGs comes from work with vertebrates, insect examples exist, e.g., *Drosophila* homologues of the *Arc* gene have been identified (Mattaliano et al., 2007). One could imagine that genes downstream of *CREB*, which take part in the structural changes accompanying synaptic plasticity, are potential links in the chain from stimulus to memory where species-specific differences arise. Similarly, genes upstream of *CREB* have been shown to regulate memory formation efficacy by acting as 'memory suppressors': for example, the *A. californica* gene *ApCREB2* can repress *ApCREB1*-mediated transcription. This suggests that the threshold for long-lasting memory can be regulated, and requires not only the activation of ApCREB1, but also the relief of ApCREB2-mediated repression (Abel et al., 1998). Another suppressor gene is protein phosphatase 1 (PP1) (Genoux et al., 2002).

Although it is widely accepted that new memories are encoded as neural activity-induced changes in synaptic efficacy, which require *de novo* protein synthesis (Miyashita et al., 2008), the processes that allocate individual memories to specific neurons have remained elusive (Won and Silva, 2008). From several recent studies (reviewed in Won and Silva, 2008), a picture is now emerging that shows how memory allocation in neural networks may be modulated. For example, levels of CREB can affect the probability of a neuron to be recruited into a given memory representation (Han et al., 2007; Won and Silva, 2008). Future studies addressing the identification of genes involved in learning and memory in *Cotesia* and other insects should take these exciting developments into account. Moreover, CREB activation triggers an autoinhibitory feedback loop, which is a process that could be used to allocate memories to cells other than those that have been recently involved in memory. The identification of IEGs may be very valuable in this context as well; a gene such as *Arc* can be visualised by fluorescence in situ hybridisation (FISH). Neuronal activity induces a rapid and transient increase in *Arc* transcription,

and thus nuclear-localized *Arc* mRNA can serve as a molecular signature of a recently active neuron (Guzowski et al., 1999). *Cotesia* brains can easily be dissected at appropriate times after conditioning and are suitable for immunofluorescence studies and confocal imaging (chapter 7, Bleeker et al., 2006b). The availability of sequence data of genes putatively involved in synaptic changes could potentially provide a wealth of information on how gene expression and competition in neural networks acts in the way memories are formed and allocated.

In the cricket *Gryllus bimaculatus*, olfactory memory was impaired through RNA interference (RNAi) acting on the nitric oxide synthase (NOS) gene (Takahashi et al., 2009). This study showed that in insects, an injection of double-stranded RNA (dsRNA) in the hemolymph suffices to do deliver these molecules to the brain. Clearly, the sequence data that I obtained for the *Cotesia CREB* genes and their splice variants provides a great opportunity to use RNAi and test whether a specific splice variant acts as an activator or as a repressor. Moreover, these sequence data can be used to assess the spatial localisation of the different splice variants in *C. glomerata* and *C. rubecula* brains, using FISH or the *in situ* PCR technique (Abdel-Latief, 2007).

At the cellular level, the activity of *Cotesia* homologues of identified neurons in other insects may prove to be enlightening. Hammer (1993) has shown that the ventral unpaired median (VUM) neuron VUMmx1 acts as the neural substrate of the US, and innervates the olfactory (CS) pathway. Such VUM neurons are common in insects and were also shown to be present in *C. glomerat*a and *C. rubecula* (Bleeker et al., 2006b). Possibly, the electrophysiological characteristics of these neurons differ between the two species. For example, a *C. rubecula* VUM neuron (or other reward-sensitive neuron) may need more stimulation before it triggers 'downstream' activity and acts in coupling of the US and the CS. Possibly, *Cotesia* wasps possess neurons similar to, e.g., the GABAergic anterior lateral paired (ALP) neurons in *Drosophila*, which were shown to suppress olfactory learning (Liu and Davis, 2008), or the dorsal paired median (DPM) neurons in the same animal, which form an amnesiac-dependent memory trace after aversive odour training (Yu et al., 2005).

More 'why': possible avenues for bidirectional selection and behavioural work

I have shown that a high learning rate is associated with constitutive costs (chapter 7). More insight is needed, however, in how life-history parameters such as longevity change over time, in terms of trade-offs. Future studies with bidirectionally selected parasitic wasps should aim to gather as many data as possible from each generation of the selection lines, e.g., lifespan, adult body size, egg load, and other traits, in order to 1) visualise trends with a higher temporal resolution than I obtained in this thesis, and 2) to be able to statistically correlate traits in terms of trade-offs. Furthermore, it would be very interesting to investigate whether bidirectionally selected wasps also show operating costs of learning (i.e., costs that are only paid when an animal actually learns).

Time limitations prevented me from simultaneously rearing and maintaining bidirectionally selected *C. rubecula* lines, but it would be very interesting to see whether it is possible to increase the learning rate in *C. rubecula* (and/or decrease it even further), and ask some of the same questions that I now (partially) answered for *C. glomerata* (chapters 5, 6, and 7). It would also be very interesting to test the performance of bidirectionally selected wasps under (semi)-field conditions, and compare the results with those from unselected wasps.

Tempel et al. (1983) showed that the duration of the ASM phase is different, depending on the type of reinforcement that was used. In line with this, the long ASM phase that I found to exist in *C. glomerata* (chapter 6) may be a specific feature of oviposition-reward learning. It would be worthwhile to further investigate this with different species of parasitic wasps.

Examples of other intriguing questions that one could ask are: do fast learners perform better in a *C. glomerata* / *P. brassicae* trophic system (i.e., gregarious generalist/gregarious host). Do slow learners perform better in a *C. rubecula* / *P. rapae* trophic system (i.e., solitary specialist/solitary host)? Are large brains required for a high learning rate? Are large brains required for the maintenance of multiple long-lasting memory traces? And linking behavioural work with molecular work: do wasps with *C. glomerata*-like *CREB* expression perform better in a setting where host caterpillars occur in clusters? Do wasps with a *C. rubecula*-like *CREB* expression perform better in a setting with single, scattered hosts? Is *CREB* expression different in the decreased-learning and increased-learning lines?

Epilogue

Clearly, our model system provides us with a plethora of possibilities to investigate how tailor-made memories arise and function. It enabled us to directly compare species-specific differences (as they occur under natural circumstances) representing long-term adaptive changes (chapters 3 and 4, (Bleeker et al., 2006a; Bleeker et al., 2004; Bleeker et al., 2006b; Geervliet et al., 1998b; Smid et al., 2003; Smid et al., 2002; Smid et al., 2007). Moreover, I have shown that variation within one of the species can be used to observe how changes underlying such long-term adaptations are initiated in response to selection. When the level of fitness associated with a certain learning rate is altered, changes in memory dynamics occur and trade-offs become apparent (chapters 5, 6, and 7).

The more traditional studies of single model organisms are powerful but somewhat one-dimensional, e.g., transgenetic overexpression or gene knockouts. The advantages of a comparative approach (combined with ecologically relevant learning paradigms) over

such traditional approaches are evident. No matter how much knowledge is gathered on the fundamental aspects of learning and memory, ultimately, for biologists these facts only make sense in a natural context. All organisms interact with their biotic and abiotic environments, a fundamental issue which is not fully appreciated when solely studying how molecules, cells, and structures function in an organism. One also needs to know why such processes are initiated, terminated, and modulated. Although these ideas are certainly not new, studies on learning and memory that actually use this approach have only begun to appear fairly recently (Bleeker et al., 2006a; Mery et al., 2007a; Raine and Chittka, 2008; Smid, 2006; Smid et al., 2007).

I believe that the value of the comparative approach lies in the fact that it may prove that species-specific differences found in behavioural traits such as learning are adaptive. Conceptually, these ideas fit into the field of neuroecology, which has emerged in recent years, and which can be defined as the study of the neural mechanisms of behaviour guided by functional and evolutionary principles (Bolhuis and Macphail, 2001). Bolhuis and Macphail (2001) have expressed their reservations concerning the neuroecological approach of learning and memory, however: 'In general, there are at least three ways in which neuroecological interpretations of learning and memory are problematic: 1) in the attempted localisation of a specific brain region as the neural substrate of some adaptive specialisation of learning or memory; 2) in treating failures of laboratory experiments to support behavioural predictions of the adaptive specialisation view; and 3) in demonstrating causal links between correlated behavioural and neural differences' (Bolhuis and Macphail, 2001). Although I do not deny the validity of the problems in neuroecological interpretations listed by Bolhuis and Macphail, this thesis shows that such problems can be overcome by using a multidisciplinary and comparative approach. In addition, the use of insects, with their short generation time and high throughput possibilities offers significant advantages in terms of experimental conditions and prerequisites. Finally, combinations of molecular, histological and behavioural experiments under laboratory and field conditions experiments can provide evidence that differences in learning rate (caused by selection or changes in gene expression) are adaptive in specific ecological settings. This thesis provides a solid basis to reach this goal.

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List of abbreviations

AL	antennal lobe
ALP	anterior lateral paired (neuron)
AMN	amnesiac (protein)
ANI	anisomycin
ARM	anaesthesia-resistant memory
ASM	anaesthesia-sensitive memory
BSA	bovine serum albumin
bZIP	basic leucine zipper (domain)
cAMP	cyclic adenosine monophosphate
cDNA	complementary DNA
cGMP	cyclic guanosine monophosphate
CODEHOP	consensus-degenerate hybrid oligonucleotide primer
CRE	cAMP response element
CREB	cAMP response element binding (protein)
CREM	cAMP response element modulator
CS	conditioned stimulus
CT	threshold cycle
DLL	decreased-learning line
DNA	deoxyribonucleic acid
DPM	dorsal paired median (neuron)
dsRNA	double-stranded RNA
eIF	eukaryotic initiation factor
eLTM	early long-term memory
eSTM	early short-term memory
FISH	fluorescence in situ hybridisation
GLM	general linear model
GPA	glutaraldehyde-picric acid (fixative)
HIPV	herbivore-induce plant volatile
HTL	hind tibia length
IEG	immediate-early gene
ILL	increased-learning line
ITI	intertrial interval
KC	Kenyon cell
KID	kinase-inducible domain
lLTM	late long-term memory
lSTM	late short-term memory
LTD	long-term depression
LTF	long-term facilitation
LTM	long-term memory
LTP	long-term potentiation

MB	mushroom body
mRNA	messenger RNA
MTM	mid-term memory
NGF	nerve growth factor
NO	nitric oxide
NOS	nitric oxide synthase
OR	olfactory receptor (protein)
ORF	open reading frame
ORN	olfactory receptor neuron
PBS	phosphate-buffered saline
PBS-T	phosphate-buffered saline + Triton
PCR	polymerase chain reaction
pCREB	phosphorylated CREB
PER	proboscis extension response
PKA	protein kinase A
PKC	protein kinase C
PN	projection neuron
PP1	protein phosphatase 1
Q1/2	glutamine-rich domain
qPCR	(real-time) quantitative PCR
QTL	quantitative trait loci
RACE	rapid amplification of cDNA ends
RNA	ribonucleic acid
RNAi	RNA interference
SNR	signal-to-noise ratio
SOG	suboesophageal ganglion
STM	short-term memory
US	unconditioned stimulus
VUM	ventral unpaired median (neuron)

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SAMENVATTING

Leren en geheugenvorming worden vaak gezien als eigenschappen met louter voordelen, maar er zijn ook kosten aan verbonden, bijvoorbeeld in termen van metabolisme. Naar verwachting verschillen de kosten en baten van leren en geheugenvorming tussen diersoorten, en daarom zal het gemak en de snelheid waarmee een stabiel, 'geconsolideerd' langetermijngeheugen (long-term memory, LTM) wordt gevormd ook verschillen tussen deze soorten. Afhankelijk van de ecologische niche waarin een dier leeft, kunnen zowel 'langzaam' als 'snel' leren voordelige evolutionaire aanpassingen zijn. Als een dier gedurende zijn leven te maken heeft met een relatief voorspelbare omgeving is snel leren een goede strategie. Als de omgeving daarentegen onvoorspelbaar is, loont het om meer tijd te investeren in het evalueren van informatie en om meerdere ervaringen op te doen, alvorens informatie op te slaan in de vorm van langetermijngeheugen. Dit concept noemen we tailor-made memories ('op maat gemaakte herinneringen'): elk dier leert op de manier die onder de heersende omstandigheden het meest voordelig is. Om onderzoek te kunnen doen naar hoe dergelijke, op maat gemaakte herinneringen evolueren heb ik gebruik gemaakt van een multitroof modelsysteem, bestaande uit 1) twee nauwverwante parasitaire wespen (*Cotesia glomerata* en *C. rubecula*) die sterk verschillen in de snelheid waarmee ze leren, 2) de rupsen van het grote en het kleine koolwitje (*Pieris brassicae* en *P. rapae*) waarin de wespen hun eitjes leggen, en 3) de waardplanten spruitkool (*Brassica oleracea* var. Gemmifera) en Oost-Indische kers (*Tropaeolum majus*).

In mijn experimenten leerden de wespen associatief geuren herkennen van de plantensoort waarop geschikte gastheerrupsen te vinden waren, door eén of meerdere eilegervaringen ('trainingen') te ondergaan op zo'n plant. Eerdere was al aangetoond dat *C. glomerata* slechts eén training nodig heeft om LTM te vormen, terwijl *C. rubecula* daarvoor drie trainingen nodig heeft, die met tussenpozen gegeven worden. Naast LTM bestaat er nog een andere vorm van geconsolideerd geheugen, dat bestand is tegen anesthetische behandelingen (anaesthesia-resistant memory, ARM). Zowel LTM als ARM zijn bestand tegen anesthesie, die kan worden opgewekt door de wespen na het trainen te koelen op ijs. In tegenstelling tot LTM echter, is ARM niet afhankelijk van de synthese van eiwitten, en in die zin is ARM een 'goedkope', maar minder stabiele vorm van langetermijngeheugen. Het geconsolideerde geheugen van *C. glomerata* lijkt volledig uit LTM te bestaan, terwijl het in *C. rubecula* een combinatie lijkt te zijn van zowel ARM als LTM.

De vorming van LTM is afhankelijk van eiwitsynthese. In dit proces speelt de transcriptiefactor cAMP response element-binding protein (CREB) een sleutelrol. Het CREB-eiwit komt voor in verschillende vormen die isovormen genoemd worden, en die van elkaar verschillen doordat ze vertaald zijn vanaf mRNA-transcripten (van eén gen) waar verschillende stukjes uit zijn geknipt. Het is aangetoond dat CREB-isovormen in modelorganismen zoals de fruitvlieg *Drosophila melanogaster*, de zeehaas *Aplysia californica*, en ook in zoogdieren zoals muizen en de mens, de transcriptie van andere genen zowel kunnen activeren als kunnen remmen. Dit heeft geleid tot de hypothese dat de verhouding tussen activerende en remmende CREB-isovormen in feite functioneert als

een soort moleculaire schakelaar die bepaalt of er LTM gevormd wordt. Een dergelijke schakelaar zou verantwoordelijk kunnen zijn voor soortspecifieke verschillen in leren en geheugenvorming. Ik heb het *CREB*-gen van *C. glomerata* en *C. rubecula* gekloneerd, de DNA-sequenties bepaald, en negen isovormen geïdentificeerd die voorkomen in beide *Cotesia*-soorten. Twee van de negen isovormen verschillen significant in de mate waarin ze voorkomen in de twee wespensoorten; de rest verschilt daarin niet. Het ondergaan van een training echter, lijkt veranderingen te induceren in de expressie van de meest voorkomende isovormen, waaronder de isovormen waarvan ik verwacht dat ze activerend werken. Dit suggereert dat het leerproces zélf een isovormverhouding bewerkstelligt die bepaalt of LTM al dan niet gevormd wordt.

Hoewel zulke moleculaire mechanismen heel snel in werking kunnen treden, kan het soms toch dagen of zelfs weken duren voor informatie daadwerkelijk is opgeslagen als langetermijngeheugen. Om het optreden van zulke verschillen in de dynamiek van geheugenvorming te verklaren, is inzicht nodig in wat er precies gebeurt wanneer natuurlijke variatie in leersnelheid wordt onderworpen aan selectie. Om dit te onderzoeken, heb ik 'tweerichtingsselectie' toegepast en twee *C. glomerata*-lijnen gekweekt die significant verschillen in leersnelheid: een langzamer lerende lijn (decreased-learning line, DLL) en een sneller lerende lijn (increased-learning line, ILL).

Door vóór het trainen de eiwitsyntheseremmer anisomycine (ANI) aan de wespen te voeren, en na het trainen de geheugenretentie te bepalen, kon ik aantonen dat de dynamiek van de vorming van geconsolideerd geheugen verschilde tussen de twee lijnen. De DLL consolideerde helemaal geen LTM meer, terwijl de ILL dat wel deed. Door deze resultaten te combineren met die van een experiment waarin ik geheugenverlies induceerde door de wespen te koelen op ijs, kon ik laten zien dat in *C. glomerata* het koudegevoelige kortetermijngeheugen direct consolideert tot LTM, zonder tussenliggende ARM-fase. ARM is een goedkope vorm van langetermijngeheugen en wordt daarom geacht voor te komen bij dieren die meer tijd nodig hebben om informatie te evalueren alvorens deze definitief op te slaan (zoals *C. rubecula*). Het feit dat *C. glomerata* geen ARM kan vormen is kostbaar, in de zin dat dit kan leiden tot het verspillen van energie aan de premature opslag van onbetrouwbare informatie.

Een vergelijking van beide lijnen liet zien dat een hoge leersnelheid gepaard gaat met kosten. De levensduur van de DLL was significant langer dan die van de ILL. Verder hadden vrouwtjes van de ILL significant grotere hersenen dan die van de DLL, bij een gelijke lichaamsgrootte. Deze bevindingen tonen aan dat er zogenaamde trade-offs optreden (verschuivingen in de investering van energie in bepaalde eigenschappen, zoals in dit geval grotere hersenen en een kortere levensduur) als gevolg van de selectieprocedure die ik heb gehanteerd. Bovendien lijken deze kosten constitutief van aard te zijn. Dit betekent dat dieren die in staat zijn snel te leren de kosten hiervoor betalen door een groot, energetisch duur brein te handhaven en een verminderde levensduur te hebben, zelfs als het leervermogen niet daadwerkelijk aangesproken en gebruikt wordt.

De resultaten van mijn werk geven aan dat vergelijkend onderzoek, met behulp van een modelsysteem dat bestaat uit twee nauwverwante soorten die van nature sterk verschillen in leersnelheid, unieke inzichten kan verschaffen in hoe verschillen in leren en geheugen ontstaan. Het gebruik van een dergelijk modelsysteem verdient dan ook de voorkeur boven het gebruik van 'traditionele' modelorganismen. De aanpak die ik gebruikt heb maakt het mogelijk verschillende hypotheses te testen met behulp van een in ecologisch opzicht relevant leerparadigma. Onderzoek op het gebied van de (neuro) biologie zou zeer gebaat zijn bij een toename in het gebruik van modelsystemen die bestaan uit nauwverwante organismen die verschillen in eén of meer eigenschappen. Het werk dat beschreven is in dit proefschrift laat zien hoe vruchtbaar een dergelijke aanpak kan zijn.

NAWOORD

Ah, het nawoord. Dit is samen met mijn c.v. waarschijnlijk het enige stukje tekst in dit proefschrift dat nog volledig mijn eigen persoonlijkheid weerspiegelt. Natuurlijk ben ik degene geweest die dit boekje heeft geschreven, maar gelukkig is in de wetenschappelijke honderd-en-zoveel pagina's van dit werk ook de hand van de meesters duidelijk te herkennen. Tijdens het proces van schrijven, schrappen, en (reviseren)[2] hebben mijn (co-)promotoren mijn schrijven weten te stroomlijnen en optimaliseren, en laat ik er maar gewoon voor uitkomen, ze hebben fantastisch werk verricht! Niet alleen op redactioneel gebied trouwens. Het is dus niet meer dan gepast om dit nawoord te beginnen met woorden van dank aan:

Marcel. De goeroe van de insect-plant interacties, en de man die al jaren in staat is gebleken van het Lab voor Entomologie een plaats te maken waar fantastische wetenschap wordt bedreven. Marcel, bedankt voor de mogelijkheid om bij Ento te promoveren! Gedurende de hele periode, maar met name in de afrondende fase, heb je goed gestuurd op een realistische tijdsplanning, en je revisies van alle hoofdstukken kwamen in een hoog tempo bij me terug. Dit is de kwaliteit van mijn promotie en mijn proefschrift zeer ten goede gekomen.
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Joop. De man met Kennis Van Zaken. Tijdens de eerste fase van mijn promotie kon ik bouwen op jouw inzichten in electrofysiologie, neurobiologie, insect-plant interacties, tja… in wat niet eigenlijk? Hoewel je in de moleculaire fase van mijn promotie niet meer direct betrokken was bij mijn werk, wil ik je hier toch graag van harte bedanken voor je inzichten en, dit mag niet onvermeld blijven, je droge humor!
Hans. Over goeroes gesproken. Ik denk dat we veilig kunnen stellen dat het Lab voor Entomologie niet zonder jouw kennis en kunde kan, en dat gold voor mijn promotie en mijn proefschrift in het bijzonder! Je bent zowel in professioneel als in persoonlijk opzicht een geweldige dagelijkse begeleider geweest, en de waarheid is: zonder jouw ideeën, stimulerende discussies, en immer positieve kijk op de zaken was dit proefschrift er niet gekomen. Punt. Ik hoop dat je de komende jaren je wetenschappelijke plannen kunt realiseren, laat het beurzen regenen!

Komen we bij het Lab. De afgelopen vier jaar zijn zo'n beetje voorbij gevlogen, en dat komt niet in het minst omdat Ento gewoon een hele gezellige club mensen herbergt. Op het sociale vlak heb ik minder van mezelf laten zien dan met alle miljoenen Beerhives, labuitjes en andere activiteiten had gekund; dat lag de eerste jaren vooral aan het feit dat ik nog in Utrecht woonde en leefde, en het laatste jaar met name aan de grote bergen werk die ik nog moest verzetten om dit boekje te realiseren. Maar dat betekent niet dat ik het niet naar mijn zin heb gehad! Ik zal niet iedereen met naam en toenaam noemen, maar eervolle vermeldingen zijn er voor Tjeerd en onze gedeelde muzieksmaak en enigszins verknipte kijk op de wereld, mijn kamergenoten Karin, Valentina, en Erik

cum laude Poelman (eeuwig onverstoorbare topwetenschapper met geheime liefde voor duivelsche muzak). Verder heb ik altijd erg genoten van de eigen kijk op de zaken van Jeroen, Rieta en Yde. Ik ben DJ Patrick V. (man van vele talenten) erg dankbaar voor de lessen moleculair-biologische technieken, de storende radio op het lab en de gezellige samenwerking. Roland, Sander, Renate, Yu Tong en Si-Jun, vriendelijke postdocs die jullie zijn! Bedankt voor de gesprekken tussen alle bedrijven door. En dan natuurlijk nog wat lovende woorden voor Maaike, Ties, Nina, Tibor en Gabriella: niet alleen zijn jullie leuke mensen, maar jullie hebben ook nog eens prachtige gezinnetjes gesticht! Alle andere mede-AIO's die ik nu niet met name noem, ik zal de PhD trip naar Engeland niet vergeten, dat was echt een toffe ervaring! Dank daarvoor.

Leo, André, Frans, bedankt voor jullie werk 'achter de schermen'. Wat is een Lab voor Entomologie zonder insecten? Daar zijn we nooit achter gekomen want dankzij jullie hebben mijn collega's en ik altijd meer dan voldoende beestjes gehad om mee te werken. Onbetaalbaar werk doen jullie, in mijn ogen.

Onbetaalbaar werk werd ook verricht door de enige MSc student die het werk aan leren en geheugenvorming de laatste jaren interessant leek te vinden (in ieder geval de enige die bij mij een afstudeervak heeft gedaan). Loes, je bent een kei! Zonder jouw ontzettend harde werk was de basis voor een belangrijk deel van mijn proefschrift er niet geweest. Dank je wel!

Dan wat betreft mijn leven buiten het werk… er zijn behoorlijk wat mensen die ik hier wel even bij naam ga noemen, gewoon omdat het kan (het is mijn proefschrift!): allereerst de capoeiraclub. De afgelopen jaren zijn jullie voor mij heel belangrijk geweest: professor Bram, Arjan, Rik, Saskia, Marieke, Anne, Mathijs, Mirella, Mira, Bram(metje), Jasper, Brenda, Sanne, Frank, Andrej, Tom, Renske, Susanne, Emile, Jackson, Ellen, Lara, Madelon, Mireille, en de andere leerlingen van Bram, de rest van Grupo Capoeira Brasil, en de andere capoeiristas in Nederland. Altijd een genoegen om met jullie te trainen, te socialiseren, muziek te maken, en te feesten, en ik hoop dat we dat nog lang blijven doen! Ook mijn kersverse capoeiraleerlingen in Wageningen noem ik hier kort: ik vind het supertof om mijn passie nu op jullie te kunnen overdragen!

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ik veel aan heb gehad.

Ter afsluiting wil ik graag mijn respect betuigen aan mijn naaste familie: mijn moeder Tineke, mijn vader Frank (R.I.P.), mijn zus Mirjam (en Bas) en mijn geweldige nichtje Lola! En mijn 'schoonouders' Arie en Ineke, 'schoonbroer' Edward, en 'schoonoma', niet te vergeten.

En dan blijft er natuurlijk nog eén persoon over: Annette… jij bent absoluut het allerbelangrijkste en het allermooiste dat mijn AIO-tijd me heeft gebracht. Bedankt voor alle liefde, plezier & flauwe humor, support, muziek, natuur, en onze gedeelde interesses en kijk op de dingen. Ik ben superblij dat we zijn gaan samenwonen! De volgende stap is jouw promotie, en daarna? Geen idee, maar het wordt vast leuk... lieverd, ik hou van jou!

Michaël
Wageningen, voorjaar 2009

CURRICULUM VITAE

I was born on August 15th, 1974 in the small harbour town of Terneuzen, in the South-western delta area of the Netherlands. After finishing secondary school and spending some time in chemistry/laboratory education I decided to study biology, either in Utrecht, Wageningen or Nijmegen. Because of the good atmosphere and the variety of subjects in the curriculum I chose for Wageningen University. I have always had a broad range of interests, and during my studies this was not different; I graduated with a curriculum focusing on the organismal biology of plants and animals, (molecular) embryology, and taxonomy. My first hands-on experience with a research project came at the department of Experimental Zoology, where I worked with the zebrafish *Danio rerio*. This animal is a well-known model organism in the area of developmental biology. My supervisors were modelling embryonic development and tried to predict the onset of angiogenesis, i.e., at what stage the size of the embryo would necessitate the development of a vascular system in order to provide all tissues with oxygen. The embryo was modelled as a sphere, which if course does not reflect the actual shape. Therefore, I made 3D reconstructions of actual zebrafish larvae and I calculated the volumes of the yolk mass and the embryo itself. These data could then be fed into the model. I enjoyed the histology/confocal microscopy/image processing parts very much, but for my second project I wanted to do something completely different. Via the Lab of Entomology I came into e-mail contact with professor Louise Vet. She proposed I could do a literature study for the Netherlands Institute of Ecology (NIOO-KNAW) in Heteren, under supervision of dr. Martijn Bezemer and prof. Wim van der Putten. The interest in below-ground multitrophic interactions was growing rapidly at that time and my assignment was to make a 'meta-analysis' of the available body of literature and see which organisms, which interactions, which chemicals, etcetera, had been described in below-ground multitrophic interactions. Again, this was an interesting and stimulating project. At the time of my graduation, job opportunities for academics were scarce so I spent several years working in a non-academic environment, until I got a PhD position at the Lab of Entomology in the spring of 2005. This project focused on associative learning in parasitic wasps, and the results of my research at Entomology are presented in this thesis. As of April 2009, I occupy a position at the Plant Protection Service of the Netherlands, as a 'senior specialist validation and diagnostic techniques'.

Most of my leisure time is spent playing and teaching capoeira, having a social life, reading popular science, thrillers, and fantasy books, listening to music and playing guitar, drawing, watching movies, hiking and enjoying nature.

EDUCATION STATEMENT

PE&RC PhD Education Certificate

With the educational activities listed below the PhD candidate has complied with the educational requirements set by the C.T. de Wit Graduate School for Production Ecology and Resource Conservation (PE&RC) which comprises of a minimum total of 32 ECTS (= 22 weeks of activities)

Review of Literature (5.6 ECTS)

- Assessing natural variation in olfactory learning and memory formation (2009)

Laboratory Training and Working Visits (4.2 ECTS)

- Electrophysiology; University of Southampton (2006)
- Visiting labs in related areas of expertise (with a group of PhD students); several universities and institutes in the London area (2007)

Post-Graduate Courses (2.8 ECTS)

- Spring School Chemical communication: from gene to ecosystem; FRONTIS and PE&RC (2005)
- Bioinformatics; EPS (2007)

Deficiency, Refresh, Brush-up Courses (5.6 ECTS)

- Functional neurobiology (electrophysiological methods); Utrecht University (2005)
- Basic statistics; PE&RC (2007)

Competence Strengthening / Skills Courses (3 ECTS)

- PhD Competence assessment; WGS (2005)
- Time planning and project management; WGS (2006)
- Techniques for writing and presenting a scientific paper; PE&RC (2006)

Discussion Groups / Local Seminars and Other Scientific Meetings (9.2 ECTS)

- Entomology PhD lunch meeting (2005-2009)
- 19e Entomologendag (2008)
- NERN (2009)

PE&RC Annual Meetings, Seminars and the PE&RC Weekend (1.2 ECTS)

- PE&RC day: scientific publishing (2005)
- PE&RC day: the scientific agenda (2006)
- PE&RC day: collapse (2007)
- Current themes symposium: plants, insects and microbes (2008)

International Symposia, Workshops and Conferences (3 ECTS)

- BEPAR (2008)

Courses in Which the PhD Candidate Has Worked as a Teacher

- Biology and management of plant pathogens, insects and weeds; Entomology (2005-2008)

Supervision of MSc Student(s)

- Artificial selection on learning rate in *Cotesia glomerata*; 30 days; 1 student

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