Natural variation in memory formation among *Nasonia* parasitic wasps

from genes to behaviour

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Chapter 1

General introduction

Katja M. Hoedjes

LEARNING AND MEMORY FORMATION AMONG ANIMAL SPECIES

Learning can be defined as a process in which information on the environment is acquired and stored in the brain; memories are the neuronal representations of this stored information (Dukas, 1998a). Humans and animals alike have to cope with an ever-changing environment in the ultimate quest to survive and reproduce. The ability to learn and form memories is an indispensable trait that enables animal species to adapt to novel and changing environments throughout their lives (Dukas, 1998b). Animals can use learned information or skills to change and optimize their behaviour, which can improve their abilities to find food, mates or hosts (Papaj & Vet, 1990; Dukas, 2004; Raine & Chittka, 2008). It is not surprising that the ability to learn has been observed in animals ranging from vertebrate species, such as ourselves, to relatively simple invertebrates. The cues and skills that species can learn are diverse: most bird species can learn complex songs and some species, for example jays, can remember the location of thousands of food caches (Clayton *et al*., 2003; Brenowitz & Beecher, 2005), bees learn the location of their hive, they can learn colours and odours of profitable flowers and how to handle these flowers (Menzel *et al*., 2006; Chittka & Raine, 2006), and the marine snail, *Aplysia californica*, can learn to withdraw its gill when lightly touched (Bailey & Kandel, 2008).

Different types of learning can be distinguished, including habituation, sensitization, associative learning, spatial learning and social learning (Shettleworth, 2010). Associative learning is the form of learning that has been studied most intensively among distant animal phyla and it is the type of learning that I study in this thesis. Associative learning can be studied using two different conditioning approaches. (1) In classical (Pavlovian) conditioning, animals learn to associate a stimulus (the conditioned stimulus, CS) with a reinforcing stimulus (the unconditioned stimulus, US), e.g. a reward or a punishment. The animal will subsequently demonstrate an altered conditioned response towards the CS (Rescorla, 1988). An example of classical conditioning is the widely used odour-shock conditioning paradigm used to condition fruit flies, in which these animals learn that an odour predicts a noxious electric shock. The flies will subsequently avoid the odour associated with the electric shock (Tully & Quinn, 1985). (2) In operant conditioning, animals associate their own behaviour with a reinforcing stimulus (Brembs, 2003). An example is the heat box-paradigm for fruit flies in which the animal will learn to avoid a heated area in a chamber after walking onto it (Wüstmann *et al*., 1996). In both classical and operant conditioning procedures an animal will learn that specific cues or behaviours predict a reward or punishment and they will change their behaviour accordingly. Although there is variation in the skills or the type and complexity of information that different animal species can learn, there are many similarities in behavioural properties of

associative learning and subsequent memory formation (Dubnau, 2003).

A large number of studies in the field of learning and memory have focussed on proximate factors that regulate these processes. The cellular mechanisms underlying associative memory formation have been studied intensively in a small number of model organisms, most importantly mice and rats (Chen & Tonegawa, 1997), fruit flies and honeybees (Margulies *et al*., 2005; Eisenhardt, 2006), and the marine snail *Aplysia californica* (Bailey & Kandel, 2008). These studies have revealed that genetic and neural pathways involved in memory formation are highly conserved among animal species (Dubnau & Tully, 1998). A learning experience results in the formation of distinctly different types of memories. These types can be distinguished on a temporal scale (e.g. short-term memory and long-term memory), but also on the basis of genetic and neural pathways involved (Davis, 2005). Especially research on memory formation in invertebrate species has been highly valuable to identify cellular pathways that underlie these processes, which was acknowledged by awarding the Nobel Prize in physiology or medicine to Eric Kandel in the year 2000 for his pioneering work on synaptic plasticity in *Aplysia*. Invertebrate species have a simpler neuronal network than vertebrate species, which facilitates studies on individual or small groups of neurons. In combination with pharmacological or genetic manipulation, this provides opportunities to identify neural and genetic pathways involved in learning and memory formation. Indeed, many mechanisms of learning and memory formation were first identified in invertebrate species, and were later found to be present in vertebrate species as well (Dubnau & Tully, 1998; Bailey & Kandel, 2008; Alberini, 2009). Currently, invertebrate species are considered important model species for neurodegenerative diseases, next to the more traditional mammalian model species (Price *et al*., 1998; Bilen & Bonini, 2005; Farooqui, 2007).

Research on learning and memory formation has not only focussed on the mechanisms that underlie this trait; numerous studies have investigated evolutionary and ecological aspects of this behaviour (e.g. Vet *et al*., 1995; Dukas, 1998b; Kawecki, 2010; Shettleworth, 2010). Interestingly, a number of studies reveal subtle intra- and interspecific variation in learning and memory formation of animals (Brenowitz & Beecher, 2005; Papaj & Snell-Rood, 2007; Smid *et al*., 2007; Huigens *et al*., 2009; Ings *et al*., 2009). The characteristics of different types of memory are highly conserved, but there is variation in spatial expression of different memory types, e.g. long-term memory can form within a matter of hours in some species, but requires days in other species (Smid *et al*., 2007). The conditioning procedure required to induce formation of a specific type of memory is also variable between and within species, e.g. some species require more conditioning trials to form a long-term memory than other

species (Smid *et al*., 2007). Variation in memory dynamics has been hypothesized to be the result of species-specific differences in the ecology of animal species (e.g. Vet *et al*., 1995; Menzel, 1999), although the exact mechanisms that determine memory dynamics, both ultimate and proximate, remain to be elucidated (Kawecki, 2010). Variation in memory dynamics is discussed more in detail in **chapter 2**. In this thesis, I investigate inter-specific variation in the number of conditioning trials required to induce long-term memory formation. My aim is to elucidate both ecological factors that can explain species-specific differences in long-term memory formation, as well as the cellular mechanisms that underlie variation in this trait. I have studied variation in long-term memory formation in parasitic wasp species of the genus *Nasonia*.

THE STUDY SYSTEM

I have used parasitic wasp species of the genus *Nasonia* as my model system to study variation in long-term memory formation. These small (~ 2 mm in length) parasitic wasps lay their eggs inside the puparium of various fly species and offspring will typically develop and emerge within 2 to 3 weeks (Werren & Loehlin, 2009). The genus *Nasonia* (Hymenoptera: Pteromalidae) consists of 4 described species: *N. vitripennis*, *N. longicornis*, *N. giraulti* and *N. oneida* (Darling & Werren, 1990; Raychoudhury *et al*., 2010). *Nasonia vitripennis* was first described in 1836 and several aspects of its ecology, behaviour and physiology have been studied in detail. Genetic studies have been carried out since the 1950s (Whiting, 1967). This species parasitizes fly pupae of the families Sarcophagidae (flesh flies), Calliphoridae (blow flies) and Muscidae (house flies) occurring in a number of distinct habitats, including carcasses, bird nests and manure (Darling & Werren, 1990; Peters & Abraham, 2010). *Nasonia vitripennis* has a worldwide distribution, likely because of its association with human-associated flies (Werren & Loehlin, 2009). *Nasonia longicornis* and *N. giraulti* were first described in the 1990s. Both species occur only in Northern America, respectively in the west and the east, and they have a more restricted host range than *N. vitripennis*. Both preferentially parasitize *Protocalliphora* spp. (Diptera: Calliphoridae) in nature, which are blood-feeding blowflies that occur in bird nests (Darling & Werren, 1990). *Nasonia oneida* was described recently and is closely related to *N. giraulti* (Raychoudhury *et al*., 2010).

In recent years, the genus *Nasonia* has emerged as a model system with unique opportunities for genetic research (Werren & Loehlin, 2009). These species can be reared and handled easily in a laboratory, they can produce large numbers of offspring and they have a short generation time. In addition, *Nasonia* species have a haplodiploid mating system, similar to other hymenopteran species: females develop from fertilized eggs and are diploid, whereas

males develop from unfertilized eggs and are haploid. This makes males particularly suitable for studies on recessive traits (Werren & Loehlin, 2009). Infection with *Wolbachia* bacteria prevents interspecific hybridization in nature, but the four species can interbreed when they are cured from their infection (Breeuwer & Werren, 1990; Bordenstein *et al*., 2001). The unique opportunity to interbreed *Nasonia* species allows for introgression studies in which genes or specific traits are backcrossed from one species into the genetic background of another species (e.g. Desjardins *et al*., 2010; Loehlin & Werren, 2012). Furthermore, a number of genomics tools were developed for *Nasonia*, which include a sequenced and annotated genome, detailed genetic maps and microarrays (Werren *et al*., 2010; Desjardins *et al*., 2013). The combination of these characteristics makes *Nasonia* an excellent model genus for genetic research and the number of research groups that study these parasitoid species has increased in the last decade. The phenotypes studied are related to sex ratio, sex determination, sex pheromones and courtship behaviour (e.g. Beukeboom & van den Assem, 2001; Verhulst *et al*., 2010; Pannebakker *et al*., 2011; Niehuis *et al*., 2013), diapause induction (Paolucci *et al*., 2013), wing size (Loehlin *et al*., 2010b; Loehlin & Werren, 2012), development (Lynch *et al*., 2006), hybrid incompatibilities (Niehuis *et al*., 2008), and the effects of the gut microbiome on *Nasonia* fitness (Brucker & Bordenstein, 2013). Also learning and memory formation have been studied already in *N. vitripennis* (Oliai & King, 2000; Baeder & King, 2004; Schurmann *et al*., 2009; Schurmann *et al*., 2012), providing information on how these wasps can be conditioned and on their memory dynamics. I argue that the *Nasonia* model system also provides excellent opportunities for studies on interspecific differences in learning and memory formation. Both ecological and genetic aspects of variation in learning and memory can be investigated in these species.

THESIS OUTLINE

Chapter 2 reviews oviposition learning in parasitic wasp species, which is a type of associative learning in which finding and parasitizing a host is the rewarding stimulus. There is substantial variation in memory formation among closely related species of parasitic wasps and I argue that parasitic wasps are excellent model organisms to study both ultimate and proximate factors involved in this variation.

I have studied variation in memory formation using parasitic wasp species of the genus *Nasonia*. High-throughput methods for olfactory conditioning and memory retention testing are necessary to study this behaviour and were developed for *Nasonia* species. These methods, which are described in **Chapter 3**, were successfully used to study learning and memory formation in *Nasonia* parasitic wasps and demonstrated interspecific differences

in memory retention among the species *N. vitripennis*, *N. giraulti* and *N. longicornis*.

In **Chapter 4**, I address long-term memory formation in the species *N. vitripennis* and *N. giraulti*. The effects of conditioning procedure on memory retention, i.e. a single versus multiple conditioning trials, were investigated. Furthermore, I have characterized the dynamics of long-term memory formation by inhibiting the formation of this type of memory by using protein synthesis inhibitors and measuring the effects on memory retention. This characterization of long-term memory formation was essential for further experiments in which I aimed to elucidate ultimate and proximate factors that control memory formation.

The host is the rewarding stimulus in oviposition learning and variation in this reward has been hypothesized to be an important ecological factor that determines memory formation in parasitic wasps. In **Chapter 5**, I address the effects of different host species on memory retention both *N. vitripennis* and *N. giraulti*. The host species differed in their quality as a host in terms of numbers and size of offspring.

Chapter 6 addresses quantitative trait loci that are responsible for the inter-specific difference in memory formation between *N. vitripennis* and *N. giraulti* by backcrossing the memory phenotype of *N. giraulti* into the background of *N. vitripennis*. Genomic factors that are responsible for variation in long-term memory formation can be detected using this introgression approach, which is a unique feature of the *Nasonia* model system and a novel approach to study interspecific variation in memory formation.

A second approach to study the genetic basis of long-term memory formation was used in **Chapter 7**. HiSeq sequencing of RNA from the brains of *N. vitripennis* and *N. giraulti*, collected before and after a learning experience, was used to study differential gene expression and identify differences between the two species. This approach allows for the identification of genes and pathways that are associated with long-term memory formation and the results are complementary to the introgression experiment that is described in the previous chapter.

In **Chapter 8**, I discuss the results of this thesis in the light of recent insights in the field of learning and memory formation and I describe future perspectives for studies on variation in memory formation in parasitic wasps.

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Chapter 2

Natural variation in learning rate and memory dynamics in parasitoid wasps: opportunities for converging ecology and neuroscience

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ABSTRACT

Although the neural and genetic pathways underlying learning and memory formation seem strikingly similar among species of distant animal phyla, several more subtle inter- and intraspecific differences become evident from studies on model organisms. The true significance of such variation can only be understood when integrating this with information on the ecological relevance. Here, we argue that parasitoid wasps provide an excellent opportunity for multi-disciplinary studies that integrate ultimate and proximate approaches. These insects display interspecific variation in learning rate and memory dynamics that reflects natural variation in a daunting foraging task that largely determines their fitness: finding the inconspicuous hosts to which they will assign their offspring to develop. We review bioassays used for oviposition learning, the ecological factors that are considered to underlie the observed differences in learning rate and memory dynamics, and the opportunities for convergence of ecology and neuroscience that are offered by using parasitoid wasps as model species. We advocate that variation in learning and memory traits has evolved to suit an insect's lifestyle within its ecological niche.

INTRODUCTION

Parasitoid wasps lay their eggs in or onto other insects that eventually will be killed by the developing parasitoid larvae. There are parasitoids that develop in either eggs, larvae or pupae, generalists and specialists, gregarious (i.e. laying several eggs into one host) and solitary species; and there is a similar diversity in the ecology of their host insects and their respective food sources. Finding a host insect is a daunting task for all female parasitoids, because hosts are under strong selection to remain inconspicuous to their natural enemies, irrespective of whether the host is in the egg, larval or pupal stage. The use of indirect, chemical information is an important solution for parasitoid wasps to solve this detectability problem. For instance, parasitoids of dipteran hosts use odours of decaying substrates like mushrooms, fruits or carcasses to find host fly larvae, and parasitoids of herbivorous larvae use odours of the plant their host is feeding from. Alternatively, parasitoids can rely on information from other, more conspicuous host stages than the one under attack, such as egg parasitoids that use the pheromones of the adult host stage (Vet & Dicke, 1992). These odours are very complex blends, however, and minute differences between these blends may signal the presence of suitable hosts (Takabayashi *et al*., 2006). Dukas (1998a) proposed that generalist species may need to divide their attention over a wide variety of cues, which reduces searching efficiency owing to limited brain capacity for simultaneous processing of information. This may be a reason why so many insects are specialists, since these specialist species can focus on a more reduced set of stimuli compared with generalists (Bernays, 2001). Learning may provide a more flexible way of specialization. For instance, parasitoid wasps that experience the current presence of a certain host species, thereafter narrow their (olfactory) 'search image' by learning, as a form of temporal specialization (Ishii & Shimada, 2010). Although learning was considered as a trait that is more important for generalists that specialists, it has become clear that learning is also important as for specialist parasitoid wasps (Steidle & van Loon, 2003). Indeed, although female parasitoids have an innate preference for certain odours, in most investigated species associative learning optimizes their foraging efficiency (Turlings *et al*., 1993; Vet *et al*., 1995).

Recent research unveiled remarkable natural variation in learning rate and in the dynamics of memory formation between closely related parasitoid wasp species (Geervliet *et al*., 1998b; Bleeker *et al*., 2006a; Tamo *et al*., 2006; Smid *et al*., 2007; van den Berg *et al*., 2011). These studies suggested that this variation reflects adaptations to species-specific ecological constraints. The rich behavioural diversity among parasitoid wasp species offers a wealth of possibilities for a comparative approach to address both ultimate and proximate questions on the evolution of learning rate and memory formation. As we will argue in the last part of this paper, such a comparative approach creates unique opportunities for the

convergence of ecology and neuroscience.

LEARNING IN PARASITOID WASPS

Finding suitable hosts is a difficult task for female parasitoid wasps, but inexperienced wasps by no means search randomly. They respond innately to stimuli that are derived from their hosts or that indicate suitable hosts (Turlings *et al*., 1993; Vet *et al*., 1995), comparable to innate colour preferences in pollinators (Riffell *et al*., 2008; Ings *et al*., 2009). Parasitoids can change their innate preferences for odour cues that guide them to patches with hosts after an oviposition experience. This allows female wasps to find hosts faster, thus probably increasing their lifetime foraging success (Papaj & Vet, 1990). This oviposition learning of long-range odour cues is the main focus of this review.

In parasitoids, learning of odour cues has been studied most extensively (e.g. Vet *et al*., 1995; Smid *et al*., 2007), but parasitoids are also able to learn other cues such as colours, shapes, patterns and spatial information (e.g. Turlings *et al*., 1993; Wäckers & Lewis, 1999; van Nouhuys & Kaartinen, 2008). Parasitoid wasps also learn information about the availability of suitable hosts and use that information during subsequent visits to other patches. For parasitoids of dipteran larvae, it was found that the lower the quality of hosts in previously visited patches, the longer they search on patches with high-quality hosts (Thiel & Hoffmeister, 2009). Similarly, previous experience with unparasitized hosts reduces the acceptance of subsequently encountered hosts that already have been parasitized (Thiel & Hoffmeister, 2009). Addressing oviposition learning is highly relevant from an evolutionary ecological point of view, because the success of a female parasitoid to find and parasitize a host is directly linked to its Darwinian fitness (Papaj & Prokopy, 1989; Dukas & Duan, 2000).

Oviposition learning

In nature, a parasitoid first has to localize the microhabitat that probably contains hosts by using long-range odours (long-distance search, in most cases flight) and subsequently it has to localize the host within this microhabitat. It is only during this local search that the parasitoid encounters host-derived cues, such as faeces, silk, saliva and plant damage. These host cues provide direct information on host presence and suitability and initiate the actual oviposition behaviour; the ovipositor is inserted in the host and eggs are laid. In laboratory set-ups, oviposition-learning bioassays are used to study parasitoid learning and memory formation in ecologically relevant simulations of the natural situation. In most oviposition learning bioassays, researchers immediately bring the parasitoid in contact with host-derived cues, thereby bypassing the parasitoid's long- and short- distance

searching behaviour. In those cases, in which a conditioned stimulus (CS) (an odour) is directly followed by a reward, the conditioning procedure can be considered as a form of classical (Pavlovian) conditioning, where an association is formed between the odour and the oviposition reward. The unconditioned stimulus (US) in oviposition learning consists of two main components, i.e. contact with the host traces and the oviposition in the host, whereas complex natural odour blends are usually employed as CS (see Supplementary Information for further description of cues involved in oviposition learning). After oviposition, the wasp is gently removed, thus ending the conditioning trial.

Different set-ups are used for testing memory retention in parasitoids; all are olfactometers in which a wasp can fly or walk towards the learned odour (Geervliet *et al*., 1998b; Collatz *et al*., 2006; Huigens *et al*., 2009). In a two-choice odour preference test, wasps can choose between the learned odour and a reference odour. Memory retention is calculated for groups of wasps and considered to be present if there is a shift in preference: i.e. when the percentage of trained wasps choosing the 'learned' odour is increased compared with naive wasps. The strength of the innate responses to each of these odours has to be carefully considered to avoid masking of memory retention (Vet *et al*., 1990).

VARIATION IN LEARNING RATE AND MEMORY DYNAMICS

In parasitoid wasps, we encounter interesting variation in learning between closely related species and we argue that learning rate (defined here as the number of trials required for long-term memory (LTM)) and memory dynamics are functional traits involved in the optimization of the foraging task, and shaped by the balance between costs and benefits of these traits.

First of all, learning has several ecological costs. For instance, it would be costly for a parasitoid to change a valuable innate preference after a single oviposition experience on a plant species on which its hosts rarely occur. To prevent such maladaptive associations, animals usually require repeated learning experiences, spaced in time, before they form long-lasting memories but there are also animals that have a high learning rate, i.e. that learn instantly after only one learning experience (Collatz *et al*., 2006; Smid *et al*., 2007; Krashes & Waddell, 2008; Huigens *et al*., 2009). Second, and as a consequence of the previous, learning takes time. During the learning process animals behave suboptimally, which infers a cost to the individual (Laverty & Plowright, 1988). Furthermore, there are costs in terms of energy for maintenance and signalling in the nervous system (Laughlin, 2001) and for memory formation itself (Mery & Kawecki, 2003; Mery & Kawecki, 2005; Burger *et al*., 2008). The sum of all these different costs explains why every insect does not learn instantly: the possible benefits of a high learning rate may just not outweigh the costs.

Parasitoid wasps, with their range of interspecific variation in learning rate and memory dynamics, can be instrumental in elucidating which ecological factors are important in this context.

Innate and learned behaviour

It is important to consider how learning changes innate behaviour. Vet *et al*. (1990) proposed that learning affects innate responses depending on their strength or evolutionary importance: strong innate responses are less affected by experience than weak innate responses, and the ranking of the importance of cues that evoke these responses may be altered by experience. In this concept, stimuli can become more or less important after experience. This process is regarded as a shift in preference. For instance, *Cotesia glomerata*, a parasitoid of cabbage white caterpillars, has a low innate preference for odours of nasturtium, a less common food plant of its hosts (Geervliet *et al*., 1998b). One or multiple ovipositions in hosts on nasturtium will, however, increase the preference towards nasturtium, resulting in a higher percentage of wasps that choose nasturtium over the innately preferred cabbage in a two-choice bioassay. The innate preference for cabbage odours returns after 4 days, however (Geervliet *et al*., 1998a). Innate responses can also be highly persistent. *Leptopilina fimbriata*, a specialist of fly larvae in decaying plant substrates can learn to respond to other substrates, such as fermenting fruits. In the presence of its innately preferred substrate, the majority of the wasps still choose the innately preferred substrate, however. Only when testing these wasps on the learned substrate, it becomes clear that their response to this substrate has increased (Poolman Simons *et al*., 1992). Thus, innate responses and learned responses should not be considered as two separate traits, but as two dynamically interacting components of insect behaviour.

Memory dynamics

Learning rate cannot be considered separate from memory dynamics. The impact of learning is determined by the way the learned information is stored in memory, whereas the type and number of conditioning events determine what type of memory will be formed. To study this aspect, it is essential to discriminate between different forms of memory, since these forms vary in terms of energy consumption, stability and duration.

Eisenhardt (2006) reviewed memory types in the honeybee, whereas a comparable classification exists for the fruit fly, *Drosophila melanogaster* (Margulies *et al*., 2005); both classifications are based on highly similar mechanisms (Stough *et al*., 2006), but the used terminology is somewhat different. First, there is early short-term memory (eSTM or working memory) and late STM (lSTM). These types of memory can be disrupted with anaesthesia, such as a brief exposure to a cold shock in insects, and are known as forms of

anaesthesia-sensitive memory (ASM) (Erber, 1976; Xia *et al*., 1999). ASM lasts, depending on the investigated species, for several minutes up to a few hours (Erber, 1976; Xia *et al*., 1999; van den Berg *et al*., 2011). During the ASM phase, the formation of long-lasting memory types starts, a process called memory consolidation (Margulies *et al*., 2005). Two main forms of consolidated memory can be distinguished based on their sensitivity to protein synthesis inhibitors. LTM requires protein synthesis and can consequently be disrupted by feeding insects a protein synthesis inhibitor. The other type, called mid-term memory in honeybees, is resistant to cold shock but not dependent on protein synthesis, hence it is a form of anaesthesia-resistant memory (ARM). ARM is based on changes in existing proteins, in contrast with LTM consolidation (Tully & Quinn, 1985). ARM and LTM may occur in parallel, and the process of their consolidation may last for hours to days (Smid *et al*., 2007). ARM is less stable and durable than LTM, but it does not require protein synthesis, and is, therefore, regarded as 'cheaper' memory than LTM; flies that consolidated ARM lived longer than those consolidating LTM (Mery & Kawecki, 2005). The type of memory that is consolidated depends on both the number of conditioning trials and the intertrial interval. In aversive conditioning of the fruit fly, single trainings or 10 trainings given without intertrial interval (massed conditioning) induced only ARM formation; LTM was typically formed only after 10 spaced trainings (Tully *et al*., 1994). Studies with bees have shown that conditioning procedures with an intertrial interval of 10 min are sufficient to be regarded as a spaced training protocol (Menzel *et al*., 2001).

Menzel (1999) explained the adaptive value of these different memory types by correlating them to the different use of memory during foraging decisions of a honeybee. In this view, eSTM (in the range of seconds, see Raine & Chittka, 2007 and references therein) is used for intra-patch decisions such as whether to stay or leave a patch of similar flowers, whereas lSTM (in the range of minutes up to 1 h (Eisenhardt, 2006; Menzel, 1999) is used to store information from different patches of flowers that can either be more or less rewarding. Memory-inhibiting genes (see Genes involved in learning and memory) could prevent early memories from being consolidated unless several learning trials, stored in lSTM provide the required 'spaced training'. After a return visit to the hive, memory of the previous foraging bout can be retrieved from ARM, or memory of previous days can be retrieved from LTM, and used to evaluate the quality of subsequent flower patches. Thus, the temporal dynamics of the different memory types serve a specific role in time- and event-specific behaviour of the honeybee. Likewise, the evolution of learning and memory of parasitoid wasps may also be driven by resource distribution.

The dynamics of time- and event-specific learning experience can be expected to vary immensely between species that forage for instance on solitarily versus gregariously feeding hosts, and between specialist and generalist parasitoid species. Wasps that are

foraging for solitarily feeding hosts, for instance, experience many single conditioning trials spaced in time. By contrast, wasps foraging for gregariously feeding hosts may lay half of their eggs when finding a single patch with many hosts, which represents only one, massed-conditioning cycle. Hence, variation can be expected in memory dynamics between closely related species that differ in this ecological aspect. It is clear that the timing of a memory test must be carefully determined and the type of memory that is formed should be known for proper interpretation; information stored in STM has a different function than information stored in LTM.

Ecological factors determining variation in learning rate and memory dynamics

Several factors can be proposed as causes of variation in learning rate and memory dynamics. Here, we first describe different factors in the context of memory dynamics, and then give examples of how a combination of factors can result in different learning and memory dynamics.

Stephens (1993) argued that variability of the environment can determine the value of an innate response, and therefore its susceptibility to be altered by learning. In the case of a parasitoid wasp that experiences the temporal contingency of a cue with an oviposition reward, the variability determines whether that cue reliably predicts subsequent oviposition opportunities. If the variability in that cue is high within a generation, learning can be adaptive but memory must be stored in forms that can be changed easily, so in STM or ARM, not in LTM, because of the risk of learning irrelevant information. In that case, the reliability of a single experience is low; hence information can be only stored as LTM after several repeated conformational experiences. If variability changes slowly over the season, but is stable within a generation, reliability of learned information is high. This could result in a high learning rate: a single experience would be reliable and can then result in LTM formation. However, if between-generation variability is low, the value of innate responses is high, resulting in a strong innate response and a low learning rate. For instance, if hosts can always be found on the same host plants throughout the year, the value of innate preferences is higher than under more variable conditions (Vet *et al*., 1990; Vet & Dicke, 1992; Stephens, 1993). Indeed, both in parasitoids and in bumblebees it was found that strong innate preferences are less likely to be changed after experience (Poolman Simons *et al*., 1992; Ings *et al*., 2009).

The reward value is an important factor that influences learning; stronger rewards increase learning rate (Rescorla, 1988). In the case of oviposition learning in parasitoid wasps, the reward value may also vary considerably. First, hosts can occur solitarily or in clusters; obviously, a cluster of host eggs or larvae will be a much larger reward for a parasitoid wasp. Second, the quality of a host influences the reward value, a larger host

species may, for example, allow for the deposition of more eggs and may result in better quality offspring (Brodeur *et al*., 1998). Some host species may have reduced immune responses compared with other species, resulting in increased survival of the parasitoid's larvae (Thiel & Hoffmeister, 2009). Third, if hosts have been parasitized already by another parasitoid, their value as a reward can be experienced as lower than the value of unparasitized hosts (Thiel & Hoffmeister, 2009). In fact, the value of a reward also depends on the reliability of the learned cue (see above), but if the reward value is high, a less reliable cue may still be valuable to remember.

Roitberg *et al*. (1993) proposed that the number of lifetime learning experiences may influence the value of learning. Indeed, an animal that has only few lifetime learning experiences has less opportunity to spend several experiences to optimize its behaviour, thus slow learning may not be feasible in such a case. This may result in absence of learning ability (Potting *et al*., 1997), but also in a high learning rate (Dukas, 1998b).

Learning and memory dynamics in parasitoid wasps

Research on a number of species of the genera *Cotesia*, *Leptopilina* and *Trichogramma* is described here more in detail, to assess the differential effects of ecological factors on learning rate and memory dynamics.

Genus Cotesia

Four species of the genus *Cotesia*, each with a very different ecology, have been investigated extensively with regard to learning and memory formation. *Cotesia marginiventris* is a highly generalistic species that parasitizes larvae of many lepidopteran species on a number of plant species (Turlings *et al*., 1989). The availability of different host species changes over time and learning may allow *C. marginiventris* to search for the currently most abundant host species (Turlings *et al*., 1993; Tamo *et al*., 2006). By contrast, *Cotesia flavipes*, does not shift its preference after a host encounter (Potting *et al*., 1997). This species parasitizes several species of stemborer larvae that typically occur in large fields of perennial grasses. This is an example of a highly constant and predictable environment in which innate preferences would suffice (Stephens, 1993). Furthermore, the average number of oviposition experiences is low, because the gregarious *C. flavipes* female attacks its host larvae by entering the stemborer tunnel, causing a high mortality rate of the parasitoid owing to the chance of being crushed between the host and the wall of the tunnel. Learning may, therefore, be of little use in this situation (Potting *et al*., 1997).

Cotesia glomerata and *Cotesia rubecula* are closely related parasitoid species of cabbage white caterpillars (*Pieris* spp.) that differ not only in learning rate, but also in memory dynamics (Smid *et al*., 2007). *Cotesia glomerata* formed LTM after a single conditioning

trial, whereas *C. rubecula* needed three trials spaced in time before it formed LTM; massed experiences did not cause such an LTM, as has also been shown in fruit flies (Margulies *et al*., 2005). After both single and multiple conditioning trials, *C. glomerata* consolidated LTM within 4 h, as determined from the maximum effect achieved from application of a protein synthesis inhibitor. By contrast, a single oviposition or three massed ovipositions resulted in ARM formation in *C. rubecula*, which lasted 8 h but had waned after 24 h (van den Berg *et al*., 2011). Three spaced ovipositions did result in LTM consolidation, but this process was completed only after 3 days in *C. rubecula*, suggesting that ARM was present in parallel. These results suggest that both learning and memory formation in *C. rubecula* occur slower than in *C. glomerata* (Smid *et al*., 2007; van den Berg *et al*., 2011). There are a number of reasons why this slow learning and memory consolidation may be adaptive for *C. rubecula*, but not for *C. glomerata*. *Cotesia rubecula* is a solitary parasitoid and a specialist on the solitary caterpillar *Pieris rapae*. *Cotesia glomerata*, on the other hand is a gregarious species and its preferred host, *Pieris brassicae* is a gregariously feeding caterpillar. First, the value of the reward may differ for the two species. The fact that *P. brassicae* occurs in clusters means that finding these hosts provide a larger rewarding value compared with finding a single *P. rapae* caterpillar. The second factor relates to the distribution of caterpillars over plant species. *Pieris brassicae* lays clusters of eggs on dense stands of similar plants. By contrast, *P. rapae* randomly distributes single eggs over different host plant species and travels rather long distances between two oviposition events (Root & Kareiva, 1984). The association between the plant odour and host presence is, therefore, expected to be less reliable for *C. rubecula*. The third factor that differs is the lifetime number of learning experiences, which is low in *C. glomerata* compared with *C. rubecula*. *Pieris brassicae* caterpillars occur in groups on a single leaf, allowing *C. glomerata* to deposit hundreds of eggs at once, which is a large part of its lifetime fecundity, in what is in fact one (massed) conditioning trial. In *C. rubecula*, on the other hand, each oviposition constitutes a single learning trial. The lower lifetime number of learning experiences allows *C. glomerata* to consolidate LTM after a single encounter with *P. brassicae*, whereas such instant learning would be costly for *C. rubecula*. Both the lower learning rate and slow consolidation of memory allow *C. rubecula* wasps to assess the reliability of the information over a longer time window.

Genus Trichogramma

Wasps of the genus *Trichogramma* are minute egg parasitoids of lepidopteran eggs. These gregarious parasitoids have a limited control over flight direction and may instead hitch-hike on female butterflies to the site where they lay their eggs. Two closely related *Trichogramma* species, *Trichogramma brassicae* and *Trichogramma evanescens*, exploit

species-specific anti-aphrodisiac pheromones of two of their hosts, the gregarious *P. brassicae*, and the solitary *P. rapae*. These pheromones are transferred from male butterflies to females during mating to render them less attractive to conspecific males (Andersson *et al*., 2003). When *T. brassicae* detects an anti-aphrodisiac, it innately mounts on a mated (and thus egg-laying) female butterfly and hitch-hikes to a plant where the wasp parasitizes the butterfly's freshly laid eggs (Fatouros *et al*., 2005; Huigens *et al*., 2010). *Trichogramma evanescens* exploits the anti-aphrodisiacs in a similar way, but only after learning. A single operant conditioning trial, where approaching and mounting of a mated female *P. brassicae* butterfly upon the odour stimulus is followed by oviposition in a butterfly egg, induces LTM formation within 24 h (Huigens *et al*., 2009). *Trichogramma evanescens* is expected to have a wider range of host species than *T. brassicae* (Huigens *et al*., 2009; Huigens *et al*., 2010), and although it innately climbs onto butterflies, it does not discriminate between mated female, virgin female and male *Pieris* butterflies. LTM formation after one successful ride, especially on a gregarious, mated female *P. brassicae* butterfly, is adaptive for *T. evanescens* as a few limited opportunities to hitch-hike with such females should be enough to lay all the eggs a female wasp produces during her short lifespan (Doyon & Boivin, 2005). This is similar to the situation for *C. glomerata*.

Genus Leptopilina

Species of the genus *Leptopilina* parasitize *Drosophila* larvae, which they find by probing in very different substrates such as fermenting fruits, decaying mushrooms or decaying plant material. Some species of *Leptopilina* are generalist species that attack multiple drosophilid species in several habitats, whereas other species have a more restricted host and/or substrate range. Poolman Simons *et al*. (1992) compared the generalist *Leptopilina heterotoma* and the specialist *Leptopilina boulardi* parasitoids and showed that both species shifted their preference towards a learned odour after a single oviposition experience. Other studies have found that a preference shift was maintained up to 3 days in *L. heterotoma* (Vet & Schoonman, 1988) and approximately 1–2 days in *L. boulardi* (Poolman Simons *et al*., 1992; Kaiser *et al*., 2009). An important difference between *L. heterotoma* and *L. boulardi* that affects their foraging behaviour is observed in innate preferences, as *L. boulardi* responded invariably strongly to innately preferred apple substrate, regardless of previous experiences, whereas the response of *L. heterotoma* depended on previous experience in all cases tested (Vet & Schoonman, 1988; Poolman Simons *et al*., 1992). This result highlights the importance of addressing learning as well as innate behaviour to understand foraging behaviour of a parasitoid wasp. Interesting questions that remain are whether differences in memory dynamics exist and how different training regimes affect these memory dynamics. Species of the genus *Leptopilina* are well suited to investigate

such questions using a comparative approach.

Using a comparative approach to study learning rate and memory dynamics

Although many studies have focused on learning and memory in parasitoid wasps, only few of these have used a comparative approach. The above mentioned studies on wasps of the genera *Cotesia*, *Leptopilina* and *Trichogramma* have provided valuable insights into the range of variation and the ecological factors that probably contribute to the observed differences. Below we describe some additional comparative studies in this context. Tamo *et al*. (2006) have studied effects of single trial conditioning in three generalist parasitoid species, *Cotesia marginiventris*, *Campoletis sonorensis* and *Microplitis rufiventris*, and showed that the effect of conditioning was different for the three species. While *C. marginiventris* showed a preference shift towards the learned odour, *M. rufiventris* increased its innate preference and *C. sonorensis* did not show any change in its preference. This shows that host range by itself does not satisfactorily explain differences in learning rate in this case (Steidle & van Loon, 2003; Tamo *et al*., 2006). Two species of pteromalid wasps, *Nasonia vitripennis* (Schurmann *et al*., 2009) and *Lariophagus distinguendus* (Collatz *et al*., 2006) were analysed for their memory dynamics by using either an inhibitor of ARM or LTM. In *L. distinguendus*, LTM was formed after single trial conditioning, comparable to *C. glomerata*. In *N. vitripennis*, however, memory formed after a single trial waned between 4–6 days and was therefore assumed not to be LTM. Instead, this memory could be inhibited by a blocker of intermediate memory forms (presumably ARM). Such a difference can be explained by differences in distribution patterns of their respective hosts. *Lariophagus distinguendus* parasitizes stored grain beetles, which occur in large patches, so similar to the situation for *C. glomerata*. *Nasonia vitripennis* is a parasitoid of fly pupae that can be found predominantly in bird nests in low densities (mostly between 1 and 10), which would favour ARM-like memory rather than LTM after a single experience. This is an interesting finding, also because *N. vitripennis* has become a new model species for which genomics tools have been developed (see Future perspectives). Future experiments may reveal a more complete overview of memory dynamics of this species.

In order to properly compare learning rate and memory dynamics of different parasitoids wasp species, it is important to understand how different conditioning set-ups and test procedures can affect a parasitoid's response. Generally speaking, it will be most feasible to compare species that can be assayed with very similar methods, because this will reduce the likelihood that the conditioning itself, instead of the ecological factors under investigation, will influence observed differences.

Intraspecific variation in learning and memory

Besides species-specific differences, intraspecific variation in learning can also be expected. For instance, some bumblebee (*Bombus terrestris*) populations from geographically different locations had a stronger and more persistent innate preference for blue flowers than others, and had a lower learning rate (Ings *et al*., 2009). Colonies obtained from a commercial supplier differed in learning rate, and those with a lower learning rate were less efficient in foraging for nectar (Raine & Chittka, 2008). This shows that innate preferences differ profoundly between populations of the same species, and that these differences affect learning rate that may have influence on efficiency of nectar collection. Thus, at population level, specific adaptations to ecological differences are likely to be common as well as at species level. Similar studies can be performed with parasitoid wasp species that have, for instance, different hosts in geographically different populations. Two populations of *L. heterotoma*, were compared, one of which coexists with its superior competitor *L. boulardi*. This population, therefore, has a limited time window for successful parasitation, resulting in a higher innate response level than the other population. However, no differences in learning were found in this study (Kaiser *et al*., 2009).

Another way to unravel the effects of different ecological factors on learning traits is to perform artificial selection experiments. This approach can be used to test specific predictions raised by species comparisons. For instance, Smid *et al*. (2007) hypothesized that *C. glomerata* does not form ARM after a single learning trial, but only LTM. An artificial selection experiment with this parasitoid species, aimed to decrease learning rate, resulted in a low learning rate line in less than 10 generations (van den Berg *et al*., 2011). Wasps of this low learning-rate line formed normal LTM after spaced conditioning, but formed only ASM after single trial conditioning, instead of LTM, which is formed in unselected wasps. This showed that *C. glomerata* does not form ARM instead of LTM when selection pressure is used against a high learning rate. Apparently, single-trial LTM formation is 'hard-wired' in this species, when it is rewarded with *P. brassicae*. Experiments to assess costs of having a high or low learning rate are a logical next step when such selection lines are established. In *Drosophila*, it was shown that learning has both operating costs as well as constitutive costs: flies from a high learning line had a reduced larval competitive ability (Mery & Kawecki, 2003) and a reduced longevity (Burger *et al*., 2008).

OPPORTUNITIES FOR CONVERGENCE OF ECOLOGY AND NEUROSCIENCES

Ecologists have eagerly exploited parasitoid wasps to investigate the adaptive value of variation in learning rate and memory dynamics, providing insights on the ecological relevance of these differences. However, to fully understand learning and memory, a

multi-disciplinary approach is needed in which ecological and evolutionary studies are combined with mechanistic studies. The vast amount of neuroscientific knowledge of a few well-established insect model species, mainly fruit flies and honeybees, provides valuable reference information and promising candidate neurons and genes to investigate mechanisms underlying learning and memory formation (Davis, 2005; Eisenhardt, 2006).

Neural pathways involved in learning and memory

When insects learn odours, these odours are detected by olfactory receptor neurons and the resulting information is processed in the insect brain. Differences in perception or neurological processing of the odour cues may play a role in the observed differences in learning rate. An analysis of antennal responses to individual odour components emitted by cabbage plants showed that *C. glomerata* and *C. rubecula* have a comparable olfactory receptive range (Smid *et al*., 2002) and also the morphology of their antennal sensilla is similar (Bleeker *et al*., 2004). Furthermore, a three-dimensional analysis of the glomeruli in the antennal lobe (AL), which is the first brain structure in the olfactory pathway, showed that the glomerular organization is similar for both species (Smid *et al*., 2003). These first results suggest that the perception and processing of odours in these species are comparable but more research is necessary to compare different species and include higher integrative centers, like the mushroom bodies (MB) and the lateral horn (LH) (Davis, 2005).

Another possibility that may explain differences in memory dynamics of parasitoid wasps is the perception of the US. During oviposition, host traces and host haemolymph are perceived by the antennae and the ovipositor (Takasu & Lewis, 2003; van Lenteren *et al*., 2007). It is currently unknown how this information is transmitted to the brain. In honeybees and fruit flies, octopaminergic neurons were found to transmit rewarding stimuli in the brain, which is distinctly different from the dopaminergic pathway that transmits information on aversive stimuli (Schwaerzel *et al*., 2003). In honeybees, a sucrose reward is detected by receptor neurons on the mouthparts, which activate the VUMmx1 neuron. This neuron innervates the AL, MB and LH, which are, therefore, all putative locations for the convergence of the US and CS (Hammer, 1993). It is expected that neurons with similar properties transmit the host reward signals in the parasitoid's brain and differences in response characteristics or in the density of axonal endings, from which octopamine is released, may underlie the observed species-specific differences in learning rate and memory dynamics. Several octopaminergic neurons have already been identified in *C. glomerata* and *C. rubecula* (Bleeker *et al*., 2006b) and it would be interesting to investigate which neurons transmit which reward signals and whether differences in this pathway can be correlated to differences in memory dynamics.

Genes involved in learning and memory

The genetic pathways that are involved in memory formation are highly conserved, even for organisms ranging from insects to mammals (Dubnau, 2003). Research on model insects has resulted in a long list of genes that are involved in memory formation (e.g. Davis, 2005). One of the most extensively studied pathways involved in learning is the cAMP-dependent signalling pathway (Margulies *et al*., 2005; Eisenhardt, 2006). The CREB (cAMP responsive element binding protein) gene is a transcription factor in this pathway, which plays a decisive role in the initiation of LTM formation (Yin *et al*., 1994; Abel *et al*., 1998). Several different isoforms resulting from alternative splicing of CREB transcripts are known, which can act as either transcriptional activator or suppressor (Yin *et al*., 1995). It was shown that high expression levels of CREB-suppressor isoforms inhibit LTM formation in a variety of species (Yin *et al*., 1994; Bartsch *et al*., 1998). This resulted in the hypothesis that the balance between CREB activators and inhibitors acts as a molecular switch, determining the number of spaced training events that is required for LTM formation (Yin *et al*., 1995; Perazzona *et al*., 2004; van den Berg *et al*., 2010). Nine different CREB transcripts were identified in *C. glomerata* and *C. rubecula*, predicting putative CREB activator and suppressor isoforms, which are identical in both species. A first study on CREB expression in naive wasps of these two species showed the relative abundance of each of the transcripts, which was similar for the most abundant transcripts (van den Berg *et al*., 2010).

So far, the CREB gene, which is a promising candidate gene for differences in learning rate, has been the only gene investigated in parasitoids in this context. The candidate gene approach (Fitzpatrick *et al*., 2005) can be used to study other genes that may be involved in differences in learning and memory dynamics.

Future perspectives

Some exciting new developments contribute to the potential of parasitoid wasps as model organisms for multi-disciplinary studies. Three species of the genus *Nasonia* have recently been sequenced and annotated (Werren *et al*., 2010) and molecular research on other parasitoid species can benefit greatly from this genome sequence information. Furthermore, several molecular tools and resources, such as microarrays and expression array data, are available for these species and it is possible to interbreed the different *Nasonia* species, allowing backcrossing of loci of one species into the genetic background of another species. This allows for identification of quantitative trait loci (Loehlin *et al*., 2010a).

Other new tools that will probably accelerate neuroscientific research in parasitoid wasps are RNAi and next-generation sequencing. RNAi makes it possible to use a direct genetic approach in non-model organisms (Belles, 2010). It can be used to reduce the

expression of a specific gene and to subsequently investigate the direct effect of this gene on the behaviour of the insect or on the expression of other genes. Next-generation sequencing provides a genomics approach for non-model organisms (Gibbons *et al.*, 2009). It will provide information on expression levels of all genes and will allow a better understanding of the genetic networks that cause interspecific variation in memory dynamics.

Research on parasitoid wasps can provide a wealth of information on both the ecological relevance and the neural and genetic mechanisms underlying variation in learning and memory formation. Such multi-disciplinary research is necessary to understand the mechanisms that underlie naturally occurring variation, but it will also elucidate the true significance of neural or genetic variation. Both ecologists and neuroscientists can greatly benefit from a convergence of their fields. Considering the conserved genetic pathways that are involved in learning and memory formation (Dubnau, 2003), this integration may not only further the field of insect behaviour, but may also simultaneously enhance our understanding of learning and memory in higher animals.

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SUPPLEMENTARY INFORMATION

Important cues in oviposition learning

The unconditioned stimulus (US) in oviposition learning consists of two main components, i.e. contact with the host traces and the oviposition in the host. The host traces (faeces, silk, salivary, feeding damage) have three different functions which can be described analogous to the description of the proboscis extension reflex in the honeybee (Hammer & Menzel, 1995). First of all, there is a releasing function; the host traces initiate oviposition behaviour (van Alphen & Vet, 1986). Secondly, detection of host traces causes arousal and sensitization, which has also been termed priming (Turlings et al., 1993), a function denoted as the modulatory function in PER conditioning (Hammer & Menzel, 1995). This characteristic can be described as a general increase in the responsiveness of wasps to stimuli and is considered non-associative learning (Takasu & Lewis, 2003). The modulatory function of host cues is essential to "prepare" the wasp for learning; when all host cues were washed away, no increased responsiveness to odours occurred in *C. glomerata* (Bleeker *et al*., 2006a). Thirdly, contact with host traces can have a reinforcing function, a wasp could form memory when it encounters host traces only (Lewis & Martin, 1990; Vet & Groenewold, 1990; Takasu & Lewis, 2003), without subsequent oviposition, but this memory was only short-lasting compared to a complete oviposition experience in the wasp *Microplitis croceipes* (Takasu & Lewis, 2003). The actual oviposition or contact of the ovipositor with the host haemolymph had an additional reinforcing effect resulting in a stronger association of the odour with the presence of hosts (Takasu & Lewis, 2003; Collatz *et al*., 2006). A similar phenomenon is seen in PER conditioning in honeybees; sucrose stimulation of the antennae alone, or feeding sucrose alone resulted in memory that decayed faster than after sucrose stimulation of the antenna followed by sucrose feeding (Wright *et al*., 2007).

Studies of oviposition learning often use a CS consisting of natural odour blends in natural concentrations; this feature facilitates interpretation of preference shifts from innate to learned responses. Some studies suggest that a blend is perceived as a singular entity (Perez-Orive *et al*., 2002; Riffell *et al*., 2009), whereas other studies suggest that individual components can be recognized (Meiners *et al*., 2003; Reinhard *et al*., 2010). Parasitoids generalize comparable odour blends to a certain extent, but can learn to discriminate between highly identical odour blends (Vet *et al*., 1998; Meiners *et al*., 2002). This shows that learning improves the ability to detect complex rewarding blends against a background of unrewarding blends.


Chocolate




Vanilla


Vanilla



Chocolate

Chapter 3

High-throughput olfactory conditioning and memory retention test show variation in *Nasonia* parasitic wasps

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ABSTRACT

Most of our knowledge on learning and memory formation results from extensive studies on a small number of animal species. Although features and cellular pathways of learning and memory are highly similar in this diverse group of species, there are also subtle differences. Closely related species of parasitic wasps display substantial variation in memory dynamics and can be instrumental to understanding both the adaptive benefit of and mechanisms underlying this variation. Parasitic wasps of the genus *Nasonia* offer excellent opportunities for multidisciplinary research on this topic. Genetic and genomic resources available for *Nasonia* are unrivaled among parasitic wasps, providing tools for genetic dissection of mechanisms that cause differences in learning. This study presents a robust, high-throughput method for olfactory conditioning of *Nasonia* using a host encounter as reward. A T-maze olfactometer facilitates high-throughput memory retention testing and employs standardized odors of equal detectability, as quantified by electroantennogram recordings. Using this setup, differences in memory retention between *Nasonia* species were shown. In both *Nasonia vitripennis* and *Nasonia longicornis*, memory was observed up to at least 5 days after a single conditioning trial, whereas *Nasonia giraulti* lost its memory after 2 days. This difference in learning may be an adaptation to species-specific differences in ecological factors, for example, host preference. The high-throughput methods for conditioning and memory retention testing are essential tools to study both ultimate and proximate factors that cause variation in learning and memory formation in *Nasonia* and other parasitic wasp species.

INTRODUCTION

Learning and memory have been shown in a large number of animal species, with a focus on a few well-established model species, including the marine snail (a mollusc), the fruit fly and honeybee (insects), several bird species and mammals (most importantly mouse and rat) (Bottjer & Johnson, 1997; Chen & Tonegawa, 1997; Margulies *et al*., 2005; Eisenhardt, 2006; Reissner *et al*., 2006). The importance of learning is reflected by strikingly similar features of memory formation in this diverse group, such as the effects of massed or spaced conditioning, as well as highly conserved neural and genetic pathways that underlie this trait (Dubnau, 2003). Nonetheless, differences can be observed as learning is shaped by differences in ecology between animal species (Hoedjes *et al*., 2011). Furthermore, memory dynamics can vary within a species depending on the type of conditioning (Nakatani *et al*., 2009; Burke & Waddell, 2011). Variation is determined by factors such as quantity and quality of the reward or punishment, and reliability of learned cues (Hoedjes *et al*., 2011). In-depth studies on a larger number of species and on different types of conditioning are needed to understand variation in learning and memory. Parasitic wasps can be instrumental for understanding this variation.

Several wasp species are ecologically and behaviorally well studied, are known to learn environmental cues readily (Vet *et al*., 1995) and display substantial interspecific and intraspecific variation in memory dynamics (Tamo *et al*., 2006; Smid *et al*., 2007; van den Berg *et al*., 2011). Learning plays an important role in optimizing foraging efficiency, and thus reproductive success, of female wasps searching for hosts (Papaj & Vet, 1990). Parasitic wasps, therefore, feature an alternative type of appetitive conditioning, based on the reward that a female wasp experiences when finding and ovipositing in a host, so-called oviposition learning. This study is the first to show differences in oviposition learning between species of the genus *Nasonia*. Especially these species are excellent for comparative, multidisciplinary studies of variation in learning and memory. Many aspects of the biology of *Nasonia vitripennis*, *Nasonia giraulti* and *Nasonia longicornis* have been studied, and the species are known to differ in host range and host preference (Darling & Werren, 1990; Desjardins *et al*., 2010). Furthermore, their genomes have been sequenced, and there are several unique genetic tools available (Werren & Loehlin, 2009; Werren *et al*., 2010). This provides opportunities to test hypotheses on how ecological differences may result in different memory dynamics as well as studying the genetic basis of this variation.

Previous studies showed learning in *N. vitripennis* but involved laborious methods for conditioning and testing memory retention (Oliai & King, 2000; Schurmann *et al*., 2009). In *Drosophila*, the availability of standardized, high-throughput methods for both conditioning and memory testing (Tully & Quinn, 1985) was a prerequisite for the immense success of this model species. This article describes the development of both high-through-

put olfactory conditioning and a memory retention test for the *Nasonia* model system. This setup was successfully used for *N. vitripennis*, *N. longicornis* and *N. giraulti*.

MATERIALS AND METHODS

Nasonia strains and maintenance

Cultures of *Nasonia* were reared in polystyrene rearing vials (dimensions 28.5 x 95 mm) with foam stoppers (Genesee Scientific, San Diego, CA, USA) in a climate cabinet under a constant temperature (25°C) and a photoperiod of 16:8 (L/D). The wasps were reared on Calliphora vomitoria pupae, which were obtained as maggots (Kreikamp, Hoevelaken, the Netherlands) and allowed to pupate at room temperature and subsequently stored in a fridge for a maximum of 1 week. Inbred strains of *N. vitripennis* (AsymC), *N. giraulti* (RV2x(U)) and *N. longicornis* (IV7(U)) were used for the experiments (Werren *et al*. 2010). Both females and males were collected on the day of emergence to ensure mating and were kept in vials with access to honey and water. Females were used in experiments between 1 and 3 days after emergence.

Electroantennogram analysis

Odors that were expected to be neutral stimuli to *Nasonia* were chosen for the experiments. Vanilla and chocolate extract (Natural Chocolate extract and 2× Royal Brand Bourbon Vanilla extract; Nielsen-Massey Vanillas Intl., Leeuwarden, the Netherlands) are watery extracts that produce complex odor blends. Electroantennogram (EAG) analysis was performed to confirm that all three species had a similar sensitivity to these two odors. A large difference in sensitivity to one of the odors would make it more difficult for wasps to detect both odors in the olfactometer and could hinder the detection of memory retention. We used an EAG setup as described previously (Smid *et al*., 2002). Odor blends were diluted in water to 1%, 10% and 100%, and glass capillaries (Stuart SMP1/4, inner diameter: 1.3 mm, length adjusted to 30 mm; Bibby Scientific, Staffordshire, UK) were filled with these odors. The glass capillaries were subsequently placed in a Pasteur pipet and attached to the wall of the pipet with double-sided adhesive tape to ensure that both ends are exposed to the air in the Pasteur pipet. The resulting odor cartridge was then sealed with parafilm until use. *Nasonia* wasps were decapitated and the very tip of the antenna was cut with a scalpel. The tip of the antenna was then brought into contact with the glass recording electrode of the EAG setup, whereas the head, with a part of the prothorax attached, was connected to the ground electrode, as described previously. Natural almond extract (Nielsen-Massey Vanillas Intl.) 10% diluted in water was used as a standard odor. All measurements were corrected for responses to a blank odor stimulus (pure water) and normalized to the

standard odor as described previously (Smid *et al*. 2002).

Olfactory conditioning assay

This olfactory conditioning assay is a simulation of natural behavior, in which female wasps likely use odor cues to find suitable host patches and then localize hosts in the patch (Whiting, 1967). The long-range searching behavior is omitted in this assay and female wasps are placed in close contact with the hosts instead. The female wasps will immediately perceive the odor and will encounter a host quickly thereafter; for this reason, the conditioning assay is considered a form of classical (Pavlovian) conditioning. The aim of the conditioning procedure was to associate a rewarding host encounter (unconditioned stimulus, US) with one of the two odors, vanilla or chocolate (conditioned stimulus, CS+), followed by exposure to the other odor without reward (CS–). This differential conditioning procedure was carried out in a reciprocal manner, where each group of 48 wasps was divided into two parts, one receiving a CS+ with chocolate followed by a CS– with vanilla, and the other a CS+ with vanilla and a CS– with chocolate. CS+ conditioning was adapted from Schurmann *et al*. (2012) and was performed at room temperature in wells of a 12-well microtiter plate (Greiner Bio-One, Alphen aan den Rijn, the Netherlands), which allows individual observations during the conditioning procedure. Two *C. vomitoria* pupae (US) and a piece of filter paper (0.75 cm2) with 1 µl vanilla or chocolate extract (CS) were placed in a well, before one female wasp was released from an aspirator into the well, which was then closed (not airtight) with a plastic cap (protection plug, 21.7 mm diameter; Skiffy, Amsterdam, the Netherlands). A wasp can immediately smell the odor and, because of the small size of the well, will encounter a host quickly thereafter. The wasp was then left for 1 h, in which she typically drills into a host pupa, forms a feeding tube and starts to feed from it. Wasps that did not start drilling within the first 30 min of the training were noted and removed from the experiment after 1 h. All wasps that had shown drilling behavior were then gently transferred to an empty rearing vial and kept here, as a group, for 15 min. A glass capillary with one closed end (ID 1.3 mm, length adjusted to 30 mm; Fisher Emergo, Landsmeer, The Netherlands) was filled with the complementary odor (CS–) using a syringe and was then placed in the vial. Wasps were exposed to this odor without a reward for 15 min. Earlier research on *N. vitripennis* has shown that presenting the insect with an odor (CS–) after the reward (a host experience) results in decreased attraction to that odor (Schurmann *et al*., 2009). Differential conditioning with two odors (CS+ and CS–) was therefore expected to result in a stronger preference shift toward the CS+ than a conditioning with CS+ only, similar to the result in fruit flies (Tully & Quinn, 1985). When conditioning was finished, wasps were transferred to rearing vials with access to honey and water and kept in a climate cabinet under a constant temperature (25°C) and a

photoperiod of 16:8 (L/D) until testing. Groups of 48 (two reciprocal groups of 24 wasps) *N. vitripennis*, *N. longicornis* or *N. girault*i were conditioned as described above. Each reciprocal group of 24 wasps was then divided in two groups of 12 wasps at 4 (±0.5), 24 (±1), 48 (±1), 72 (±1), 96 (±1) or 120 (±1) h after conditioning to test memory retention (see below). This was repeated five times on different days, resulting in 20 groups (10 per reciprocal conditioning) per data point. In addition, groups of *N. vitripennis* were 'conditioned' with the same procedure but without host reward to assess the effects of presentation of the odors alone. These wasps were tested 4 h (±0.5) after conditioning.

Memory retention test

The T-maze olfactometer (Figure 1) designed for testing memory retention in *Nasonia* was adapted and modified from the well-established T-maze designed for *Drosophila* (Tully & Quinn, 1985). Wasps of the genus *Nasonia* are commonly observed to move by walking, making this setup suitable for this species. Differences in behavior between *Drosophilia* and *Nasonia* did require a number of modifications to the design. The T-maze for *Nasonia*

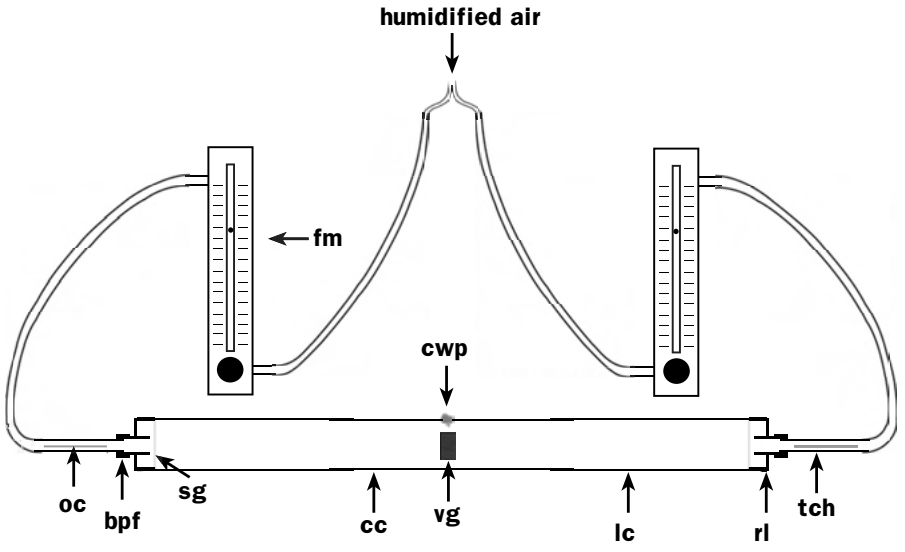

Figure 1: Schematic overview of the T-maze for *Nasonia*. The T-maze consists of a central cylinder (cc) that slides into two lateral cylinders (lc). The cylinders are connected to a Teflon capillary holder (tch) with a brass press fit (bpf) fitted on a polyvinyl chloride (PVC), removable lid (rl). The Teflon capillary holders are holding the odor capillaries (oc) for odor supply. Humidified and charcoal-filtered air is blown into the T-maze via adjustable flow meters (fm) and can leave the T-maze via the ventilation grid (vg) in the central cylinder. Wasps are released through a hole in the center of the central cylinder and are allowed to move freely in the T-maze. Escape is prevented by closing the hole in the center with a cotton wool plug (cwp), netting on the ventilation grid and netting on the side grid (sg).

was enlarged, because crowding of *Nasonia* wasps in smaller tubes resulted in fleeing from each other.

The design for *Nasonia* does not include a training tube and sliding center as training was performed in microtiter plates as described above. The T-maze was made of Plexiglas and consisted of three tubes. Two lateral tubes (length: 20 cm and diameter: 4 cm) were connected to the center tube (length: 20 cm and diameter: 3.5 cm), which were attached by sliding them into each other. The distal ends of the tubes were connected to Teflon tubing for odor supply. Charcoal filtered, moisturized air (60–70% relative humidity) was blown into the apparatus with a flow rate of 100 ml/min on each side, which can leave the setup through ventilation slits in the middle tube. Polyamide netting (Monodur, PA 250; Nedfilter b.v., Almere, the Netherlands) prevented wasps from entering the Teflon tubing and ventilation slits. Capillaries filled with odors (chocolate or vanilla, respectively, as described above) were introduced in the Teflon tubing adjacent to the connection with the lateral Plexiglas tubes. Odor supply was adjusted for each species by the number of capillaries that were placed in the Teflon tube until naïve wasps distributed themselves approximately 50:50 when given a choice for chocolate and vanilla (groups of 12 (±2) wasps were released simultaneously, and 20 groups were tested on 5 different days). For testing memory retention in *N. vitripennis* and *N. longicornis*, two capillaries of chocolate extract and two capillaries of vanilla extract were placed in the tubes. In the case of *N. giraulti*, four capillaries of chocolate extract and two capillaries of vanilla extract were placed in the tubes. The entire setup was shielded from environmental light and surrounded by white surfaces, and illumination was provided from above by LED strip illumination (Grandi 'white' 6000-6500K, 170 lm/m with 30 leds/m mounted against a white shelf 40 cm above the T-maze). During the run, a sheath of white paper (Satino, van Houtum, the Netherlands) shielded the T-maze from direct illumination.

Standard procedure involves testing memory for each of the two reciprocal pairs of up to 24 wasps in two runs to prevent crowding in the T-maze. Ten to twelve wasps were released into the middle part through a circular opening (8 mm diameter) using an aspirator, after which the opening was closed with a cotton wool plug. The memory retention test was performed at a temperature of 23.5 ± 1°C. Wasps that were released in the T-maze are allowed to move freely in the tubes for 10 min, after which their choice is recorded. Only the wasps that have entered one of the two arms were considered to have made a choice. Wasps that remain in the middle part of the T-maze are regarded as non-responding. On average, approximately 5–10% of the wasps do not respond in the test, and these wasps were ignored in the data analysis.

Experiments were performed to assess whether releasing wasps in groups of 12 (the number of wasps trained in one 12-well microtiter plate) affected the choice they made

in the T-maze compared with individually released wasps in *N. vitripennis*. A total of 12 (±2) wasps trained on vanilla and chocolate (CS+) were tested individually 4 h (±0.5) after conditioning. Two reciprocal groups were reconstructed from the results of individually released wasps. This was repeated five times on different days. Memory retention was compared with wasps that had been tested in groups of 12 (±2) wasps 4 h (±0.5) after conditioning.

Data analysis

Relative EAG responses were calculated as a percentage response compared with 10% almond. We used univariate analysis of variance (ANOVA) to test whether there were differences in odor perception between the two odors, chocolate and vanilla, for each species and whether there were differences in relative sensitivity between the species. Naïve preferences toward the odors in the T-maze were analyzed by calculating percentage of wasps choosing vanilla for each group (n = 20). These percentages were tested with a t-test with a test value of 50.

Conditioning is expected to result in a shift in preference of the two groups of wasps toward the CS+. The difference in preference between two reciprocal groups was used as a measure for memory retention and is represented by the performance index (PI), comparable to the PI described by Tully *et al*. (1994). The first group has vanilla as CS+ and chocolate as CS−, and the second group has chocolate as CS+ and vanilla as CS−. The PI is calculated by subtracting the percentage of the second group choosing vanilla (CS−) from the percentage of the first group choosing vanilla (CS+): group 1 (CS+) − group 2 (CS−). If all trained wasps choose CS+, the difference between two reciprocal groups is at its maximum and the PI would be 100. This would represent perfect memory retention. When there is no memory retention, the two groups will choose similarly; this would result in a PI of 0. In order to monitor odor bias or preference after conditioning, an analysis was performed to observe if reciprocal groups showed a similar shift in preference toward the CS+ after conditioning. This was performed by subtracting the percentage of the second group choosing chocolate (CS+) from the percentage of the first group choosing vanilla (CS+). An equal shift in preference in both groups will result in a value of 0, which means that there is no bias toward one of the odors. All values from different time points after conditioning were taken together for each of the three species (n = 60), and a t-test was performed to test for odor bias after conditioning.

Two reciprocal groups of 24 wasps that were trained on 1 day were tested in two series of 12 wasps. Two reciprocal series of trained wasps were tested immediately after each other and a PI was calculated for these wasps. The setup was then turned 180° to average out the effect of external factors, and the two remaining series of wasps were tested. This

results in two PIs per day. The experiment was repeated five times in total, resulting in 10 PIs. Normality and equal variances of the data were tested, and a t-test was used to test memory retention for each species at each time point. Univariate ANOVA was used to test whether there were differences between species, between time points and whether these factors interact. PIs of wasps released in groups or individually (*N. vitripennis*, 4 h after training) were compared with an independent samples t-test. All statistical analyses were performed in SPSS, version 19 (IBM, Armonk, NY, USA).

RESULTS

High-throughput olfactory conditioning assay for Nasonia

This study describes a conditioning assay in which female wasps of the genus *Nasonia* associate an odor (CS+) with a rewarding host encounter, in this case two *C. vomitoria* pupae. When a female wasp encounters a host pupa, she will touch the host with her antennae and she will subsequently start drilling into the puparium. In general, 85–100% of all females will initiate drilling within 30 min. When a female finishes drilling, she can build a feeding tube and feed from the host. No obvious differences were observed in drilling or feeding behavior between *N. vitripennis*, *N. longicornis* and *N. giraulti* in pilot experiments (results not shown). After associating an odor (CS+) with the rewarding host encounter, the wasps were exposed to the second odor without a reward (CS–). This conditioning step was found to improve PIs compared with training with the CS+ alone in pilot experiments (results not shown) and was therefore included in the procedure. Presentation of the odors alone, without a reward, does not result in significant memory formation (PI = −0.2 ± 5.5; n = 10, $t_9 = -0.038$, $P = 0.979$). This conditioning assay can be used for all three species making it suitable for comparative studies on *Nasonia* spp. Training wasps in microtiter plates allows simultaneous conditioning of large numbers of individual wasps, while efficient individual monitoring remains possible.

Choice and concentration of odors

All three species responded in a dosage-dependent manner to both vanilla and chocolate odor. The EAG analyses (Figure 2) showed that there was a significant effect of concentration as well as of species, showing that there are differences in relative responses between species as well as between different odor concentrations. There was, however, no significant effect of odor, which indicates that the responses to both odors are equal (odor: $F_{1,240} = 0.979$, $P = 0.323$; concentration: $F_{2,240} = 89.343$, $P < 0.001$; species: $F_{2,240} = 4.177$, $P = 0.016$; odor × concentration: $F_{2,240} = 0.733$, $P = 0.481$; odor × species: $F_{2,240} = 0.752$, $P = 0.472$; concentration × species: $F_{4,240} = 1.62$, $P = 0.170$; odor × concentration × species: $F_{4,240} = 0.449$, $P = 0.773$).

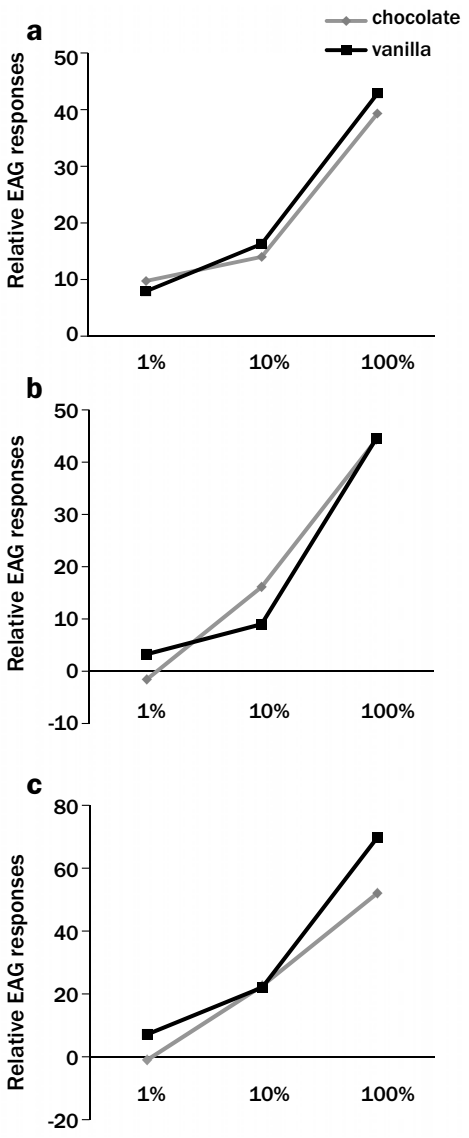

Figure 2: Electroantennogram responses. Relative EAG responses of (a) *N. vitripennis*, (b) *N. longicornis* and (c) *N. girault* to different concentrations of chocolate and vanilla odor. All responses were corrected for blank with EAG response to 10% almond odor in water (n = 17 for *N. vitripennis* and n = 13 for *N. longicornis* and *N. girault*). There was a significant effect of concentration and species, but no significant effect of odor. None of the interactions were significant.

Behavioral responses of unconditioned wasps toward vanilla and chocolate odor showed that when two capillaries of vanilla and chocolate were placed in the T-maze, both *N. vitripennis* ($t_{19} = -0.292$, $P = 0.774$) and *N. longicornis* ($t_{19} = 0.158$, $P = 0.876$) preferred the odors equally, resulting in a near 50:50 distribution. For *N. girault* ($t_{19} = 0.737$, $P = 0.470$), two capillaries of vanilla and four capillaries of chocolate resulted in a near 50:50 distribution. Both the results from EAG analyses and behavioral tests show that vanilla and chocolate are suitable odor sources for this assay.

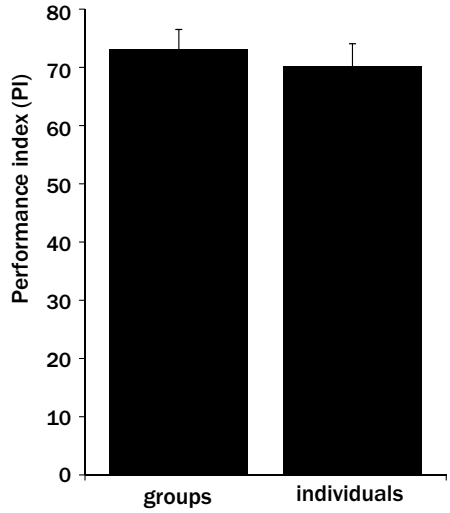

Figure 3: Effect of testing *Nasonia vitripennis* in the T-maze individually vs. a group of wasps. Memory retention of individual and groups of 10-13 *N. vitripennis* wasps was compared 4 h after conditioning to assess if testing groups of wasps simultaneously had an effect on the PIs. The PI of wasps tested in groups is 73.0 ± 3.5 (n = 10); the PI of individually tested wasps is 70.1 ± 4.0 (n = 5). There is no significant difference between these scores.

High-throughput memory retention test using the T-maze

A T-maze olfactometer was designed to allow high-throughput testing of memory retention in *Nasonia*. Groups of wasps can be tested simultaneously in this olfactometer, greatly reducing time that is needed to test a certain number of wasps. Experiments were performed to determine whether testing wasps in groups had any effect on the choice behavior in the T-maze. Although no apparent interference of wasps was observed, i.e. wasps did not appear to avoid or follow each other, it may be possible that a wasp is influenced by choices that other wasps of a group make. Comparisons between PIs calculated from reciprocal groups of 12 (±2) wasps tested either individually or as groups showed that there was no effect on PI (group tested: PI = 73.0, n = 10; individually tested: PI = 70.1, n = 5; $t_{13} = 0.517$, $P = 0.614$) (Figure 3).

In the T-maze, the wasps walk toward one of the two odor sources. *Nasonia vitripennis*, *N. longicornis* and *N. giraulti* were observed to walk readily into the two lateral tubes, and only a minority of the wasps did not leave the middle tube. This T-maze setup can be considered suitable for all *Nasonia* species.

Memory retention in Nasonia

Memory retention was tested for *N. vitripennis*, *N. longicornis* and *N. giraulti* at 4, 24, 48, 72, 96 and 120 h after one olfactory conditioning (Figure 4). All three species were found to have a significant retention of memory at 4 and 24 h after conditioning. These results show that both the conditioning assay and the memory retention test can be used successfully for these *Nasonia* species. After 48 h, *N. giraulti* had lost its memory, whereas both *N. vitripennis* and *N. longicornis* have memory up to at least 120 h after conditioning (Figure 4 and Table 1). The memory dynamics of the three species differ from each other ($F_{2,174} = 12.649$, $P < 0.001$)

Table 1: Memory retention of *Nasonia* after a single conditioning (statistics). A t-test was used to test memory retention of each species at 4, 24, 48, 72, 96 and 120 h (n = 10) after the oviposition conditioning procedure. Asterisks indicate the level of significance (***$P < 0.001$, ** $< P < 0.01$, *$0.01 < P < 0.05$, n.s. = not significant).

Hours (h)	PI	t_9	*P*	PI	t_9	*P*	PI	t_9	*P*
	N. vitripennis			*N. longicornis*			*N. giraulti*		
4	73.0	20.92	<0.001***	67.2	13.47	<0.001***	49.4	13.21	<0.001***
24	67.5	14.62	<0.001***	53.0	7.77	<0.001***	44.5	10.30	<0.001***
48	62.7	11.70	<0.001***	47.0	10.21	<0.001***	5.7	0.94	0.372 n.s.
72	44.7	8.93	<0.001***	14.1	2.88	0.018*	1.8	0.28	0.789 n.s.
96	39.3	8.28	<0.001***	14.3	3.36	0.008**	-3.9	-0.60	0.564 n.s.
120	38.4	14.42	<0.001***	17.9	2.94	0.016*	-1.9	-0.35	0.734 n.s.

and the PI decreases over time ($F_{1,174} = 177.556$, $P < 0.001$). There was no significant interaction of species and time ($F_{2,174} = 2.349$, $P = 0.098$). These results from the analyses show that both *N. vitripennis* and *N. longicornis* have a long-lasting memory retention after a single conditioning, although their PIs decrease over time. *Nasonia girault*i only has a relatively short memory retention up to 24 h after a similar conditioning.

The odor preference was 50:50 for vanilla and chocolate in naïve animals, but after conditioning a bias toward one of the two odors was found in all three *Nasonia* species (n = 60). Both *N. vitripennis* (8.9%, $t_{59} = -3.508$, $P = 0.001$) and *N. longicornis* (6.0%, $t_{59} = -2.181$, $P = 0.033$) have a slight bias toward chocolate odor after conditioning. *Nasonia girault*i (7.1%, $t_{59} = 2.493$, $P = 0.015$) has a slight bias toward vanilla odor after conditioning. This result shows that the odor preference of the three species changes after conditioning, emphasizing the importance of using a reciprocal setup for memory retention tests.

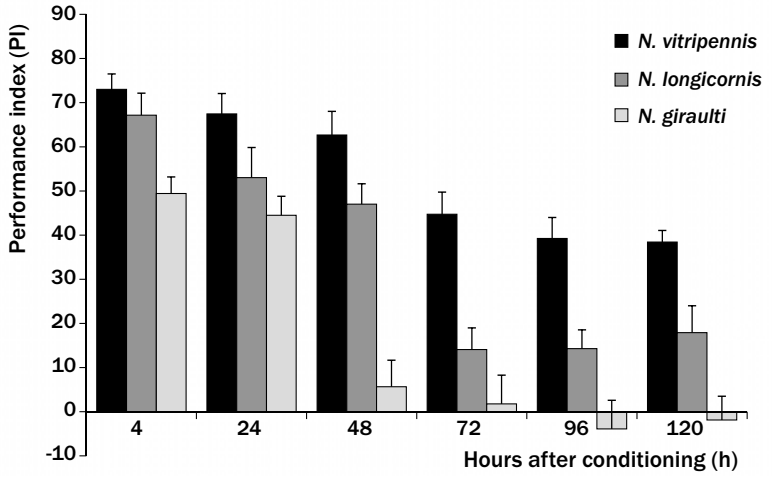

Figure 4: Memory retention of *Nasonia* after a single conditioning. Memory retention of *N. vitripennis*, *N. longicornis* and *N. girault*i was tested in the T-maze at 4, 24, 48, 72, 96 and 120 h (n = 10) after the oviposition conditioning procedure. All three species had significant memory at 4 and 24 h. Both *N. vitripennis* and *N. longicornis* have significant memory at 48, 72, 96 and 120 h after training as well, whereas *N. girault*i did not. The PIs of the three species differ from each other and differ between time points. Species and time points also interact.

DISCUSSION

This study presents a novel method for high-throughput olfactory conditioning and memory retention testing of *Nasonia*. The olfactory conditioning assay was used to investigate the association of vanilla or chocolate odor (CS+) with the reward of finding a host (US) in female wasps of *N. vitripennis*, *N. longicornis* and *N. girault*i. All three *Nasonia* species

could be conditioned using a similar protocol, allowing a good comparison of learning and memory between the species. The US consisted of multiple components in this assay; the female wasp first touches the host with her antenna and thereby will perceive chemical information inducing drilling behavior. She drills a hole in the puparium and will use her ovipositor to find the host and assess its quality; then she feeds from the pupa, which is required for egg production (Whiting, 1967). Previous studies have shown that drilling alone is sufficient for *N. vitripennis* to form an anesthesia-resistant memory that lasts up to 4 days (Schurmann *et al*., 2009). Both drilling and host feeding result in a long-term, protein synthesis-dependent memory (LTM) that lasts for at least 6 days (Schurmann *et al*., 2012). This shows that these two components of the US affect the strength of the memory differently. Access to the host for 1 h typically enables wasps to obtain multiple experiences consisting of drilling and host feeding, but actual oviposition does not occur yet (Schurmann *et al*., 2012). It will be interesting to study how drilling alone, drilling and host feeding and oviposition affect memory retention in *N. longicornis* and *N. giraulti* as well.

The T-maze olfactometer facilitates comparative memory retention tests of the three *Nasonia* species using the same odors. The odor concentrations were chosen to result in a 50:50 distribution of naïve animals. For *N. giraulti*, a higher concentration of chocolate odor was required than for *N. vitripennis* and *N. longicornis* to achieve this equal distribution. This difference between *N. giraulti* and the other two species may be the result of a slight, but insignificantly higher antennal sensitivity for vanilla compared with chocolate (Figure 2). After conditioning, the preference of *N. vitripennis* and *N. longicornis* shifts, slightly, toward chocolate odor, whereas the preference of *N. giraulti* shifts toward vanilla odor. A similar preference shift was also found in the parasitic wasp *Leptopilina heterotoma* and may be a result of a change in sensitivity in olfactory receptor neurons due to conditioning (Vet *et al*., 1990). EAG analyses before and after conditioning can elucidate this question.

Both the conditioning procedure and testing memory retention in the T-maze olfactometer are high-throughput methods compared with individual conditioning and testing of wasps (Huigens *et al*., 2009; Schurmann *et al*., 2009; van den Berg *et al*., 2011). This makes it possible to determine memory retention of a group of wasps more accurately. The average PIs in this study were calculated from a sample size of 10, and the standard errors were between 2.5 and 7. The PI is therefore a highly reproducible measure for memory allowing detection of small differences in memory retention. We expect that the T-maze can be used for many other parasitic wasps as well, especially those that are known to exhibit olfactory microhabitat and host location by walking, such as parasitic wasps of *Drosophila* (Vet, 1985; Kaiser *et al*., 2009) and *Lariophagus* (Müller *et al*., 2006). The T-maze may also be adapted to conduct high-throughput studies on olfactory responses of *Nasonia* or other parasitic wasps in the context of host location, e.g. comparable to Turlings *et al*. (2004).

Being able to perform high-throughput studies on learning and olfaction on more species of parasitic wasps will greatly accelerate studies of variation in learning and memory.

This study shows differences in memory retention between *N. vitripennis* (AsymC), *N. longicornis* (IV7(U)) and *N. giraulti* (RV2x(U)). This result may indicate that there are differences in memory retention between populations or species in this genus. More strains of these species need to be tested to investigate this variation on a wider scale. In both *N. vitripennis* and *N. longicornis*, memory was observed up to at least 5 days, although the PIs decreased over time. This may be long-term memory, which was also observed in another strain of *N. vitripennis* when conditioned with a comparable procedure (Schurmann *et al*., 2012). In contrast, no memory was present after 1 day in *N. giraulti*, showing that the memory dynamics of *N. giraulti* differ from the other two species. The significance of the differences between the three *Nasonia* strains may become clear when analyzing memory dynamics using specific memory inhibitors, as previously shown for *Cotesia* parasitic wasps (Smid *et al*., 2007).

Future studies can focus on ultimate and proximate factors that cause this variation in parasitic wasps. Ecological differences such as host range and host distribution are considered important ultimate factors that determine learning rate or memory dynamics (Hoedjes *et al*., 2011). Species or populations that have a wide host range and a wide host distribution may need to divide their attention over a wide variety of cues. Learning may be important to limit their 'search image' and focus only on a specific type of habitat or host that is available (Dukas, 1998a; Ishii & Shimada, 2010). *Nasonia vitripennis*, and to a lesser degree *N. longicornis*, are considered to be generalist species that will parasitize a wide range of fly species, most importantly from the families Sarcophagidae and Calliphoridae, in a number of distinct habitats, such as manure, decaying carcasses and birds' nests. A wider host range may explain why the strains of *N. vitripennis* and *N. longicornis* tested in this study form a long-lasting memory after only a single learning experience. *Nasonia giraulti* is considered a specialist of *Protocalliphora* spp. in birds' nest and may rely more on innate preferences, e.g. for bird nest-specific odors (Darling & Werren, 1990; Stephens, 1993; Ings *et al*., 2009). Memory dynamics can also vary depending on the encountered host species. Certain host species are reliably associated with certain cues or habitats, whereas other species do not have such a reliable association. Differences in host preference or host suitability may also result in differences in host reward value for a female wasp (Hoedjes *et al*., 2011). Several studies have addressed aspects of *Nasonia* ecology, including natural host range, host and wasp distribution, host preference and host suitability (Pimentel, 1966; Darling & Werren, 1990; Rivers & Denlinger, 1995; Peters & Abraham, 2004; Desjardins *et al*., 2010). However, few of these studies have made a comparison between different *Nasonia* species or populations, and none has evaluated the effect of ecological factors

on learning rate or memory dynamics. Such studies are necessary to understand which ecological factors shape learning and memory in *Nasonia*.

Wasps of the genus *Nasonia* are also excellent organisms to provide understanding of proximate factors that underlie differences in learning and memory. First of all, the genomes of three *Nasonia* species, *N. vitripennis*, *N. giraulti* and *N. longicornis*, are fully sequenced and partially annotated (Werren *et al*., 2010). Genetic tools for *Nasonia* include a number of arrays, such as a tiling microarray and comparative genomic hybridization mapping arrays, as well as detailed genetic and molecular marker maps. Another important characteristic of the *Nasonia* system is the possibility to interbreed different species. This allows backcrossing of loci that are involved in differences in learning and memory into another species of *Nasonia* (Werren & Loehlin, 2009). A combination of these tools will allow one to pinpoint species-specific differences in genetic pathways causing differences in learning and memory. Next to genetic differences, neural pathways may also differ. Comparative studies in *Nasonia* can focus on the organization of groups of neurons or entire brain regions similar to studies in parasitic wasps of the genus *Cotesia*. Immunolabeling may, for example, show differences in the number or arborization patterns of reward neurons (Bleeker *et al*., 2006b). Construction of 3D models of the brain and individual brain regions will provide insight into overall organization of the brains of different species (Smid *et al*., 2003). A combination of genetic and neural studies can provide extensive understanding of the mechanisms that cause differences in learning and memory in the genus *Nasonia*. It is expected that there is a large homology in genetic and neural pathways between these species and well-studied model insect species, such as fruit flies and bees (Dubnau, 2003). The large amount of knowledge gained from research on these model insects will likely benefit studies in *Nasonia*.

We argue in this study that wasps of the genus *Nasonia* offer excellent opportunities for integrative studies on ultimate and proximate factors that cause variation in learning and memory formation. The novel olfactory conditioning assay and T-maze olfactometer for testing memory retention, presented in this study, facilitate high-throughput studies in *Nasonia* wasps. This setup may be used for studies on learning or olfaction in other parasitic wasps that locate their hosts by walking as well.

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Chapter 4

Natural variation in long-term memory formation among *Nasonia* parasitic wasp species

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ABSTRACT

Closely related species of parasitic wasps can differ substantially in memory dynamics. In this study we demonstrate differences in the number of conditioning trials required to form long-term memory between the closely related parasitic wasp species *Nasonia vitripennis* and *N. giraulti* (Hymenoptera: Pteromalidae). A single conditioning trial, in which a female wasp associates an odour with the reward of finding a host, results in the formation of transcription-dependent long-term memory in *N. vitripennis*, whereas *N. giraulti* requires spaced training to do so. Memory formation does not depend on the type of reward: oviposition, which was hypothesized to be a 'larger' reward results in similar memory retention as host feeding in both *Nasonia* species. There are several genetic and genomic tools available for *Nasonia* species to identify genetic mechanisms that underlie the observed variation in the number of trials required to form long-term memory.

INTRODUCTION

The ability to learn and form memories is universal across the Animal Kingdom and both behavioural and cellular properties of memory formation are conserved among distant animal phyla (Dubnau, 2003). This makes insects relevant model species for memory research and a diverse group of insect species have been studied intensively for this reason (Margulies *et al*., 2005; Eisenhardt, 2006; Hoedjes *et al*., 2011). During an associative learning experience, an insect will associate a specific cue or behaviour with a rewarding or punishing stimulus. As a result, the insect will demonstrate, respectively, an increased or decreased preference towards that cue or tendency to perform the specific behaviour. Associative learning can thereby optimize behaviour aimed at finding food, hosts or mates (Laverty & Plowright, 1988; Papaj & Vet, 1990; Raine & Chittka, 2008).

After a learning experience, different types of memory can be formed which differ in their characteristics, as well as in cellular pathways that are involved in the formation of these memory types. Comparable classifications of memory types were made for *Drosophila melanogaster*, *Apis mellifera* and a number of parasitic wasp species (Margulies *et al*., 2005; Eisenhardt, 2006; Hoedjes *et al*., 2011). Immediately after conditioning, short-term memory (STM) is present. This type of memory lasts, depending on the investigated species, several minutes up to a few hours (Menzel, 1999; van den Berg *et al*., 2011). STM is sensitive to disruption with anaesthesia and is, therefore, also known as a form of anaesthesia-sensitive memory (ASM) (Xia *et al*., 1999). Hours to days after conditioning, longer-lasting memory types will be formed. This is a process called memory consolidation and two main types of memory are distinguished: anaesthesia-resistant memory (ARM) and long-term memory (LTM). LTM is the most stable and durable type of memory and requires protein synthesis, in contrast to ARM, which is resistant to anaesthesia but does not require protein synthesis (Tully *et al*., 1994; Smid *et al*., 2007). Generally, single or massed conditioning trials, i.e. without or with a short inter-trial interval, will result in the formation of ASM and ARM. Many animal species will only form LTM after spaced conditioning, i.e. multiple trials with a longer inter-trial interval (e.g. Margulies *et al*., 2005; Eisenhardt, 2006). There is, however, variation in the number of conditioning trials required to form LTM: some insect species will form LTM after only a single conditioning trial.

LTM formation after a single trial has been demonstrated in a number of parasitic wasp species, including *Cotesia glomerata*, *Trichogramma evanescens* and *Lariophagus distinguendus* (Collatz *et al*., 2006; Smid *et al*., 2007; Huigens *et al*., 2009). Interestingly, closely related species of these parasitic wasps, for example *Cotesia rubucula*, do require spaced conditioning trials to form LTM. Additionally, the number of trials depends on the reinforcing stimulus that is experienced during conditioning. For example, fruit flies will form LTM after a single appetitive conditioning trial, but not after a single aversive

conditioning trial (Krashes & Waddell, 2008). Memory formation in bumble bees depends on nectar concentration of the flowers and memory dynamics of parasitic wasps may depend on the value of the host (Cnaani *et al*., 2006; Kruidhof *et al*., 2012). Both the inter- and intraspecific variation in the number of trials required to form LTM are high among parasitic wasps species, which are, therefore, considered model species to study both ultimate and proximate factors involved (Hoedjes *et al*., 2011).

Parasitic wasps learn cues to find their inconspicuous insect hosts in which they lay their eggs and most studies have focused on this so-called oviposition learning (Hoedjes *et al*., 2011). Variation in LTM formation is thought to depend on the ecology of both the parasitic wasp species and its host species: factors such as the variability of the host environment, whether hosts occur gregariously or solitary, the quality of the host, and the number of hosts that a parasitic wasp will parasitize have been hypothesized to be important (Hoedjes *et al*., 2011; Kruidhof *et al*., 2012). In addition, different stimuli that are encountered during oviposition can also affect the formation of LTM. Although, oviposition is generally considered the reinforcing stimulus in oviposition learning, there are also other aspects of the rewarding stimulus. These include contact of antennae, mouth parts or ovipositor of a female wasp with host faeces, host haemolymph or other chemosensory cues that are present on the host (Takasu & Lewis, 2003; Collatz *et al*., 2006; Schurmann *et al*., 2012). Contact with host traces or host haemolymph alone can be sufficient to form a short-term memory, but oviposition was required to form a long-lasting memory in the parasitic wasp species *Microplitis croceipes* and *L. distinguendus* (Takasu & Lewis, 2003; Collatz *et al*., 2006). A strain derived from a German population of *N. vitripennis* was observed to form ARM after contact with host haemolymph, but LTM when it was also allowed to feed from this host haemolymph (Schurmann *et al*., 2009; Schurmann *et al*., 2012).

The genetic mechanisms that are responsible for variation in LTM formation are poorly understood (Hoedjes *et al*., 2011). Parasitic wasps of the genus *Nasonia* offer unrivalled opportunities to focus on both ecological and genetic aspects of variation in LTM formation. A recent study demonstrated variation in memory retention after a single conditioning trial, which waned within two days in *N. giraulti*, but lasted at least 5 days in *N. vitripennis* (Hoedjes *et al*., 2012). This difference in memory retention between *N. vitripennis* and *N. giraulti* may be explained by differences in ecological factors, as was discussed in Hoedjes *et al*. (2012). The species of the genus *Nasonia* offer unique opportunities to study the genetic basis of variation in memory formation and genomic and genetic resources are available for these species (Werren *et al*., 2010). Characterization of differences in long-term memory formation between the *N. vitripennis* and *N. giraulti* strains for which these genetic tools are available is essential to benefit from these opportunities for genetic studies.

The aim of this study was, therefore, to investigate LTM formation in *N. vitripennis* and

N. giraulti and to investigate the effect of variation in the rewarding stimulus on memory of both species. *Nasonia vitripennis* was expected to form LTM after a single conditioning trial, which was investigated using inhibitors of LTM. The ability of *Nasonia giraulti* to form long-lasting memory after multiple conditioning trials was investigated. In addition, we studied the effect of host feeding and oviposition on memory strength and memory retention in both species. Oviposition was hypothesized to be a 'larger' reward than host feeding, which would result in increased memory retention or a stronger association between the learned cue and the reward.

MATERIALS AND METHODS

Insects

Inbred strains of *Nasonia vitripennis* (AsymCx) and *N. giraulti* (RV2x(U)) were used. Both strains have a sequenced genome and genetic tools have been developed for these strains (Werren *et al*., 2010). The wasps were reared on host pupae of *Calliphora vomitoria* at 25°C and a 16L:8D photoperiod as described by Hoedjes *et al*. (2012). Pupae of *C. vomitoria* were used also used as host species during all experiments. Wasps were collected on the day of emergence and were allowed access to honey and water ad libitum, unless specified otherwise. Female wasps were used for experiments 1 to 2 days after emergence.

Oviposition behaviour

The aim of this experiment was to determine if oviposition occurs in our strains of *N. vitripennis* and *N. giraulti* during two subsequent periods of contact with a host, before subsequent experiments on oviposition learning were done. Schurmann *et al*. (2012) demonstrated that their strain of *Nasonia vitripennis* ('Hamburg' strain), will not oviposit, but only drill with their ovipositor to enable host-feeding when allowed access to the hosts for one hour. Groups of 100 *N. giraulti* and 100 *N. vitripennis* females were individually provided a single host in a well of a 24-well microtiter plate (Greiner Bio-One, Alphen aan den Rijn, the Netherlands) closed with a plastic cap (protection plug, 14.5 mm diameter, Skiffy, Amsterdam, the Netherlands) for one hour. Wasps that did not initiate drilling into the host within 30 minutes were afterwards removed from the experiment. Wasps were subsequently kept in a climate cabinet (25°C) with access to honey and water for 4 hours and were then allowed access to another single host for one hour. The hosts were kept in vials in a climate cabinet (25°C, 16L:8D) until offspring emerged in order to score the percentage of ovipositing females and the average number of offspring.

Conditioning procedure

Conditioning was done using an olfactory conditioning assay as described by Hoedjes *et al*. (2012). This is a Pavlovian conditioning assay in which female wasps will associate an odour with the reward of access to a host. Briefly, female wasps were individually allowed access to a host pupa (the unconditioned stimulus, US) for one hour in the presence of either chocolate or vanilla odour (the conditioned stimulus, CS+). Then, after a 15 minutes resting period, females were exposed to, respectively, vanilla or chocolate odour (CS-) without a reward present. Two reciprocal groups of 24 wasps were conditioned simultaneously with this procedure.

Memory retention differs between *N. vitripennis* and *N. giraulti* when wasps received a single conditioning trial in which host feeding was the reward (Hoedjes *et al*., 2012). Oviposition may be perceived as a different and possibly larger reward, than host feeding alone by female parasitic wasps during oviposition learning. This experiment aimed to assess memory retention of both species after a single conditioning trial in which host feeding was the reward (1) vs. a single conditioning trial in which oviposition is the reward (2). In addition, these wasps were compared to wasps that had received two conditioning trials spaced in time by four hours (3). Female wasps that received a single trial with host feeding as a reward (1) were conditioned as described above. These females perform host feeding, but do generally not oviposit during the conditioning period. Female wasps that received a single trial with oviposition as a reward (2) were allowed to drill into and feed from a single host for one hour (as described above) without CS+ present. This host feeding before conditioning allows these female wasps to develop eggs, so they can oviposit during a subsequent conditioning trial. These wasps were kept in a climate cabinet (25°C) with access to honey and water for 4 hours and then received a single one-hour conditioning trial, in which the majority of females indeed did oviposit. Female wasps that received two spaced conditioning trials (3) received a conditioning trial as described for (1) and were subsequently kept in a climate cabinet (25°C) with access to honey and water during a 4 hour intertrial interval. They then received a second conditioning trial similar to (2).

Memory retention test

Wasps were tested once for memory retention 24 (±1), 72 (±1) or 120 (±1) hours after conditioning in a T-maze olfactometer as described in Hoedjes *et al*. (2012). This is an olfactometer with two arms, with vanilla odour on one side and chocolate odour on the other side. A group of 12 wasps is released in the middle of the T-maze and after 10 minutes the number of wasps that has walked into each of the two arms is recorded to calculate the percentage of wasps that chose the learned odour. Immediately afterwards, a reciprocally trained groups of 12 wasps is released into the T-maze and tested similarly.

The difference in preference of these two reciprocally trained groups of wasps towards vanilla and chocolate odour is a measure for memory retention, which is represented by the performance index (PI). This method for calculating memory retention is commonly used in research on fruit flies (Tully *et al*., 1994) and PI is calculated as follows: 1st group (%CS+) – 2nd group (%CS-). If all wasps of both groups choose the odour they've been trained on (CS+), then PI will be 100, which would represent perfect memory retention. If there is no memory retention, then there will be no difference between the two reciprocal groups of wasps, which would result in a PI of 0. A previous study has shown that both *N. vitripennis* and *N. giraulti* can learn vanilla and chocolate odour equally well, and that unexperienced wasps distribute evenly over the two arms of the T-maze (Hoedjes *et al*., 2012). Two PIs per treatment were obtained per day and the experiment was repeated 5 times on different days, resulting in 10 PIs per treatment and time point.

LTM inhibition

It was expected that *N. vitripennis* would form LTM after a single conditioning trial. In this experiment we determined at which time points after conditioning LTM was expressed by inhibiting this type of memory with anisomycin (ANI) or actinomycin D (ACD), respectively a translation and a transcription inhibitor. The method for feeding these inhibitors to *N. vitripennis* was adapted from Smid *et al*. (2007). Wasps were fed ACD or ANI dissolved in sucrose solution directly before conditioning, whereas control wasps were fed sucrose solution. Female wasps were collected at the day of emergence and they were kept overnight in polystyrene rearing vials (dimensions 28.5 x 95 mm) with foam stoppers (Genesee Scientific, San Diego, CA, USA) in a climate cabinet under a constant temperature (25°C) and a photoperiod of 16L:8D without access to water and honey. The next morning, wasps were offered 0.5 µl solution containing 0.005 mM ACD (Sigma-Aldrich, Zwijndrecht, The Netherlands) in 25% sucrose. ANI (Sigma-Aldrich, Zwijndrecht, The Netherlands) was administered in a similar way, but at a concentration of 1.0 mM. Control treatment involved feeding 25% sucrose without ANI or ACD. The wasps were allowed to feed individually for 1.5 to 2 hours in wells of a 24-well microtiter plate (Greiner Bio-One, Alphen aan den Rijn, the Netherlands) closed with plastic caps to prevent escaping (protection plug, 14.5 mm diameter, Skiffy, Amsterdam, the Netherlands). The wasps generally do not eat the entire 0.5 µl solution; only individuals that were observed to feed from the solution were used for further experiments. The wasps were then transferred to polystyrene rearing vials with access to water and food and kept in a climate cabinet (25°C) for 1.5 to 2 hours, and then received a single conditioning trial (in which host feeding was the reward) as described above. Memory retention was tested in the T-maze 72 (±1), 96 (±1), 120 (±1) or 144 (±1) hours after conditioning as described above. Pilot experiments and results on another

strain of *N. vitripennis* (Schurmann *et al.*, 2012) revealed that ANI and ACD did not affect memory retention after 24 and 48 hours and these time points were therefore not included in this study.

To assess deleterious effects of ANI and ACD on *N. vitripennis*, the mortality rates of female wasps were determined. Groups of 24 wasps were individually fed 0.5 µl solution containing either 0.1 mM, 0.01 mM, 0.005 mM ACD in 25% sucrose, 1.0 mM ANI in 25% sucrose or 25% sucrose without ACD or ANI (controls). Only a single concentration of ANI was tested, because wasps refused to feed from higher concentrations of ANI. The wasps were kept in polystyrene rearing vials with access to water and food and in a climate cabinet (25°C) with a light regime of 16L:8D. The number of dead wasps was determined each day for 7 days. This was repeated 4 times. The highest concentration of the ACD and ANI that did not affect survival of the wasps and from which the wasps would feed was chosen for the experiments.

Data analysis

Normality and equal variances of the data were tested. A t-test was used to test if the PI was significantly different from 0, i.e. testing if there was memory retention. Results from *N. vitripennis* and *N. girault*i were analyzed separately using univariate ANOVA to test for effects of time (a covariate), and treatment (ANI/ACD/control) and/or conditioning procedure (procedure 1/2/3) (the fixed factor) on memory retention. The interaction between time and treatment or between time and conditioning procedure was tested and removed from the model if not significant. Pairwise comparisons were done using a Fisher's LSD test in order to determine which treatments/conditioning procedures differed from each other. Onset of memory inhibition by ANI and ACD was tested with univariate ANOVA for each time point. All analyses were done in SPSS, version 19 (IBM, Armonk, NY, USA).

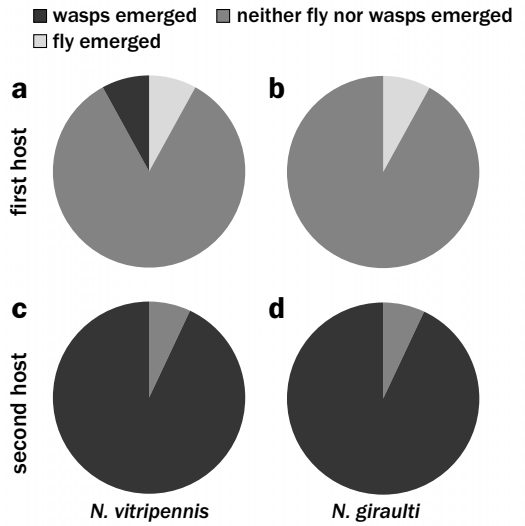

Figure 1: Oviposition behaviour. The number of parasitized ('wasps emerged') and unparasitized ('fly emerged' and 'neither fly nor wasps emerged') after a period in which a female was provided access to a single host (n = 100 females per species). Oviposition behaviour on the first and second host provided, respectively, was compared for (a, c) *N. vitripennis* and (b, d) *N. girault*i.

RESULTS

Oviposition behaviour

None of the *N. giraulti* wasps produced any offspring after their first hour of access to a host; 8% of the hosts developed into flies and 92% produced neither a fly nor wasps. Eight percent of *N. vitripennis* wasps did produce a small number of offspring (on average 3.0 offspring per ovipositing female); 8% of the hosts developed into flies and 84% produced neither a fly nor wasps. During the second period of host access, 93% of both *N. giraulti* and *N. vitripennis* produced offspring (respectively 13.2 and 11.3 offspring on average per ovipositing female). This result shows that both *Nasonia* species will mostly perform host feeding only during their first contact with a host, whereas they will mostly oviposit when offered a second host four hours later (Figure 1).

Effect of conditioning procedure on memory retention

The effect of conditioning procedure and time on memory retention (PI) was analyzed. The interaction between time and conditioning procedure was not significant for either *Nasonia* species (*N. vitripennis*: $F_{2,84} = 0.97$, $P = 0.38$; *N. giraulti*: $F_{2,84} = 0.57$, $P = 0.57$) and was, therefore, removed from the model. Memory retention depends on the conditioning procedure for both *N. vitripennis* ($F_{2,86} = 20.29$, $P < 0.001$) and *N. giraulti* ($F_{2,86} = 32.78$, $P < 0.001$) and PIs decrease over time (*N. vitripennis*: $F_{1,86} = 54.49$, $P < 0.001$; *N. giraulti*: $F_{1,86} = 89.47$, $P < 0.001$). For *N. vitripennis*, all three conditioning procedures resulted in significant PI values up to 120 hours after conditioning ((1): $t_9 = 13.87$, $P < 0.001$; (2): $t_9 = 9.82$, $P < 0.001$; (3): $t_9 = 17.27$, $P < 0.001$). The PIs of wasps that received a single conditioning trial in

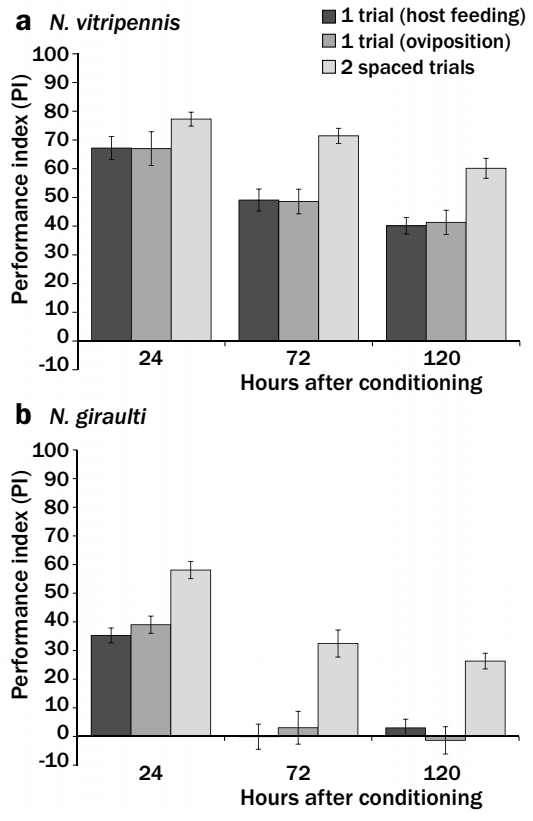

Figure 2: Effect of conditioning procedure on memory retention. Memory retention of (a) *N. vitripennis* and (b) *N. giraulti* wasps after a single conditioning trial, in which the reward is either host feeding or oviposition, and after two spaced conditioning trials (one trial with host feeding followed by one trial with oviposition) (n = 10 PIs per time point and conditioning procedure).

which host feeding was the reward (1) did not differ from wasps that received a single conditioning trial in which oviposition was the reward (2) (LSD, $P = 0.96$). Wasps that received two spaced conditioning trials (3) had significantly higher memory scores than wasps that received a single conditioning trial (1 vs. 3: LSD, $P < 0.001$; 2 vs. 3: LSD, $P < 0.001$) (Figure 2a).

For *N. giraulti* there was no difference in PIs between the two types of single trial conditioning (host feeding (1) and oviposition (2)) (LSD, $P = 0.82$); there was significant memory retention 24 hours after conditioning, but this memory had decayed after 72 hours ((1): $t_9 = -0.02$, $P = 0.987$; (2): $t_9 = 0.53$, $P = 0.608$). Two spaced conditioning trials (3), however, resulted in higher memory scores (1 vs. 3: LSD, $P < 0.001$; 2 vs. 3: LSD, $P < 0.001$) and significant memory retention up to at least 120 hours ($t_9 = 9.56$, $P < 0.001$) (Figure 2b).

LTM inhibition

The survival analysis showed that administration of 0.005 mM ACD in a 25% sucrose solution did not affect survival rate in *N. vitripennis* compared to the control treatment (Figure 3a). The survival was 100% in both cases. An increased dosage of ACD of 0.010 or 0.100 mM results in a decreased survival rate, respectively 77.1% and 3.1% of these wasps survive up to 7 days after feeding. This indicates that 0.005 mM ACD is the highest possible dosage without an effect on survival of the wasps. This concentration was, therefore, chosen for further experiments. Administration of 1.0 mM ANI did not affect survival rate in *N. vitripennis* compared to the control treatment, which was 100% in both cases (Figure 3b). This indicates that this concentration of ANI can be used for further experiments. It is possible that higher dosages of ANI can also be administered without effects on survival, but this could not be tested as

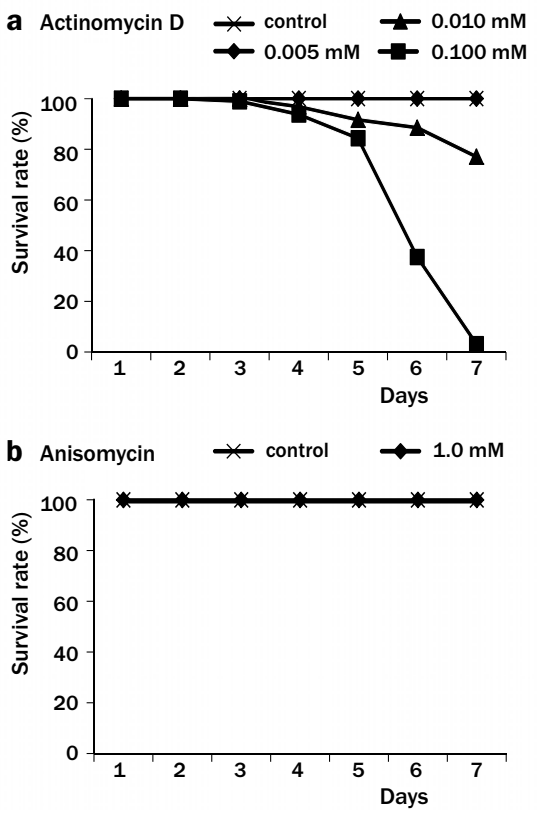

Figure 3: Survival after feeding of ACD or ANI. Survival rate of *N. vitripennis* wasps which were fed 0.5 µl sucrose solution with different concentrations of (a) actinomycin D or (b) anisomycin (n = 4 x 24 wasps per treatment).

N. vitripennis wasps refused to feed from higher concentrations of ANI.

Memory retention of *N. vitripennis* was analyzed after treatment with ANI, ACD, or the control treatment. Memory retention decreases over time ($F_{1,114} = 35.979$, $P < 0.001$), but was not affected by treatment ($F_{2,114} = 0.729$, $P = 0.484$). The interaction between treatment and time was significant ($F_{2,114} = 4.14$, $P = 0.018$), which indicates that the effect of ANI and ACD depends on the time point of the memory retention test. Both ANI and ACD inhibited memory retention in a similar manner (LSD, $P = 0.80$); the time point at which inhibition of memory becomes visible as well as the level of inhibition is similar for both inhibitors. Analysis of individual time points shows that memory inhibition by ANI and ACD is not visible 72 hours after conditioning ($_{F2,27} = 0.096$, $P = 0.91$), but starts at 96 hours ($F_{2,27} = 6.85$, $P = 0.004$) (Figure 4).

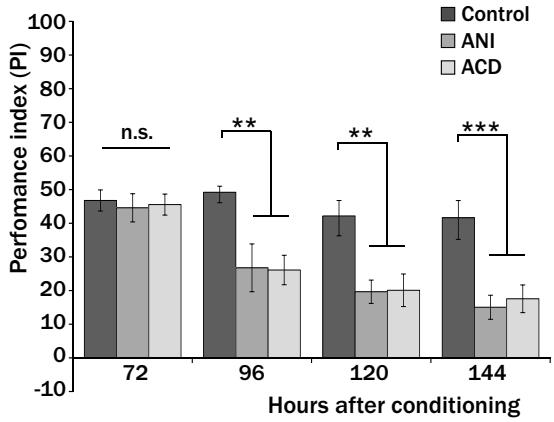

Figure 4: LTM inhibition by ACD or ANI. Inhibition of LTM after a single conditioning trial of *N. vitripennis* wasps by anisomycin (ANI) and actinomycin D (ACD) (n = 10 PIs per time point and treatment) (LSD; ***P < 0.001, ** 0.001 < P < 0.01, * 0.01 < P < 0.005, n.s. = not significant).

DISCUSSION

This study demonstrates variation in memory retrieval of *N. vitripennis* and *N. giraulti*, which we interpret as variation in memory retention between the two species. *Nasonia vitripennis* will form transcription- and translation-dependent LTM after a single conditioning trial, whereas *N. giraulti* requires two spaced trials to form long-lasting memory. The number of trials required to form LTM in these two species appears to be independent of the type of reward that was offered during conditioning, namely host feeding vs. oviposition.

It was observed that only a small percentage of *N. vitripennis* and none of *N. giraulti* will start oviposition within one hour when offered a host for the first time. *Nasonia vitripennis* require host feeding for the maturation of eggs, but few matured eggs are already present upon emergence, which may explain why a low percentage of *N. vitripennis* females will already start oviposition (Whiting, 1967). Oviposition was observed in most wasps of both species during the second host contact period of one hour, which these wasps were given after a 4 hour resting period. These results imply that the rewarding stimulus in a first conditioning trial is mostly host feeding and in a second conditioning

trial it is oviposition. We had hypothesized that oviposition would be considered a 'larger' reward than host feeding alone. However, there was no difference in the level of the PI or retention of memory when comparing these two stimuli. Host feeding by a *Nasonia* female will decrease the value of the host, as fewer resources will be available for future offspring, although *Nasonia* parasitic wasp species can use the same host for both host feeding and oviposition (Rivero & West, 2005). Possibly, *Nasonia* parasitic wasps perceive both host feeding and oviposition as a measure of reproductive success. The rewarding stimulus may also be similar in both situations: chemosensilla on the ovipositor of female wasps will likely have contact with host haemolymph before host feeding as well as before oviposition (van Lenteren *et al*., 2007), which could mean that host feeding and oviposition are not perceived as being different rewards by *Nasonia* parasitic wasps.

Both the level of PI and memory retention of *N. vitripennis* and *N. giraulti* do not depend on the type of reward during conditioning, but they do depend on the number of conditioning trials. The level of the PI increased in both species when they were conditioned twice with an intertrial interval of 4 hours, compared to a single conditioning trial. Memory retention of *N. giraulti* was, furthermore, increased up to at least 5 days, which is similar to memory retention of *N. vitripennis* after a single trial. This demonstrates that *N. giraulti* females can form long-lasting memory types only after multiple conditioning trials spaced in time, whereas *N. vitripennis* does so already after a single conditioning trial.

Nasonia vitripennis consolidates LTM after a single conditioning trial, which is then expressed 4 days after conditioning, considering that both ANI (translation inhibitor) and ACD (transcription inhibitor) inhibit memory from 4 days onwards. This suggests that the memory trace is completely dependent on translation and transcription 4 days after conditioning. This combined dependency on both translation and transcription is comparable to LTM consolidation in *Cotesia* parasitic wasps and honeybees (Wüstenberg *et al*., 1998; Smid *et al*., 2007). *Nasonia giraulti* will form a memory that will last at least 5 days after two spaced conditioning trials. Based on our results on *N. vitripennis* (Figure 4), we expect that the long lasting memory trace in *N. giraulti* is also transcription-dependent LTM.

In addition to comparing LTM formation among closely related species, *Nasonia* parasitic wasps can be compared to other parasitic wasp species. *Nasonia giraulti* can be compared to other species which require multiple conditioning trials to form LTM, such as *C. rubecula* which requires three spaced trials (Smid *et al*., 2007). *Nasonia vitripennis* forms LTM after a single trial comparable to *C. glomerata*, *L. distinguendus* and *T. evanescens*. Interestingly, the speed of LTM consolidation varies considerably among these four species: *N. vitripennis* has consolidated LTM 4 days after conditioning, whereas *L. distinguendus* and *T. evanescens* have consolidated LTM after 24 hours and *C. glomerata* already after only 4

hours (Collatz *et al*., 2006; Smid *et al*., 2007; Huigens *et al*., 2009). *Nasonia vitripennis* forms ARM, before LTM is consolidated after 4 days (Schurmann *et al*., 2009). This is comparable to memory consolidation in *C. rubecula*, which will consolidate LTM within two to three days after 3 spaced conditioning trials and also forms ARM (Smid *et al*., 2007). The rate of LTM consolidation determines the time window that an animal has to evaluate the learned information before this information is consolidated as LTM (van den Berg *et al*., 2011). Schurmann *et al*. (2012) hypothesized that a low rate of LTM consolidation would enable an animal to re-evaluate information that was learned. Memory is potentially erased if a wasp experiences conflicting information or unrewarding experiences before LTM is consolidated (Schurmann *et al*., 2012). A high rate of LTM consolidation without formation of ARM, as observed in *C. glomerata*, may only occur when the learned cue predicts host presence very reliably (van den Berg *et al*., 2011). Further experiments on the susceptibility of STM, ARM and LTM to unrewarding experiences or conflicting information are necessary to test this hypothesis. The mechanisms that regulate the number of trials required to form LTM as well as LTM consolidation rate also remain to be elucidated.

The genus *Nasonia* has emerged as a powerful model system for genetic research. Genetic resources, including genetic maps and genome sequences, were established for a purely homozygous strain of each species (Werren *et al*., 2010). We demonstrated profound variation in LTM formation between these strains of *N. vitripennis* (AsymCx) and *N. giraulti* (RV2x(U)), which allows future studies on memory dynamics to benefit from the tools available for these strains. Our results on LTM formation in *N. vitripennis* are comparable to observations on a strain from a German population of *N. vitripennis* by Schurmann *et al*. (2012). This study also demonstrated LTM expression 4 days after a conditioning trial, using different techniques to administer ACD (injection into the abdomen) and to test memory retention (a four-armed olfactometer). This confirms our results. Future studies on the homozygous strains of *N. vitripennis* (AsymCx) and *N. giraulti* (RV2x(U)) can include introgression experiments, because the species of the genus *Nasonia* can interbreed. In such an experiment, genes that regulate the number of trials required to form LTM in one species can be expressed and studied against the background of the other species in order to unravel the genetic mechanisms that regulate this trait (Werren & Loehlin, 2009). The fact that LTM consolidation after a single conditioning trial is transcription-dependent in *N. vitripennis* implies that conditioning will result in differences in expression levels of genes related to LTM formation. This opens exciting possibilities to study differential gene expression related to memory dynamics using a transcriptomics approach, which will further increase understanding of genetic mechanisms involved in LTM formation.

Conclusions

In conclusion, this study has provided valuable new insights on LTM formation of *N. vitripennis* and *N. girault*i. The two species differ in the number of conditioning trials required to form LTM, but this number is not affected by the type of reward, respectively host feeding or oviposition, which is offered during conditioning. Parasitic wasps of the genus *Nasonia* offer unique opportunities to study both ecological factors as well as genetic mechanisms underlying this variation. Such multidisciplinary research is necessary to fully understand the evolution of memory dynamics. This will not only increase our understanding of insect memory and behaviour, but also memory and behaviour in higher animals.

ACKNOWLEDGEMENTS

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Chapter 5

Unravelling reward value: the effect of host value on memory retention in *Nasonia* parasitic wasps

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Under review

ABSTRACT

Learning can be instrumental in acquiring new skills or optimizing behavior, but it is also costly in terms of energy and when maladaptive associations are formed: the balance between costs and benefits affects memory dynamics. Numerous studies have demonstrated that memory dynamics of animal species depend on the value of the reward during conditioning, even when animals are inexperienced with this reward. Reward value consists of both the perceived value of the reward to the fitness of an animal and the reliability of the learned association. The aim of this study was to study the effect of reward value on oviposition learning in parasitic wasps of the genus *Nasonia*. Host species of these parasitic wasps can differ in their value as a host, i.e. the number and size of emerging offspring, but reliability of the learned association is hypothesized to be similar for different host species. This allows studying the effects of host value only. We conditioned parasitic wasps of the species *Nasonia vitripennis* and *N. giraulti* using three different host species as a reward, which differed profoundly in their value as a host. However, for both parasitic wasp species, the resulting memory formation was independent of the value of the host. We discuss factors that may be responsible for this observation.

INTRODUCTION

Learning can optimize behaviour of an animal, but is costly in terms of time and energy, and in case that maladaptive associations are formed (Menzel, 1999; Laughlin, 2001; Mery & Kawecki, 2005). It is, therefore, not surprising that memory depends on the type and quality of the reinforcer, i.e. the reward or punishment experienced during a learning experience, or the reliability of the learned association (Rescorla, 1988; Stephens, 1993; Hoedjes *et al*., 2011).

Memory of fruit flies (*Drosophila melanogaster*) and crickets (*Gryllus bimaculatus*) decays faster when aversive conditioning is applied compared to appetitive conditioning (Krashes & Waddell, 2008; Nakatani *et al*., 2009). A conditioned response also changes when the reinforcer has an increased intensity or quality, for example in fruit flies conditioned to avoid heat (Diegelmann *et al*., 2006), male rats (*Rattus norvegicus*) that received different intensities of sexual stimulation (Camacho *et al*., 2009) and in several bird and insect species that received more nutritious or preferred food (Clayton & Dickinson, 1998; Wäckers *et al*., 2006; Wright *et al*., 2009; Burke & Waddell, 2011). These studies suggest that rewards or punishments that are perceived as more important to the fitness of an animal will result in stronger and more durable memory.

The importance of the reliability of the association between a learned cue and the reinforcer was demonstrated in a study on bumblebees (*Bombus impatiens*), which responded stronger after conditioning with an increased sucrose concentration compared to an increased volume of sucrose solution with a similar total reward. This is considered an evolutionary adaptation because a flower's nectar volume varies due to foraging activity, whereas sucrose concentration is a reliable characteristic of the species (Cnaani *et al*., 2006). Another study on magpies (*Pica pica*) has shown that these birds will respond to visual cues that reliably indicate the presence of food and will ignore less reliable spatial cues (Feenders & Smulders, 2011).

A study on two unrelated parasitic wasp species has shown that memory retention after oviposition learning, in which the reward is finding and parasitizing a host, can depend on host species (Kruidhof *et al*., 2012). *Cotesia glomerata* and *Trichogramma evanescens* both parasitize cabbage white butterflies, albeit each a different developmental stage, respectively caterpillars and eggs. Both wasp species formed long-term memory after a single conditioning trial on the large cabbage white (*Pieris brassicae*), but a short-lasting memory (<24h) after a single conditioning trial on the small cabbage white (*Pieris rapae*). Kruidhof *et al*. (2012) hypothesized that both the value of the host and the reliability of the learned cue, which together constitute 'reward value', determine memory retention. The large cabbage white has a higher host value, which is reflected by a higher number and an increased size of offspring (Salt, 1940; Harvey, 2000). The reliability of learned cues differs

due to differences in the ecology of these hosts. The large cabbage white will lay clusters of eggs on a cluster of plants of the same species (Davies & Gilbert, 1985). Learned cues that are associated with this host, such as odors of these plants are, therefore, a reliable indication for the presence of multiple hosts. The small cabbage white, on the other hand, will lay single eggs on multiple plant species (Root & Kareiva, 1984). As a result, the odor of a specific plant species is not a reliable cue for the presence of more hosts.

These studies demonstrate that the value of the reinforcer, the reliability of the association, or both can have profound effects on memory retention. In the majority of the studies described, the animals were inexperienced with the reinforcer, which implies that they were able to innately assess 'reward value' and adjusted their memory to this value. Both factors need to be considered to fully understand observed differences in memory retention in an animal. Studies that allow these two factors to be unravelled are instrumental in investigating this question.

In this study, we focussed on the parasitic wasp species *Nasonia vitripennis* and *N. giraulti* and tested how host value affects memory retention in naïve wasps. Both species can learn odors upon host encounter, but differ in their memory retention after a single conditioning trial on their host *Calliphora vomitoria*: *N. vitripennis* forms long-term, transcription-dependent memory (lasting >6 days), whereas memory of *N. giraulti* decays within two days (Hoedjes *et al*., 2012; Schurmann *et al*., 2012; Hoedjes & Smid, 2014). This interspecific difference in memory retention has been hypothesized to reflect a difference in specialisation between *N. vitripennis* and *N. giraulti* (Hoedjes *et al*., 2012). These wasp species will encounter different host species in their habitat, which differ in their suitability or value as a host (Peters & Abraham, 2010; Daoust *et al*., 2012). Reliability of learned cues is, in contrast with the study by Kruidhof *et al*. (2012), hypothesized to be similar for different host species. *Nasonia* species parasitize various species of fly pupae that occur on carcasses and in bird nests. These habitats have a highly variable community composition of fly species. Several species can co-occur and the number of available hosts can range from none or few to hundreds of hosts. Factors such as season, geography, bird species or carcass species, size, decomposition stage or decomposition type all influence the species and number of fly hosts (Anderson & Vanlaerhoven, 1996; Peters & Abraham, 2010; Castro *et al*., 2012; Daoust *et al*., 2012). Habitat specific cues are, therefore, not reliably associated with the presence of specific host species for *Nasonia*; they rather predict the presence of multiple fly species. It is, therefore, expected that the effect of host quality can be studied in the genus *Nasonia*, without inducing variation in reliability of the association at the same time.

We have varied host quality by offering *N. vitripennis* and *N. giraulti* three different host species that differ in size as a reward during conditioning: *Calliphora vomitoria*, *Lucilia*

sericata (Diptera: Calliphoridae) and *Musca domestica* (Diptera: Muscidae). The difference in host size was hypothesized to result in differences in the numbers or fitness of offspring and consequently host value (Rivers & Denlinger, 1995; Peters & Abraham, 2010). A difference in host quality was confirmed in this study. All three fly species are potential hosts for the generalist *N. vitripennis* and it was, therefore, expected to distinguish the three host species. The specialist *N. giraulti* is not adapted to these specific host species, but does accept these species as a host in laboratory rearings. These three host species were chosen, because of their suitability as a host for both *Nasonia* species and because they were commercially available in large numbers and consistent quality. If host value affects memory retention in naïve *Nasonia* species, this will be reflected by differences in the strength of the conditioned response or memory retention.

MATERIALS AND METHODS

Insects

Nasonia vitripennis (strain AsymCx) and *N. giraulti* (strain RV2x(U)) were used for the experiments; the genomes of these strains have been sequenced (Werren *et al*., 2010). The wasps were reared on *Calliphora vomitoria* pupae and were maintained as described in Hoedjes *et al*. (2012). The three host species *Calliphora vomitoria*, *Lucilia sericata* (Kreikamp, Hoevelaken, The Netherlands) and *Musca domestica* (Kreca, Ermelo, The Netherlands) were obtained as maggots and were allowed to pupate at room temperature and were subsequently stored in a fridge (4°C) for a maximum of two weeks.

Host value

Host value of the three fly species was assessed by measuring weight, length and width of 50 host pupae per species. The offspring number per female per host pupa, sex ratio and size of offspring were determined for both *N. vitripennis* and *N. giraulti* on each of the three host species. Twenty female wasps were collected at the day of emergence and were individually provided 5 hosts of the same species in a glass tube (75x12x0.08 mm; VWR, Amsterdam, The Netherlands) closed with a cotton wool plug. The wasps were removed 48 hours later and hosts were kept in a climate cabinet under a constant temperature (25°C) and photoperiod (16L:8D) until emergence of the offspring. The total number of offspring, which consists of emerged offspring, diapausing larvae and offspring that had died during development, was counted and the average number of offspring per parasitized pupa per mother was determined. Sex ratio of the emerged offspring was determined, because a decreased host size has been hypothesized to result in an increased sex ratio in parasitic wasps (King, 1987). Hind tibia length of 30 female offspring per wasp species and host

species was measured; this is a commonly used measure for adult body size of parasitic wasps (Godfray, 1994).

Olfactory conditioning assay

The olfactory conditioning assay described by Hoedjes *et al*. (2012) was used. This is a Pavlovian conditioning assay in which female parasitic wasps associate the reward (a host) with an odor. Briefly, female wasps were placed individually in close contact with two host pupae (the unconditioned stimulus, US) in the presence of either vanilla or chocolate odor (the conditioned stimulus, CS+) and they were allowed to drill into a pupa and to perform host feeding for 1 hour. Oviposition does typically not take place during this period (Hoedjes & Smid, 2014). The wasps were then exposed to respectively chocolate or vanilla odor (CS-) for 15 minutes without a reward present. Three groups of 48 wasps were conditioned simultaneously either with *C. vomitoria*, *L. sericata* or *M. domestica* pupae as reward in order to assess the effect of host value on memory retention. Conditioning was done reciprocally: each group of 48 wasps was divided and one half was conditioned with vanilla as CS+ and chocolate as CS-, whereas the other half was conditioned with chocolate as CS+ and vanilla as CS-. The occurrence of drilling within 30 minutes was also scored for each host species to assess differences in wasp behavior towards the different hosts. Wasps that did not initiate drilling within 30 minutes were removed from the experiment. Based on previous experiments (Hoedjes *et al*., 2012), *Nasonia vitripennis* was tested for memory retention either 24 (±1) or 120 (±1) hours after conditioning in order to study effects of host species on both short- and long-lasting memory types. *Nasonia giraulti* was tested either 24 (±1) or 72 (±1) hours after conditioning, because it was previously shown to form short-lasting memory types only after conditioning.

Memory retention test

All wasps were tested in a T-maze olfactometer in which vanilla odor was offered in one tube and chocolate odor in the other tube as described by Hoedjes *et al*. (2012). The standard procedure involves testing memory of each of the two reciprocal pairs of up to 24 wasps in two runs. The choice of 10-12 wasps was recorded 10 minutes after release in the T-maze and immediately afterwards a reciprocal group of 10-12 wasps was tested.

Data analysis

The different parameters for host value were analyzed separately for *N. vitripennis* and *N. giraulti*. Univariate ANOVA was used to test for differences between the host species. Pairwise comparisons were done using a Tukey-HSD test.

The percentage of wasps that initiated drilling was determined for each day of

conditioning (n=5). Univariate ANOVA was used to test for differences between the host species. Pairwise comparisons were done using a Tukey-HSD test.

Memory retention was determined by testing for a difference in preference between the two reciprocal groups of 10-12 wasps. This difference is represented by the performance index (PI) as described by Hoedjes *et al*. (2012). The PI is calculated as: group 1 (%CS+) – group 2 (%CS–). If all trained wasps choose CS+, the difference between two reciprocal groups is at its maximum and the PI would be 100. This would represent perfect memory retention. When there is no memory retention, the two groups will choose similarly; this would result in a PI of 0. A total of 10 PIs was obtained for each wasp and host species. A t-test was used to test for the presence of memory retention. Univariate ANOVA was used to test if memory retention was different between host species.

RESULTS

Host value

The weight, length and width of the three host species were determined as a measure for host size and were all found to be different between each host species (weight: $F_{2,147}$ = 951.1, $P < 0.001$; length: $F_{2,147}$ = 1073.6, $P < 0.001$; width $F_{2,147}$ = 705.2, $P < 0.001$). *Calliphora vomitoria* is the host species with the highest weight (61.17 ± 0.95 mg) and the largest length (9.36 ± 0.06 mm) and width (3.76 ± 0.03 mm). *Lucilia sericata* is intermediate for all three parameters (weight: 31.98 ± 0.39 mg; length: 7.38 ± 0.04 mm; width: 2.85 ± 0.02 mm) and *Musca domestica* has the lowest weight (24.77 ± 0.33 mg), and the smallest length (6.50 ± 0.04 mm) and width (2.66 ± 0.02 mm) (Figure 1).

The number, sex ratio and the size of offspring that emerged from each of the three

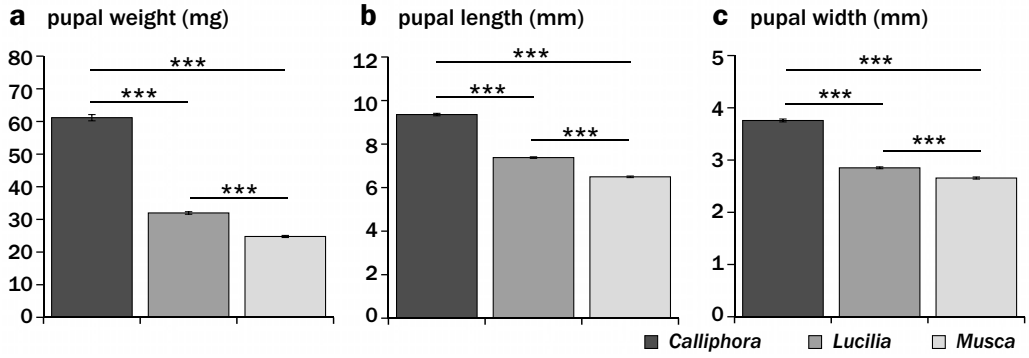

Figure 1: Characteristics of host pupae. The weight (a), length (b), and width (c) of host pupae (n=50 per host species). Asterisks indicate the level of significance (*** $P < 0.001$, ** $< P < 0.01$, * $0.01 < P < 0.05$, n.s. = not significant).

hosts were studied to determine differences in host value (Figure 2). Host species has an effect on number of emerged offspring in both *N. vitripennis* ($F_{2,57}$ = 98.0, P < 0.001) and *N. giraulti* ($F_{2,57}$ = 64.3, P < 0.001). The number of emerged offspring for *N. vitripennis* was highest for *C. vomitoria* (23.05 ± 0.87) and was on average respectively 46.9% and 50.2% lower for *L. sericata* (12.24 ± 0.48) and *M. domestica* (11.48 ± 0.54), which do not differ significantly from each other (Tukey-HSD, P = 0.69). The number of emerged offspring for *N. giraulti* was also highest for *C. vomitoria* (18.97 ± 0.72), intermediate for *L. sericata* (12.66 ± 0.56; 33.3% decreased compared to *C. vomitoria*), and lowest for *M. domestica* (8.90 ± 0.62; 53.1% decreased compared to *C. vomitoria*).

Next to emerged offspring, there were also very low numbers of diapausing larvae, which is normal for the *Nasonia* species under 16L:8D light regime. A number of offspring had died during development, which occured mostly in the pupal stage. For both *N. vitripennis* and *N. giraulti* the number of diapausing larvae per female per pupa did not depend on host species (*N. vitripennis*: $F_{2,57}$ = 2.57, P = 0.085; *N. giraulti*: $F_{2,57}$ = 1.0, P = 0.37) and the level of diapause was low (for *N. vitripennis* - *C. vomitoria*: 0.28 ± 0.10, *L. sericata*: 0.77 ± 0.21, *M. domestica*: 0.28 ± 0.16; for *N. giraulti* - *C. vomitoria*: 0, *L. sericata*: 0.04 ± 0.04, *M. domestica*: 0). The number of dead offspring per female per pupa did depend on host species for both *N. vitripennis* ($F_{2,57}$ = 4.89, P = 0.011) and *N. giraulti* ($F_{2,57}$ = 35.6, P < 0.001). The numbers of dead wasp offspring are low for *N. vitripennis* in *C. vomitoria* (0.12 ± 0.07) and *L. sericata* (0.04 ± 0.02), and this number is only significantly higher in *M.*

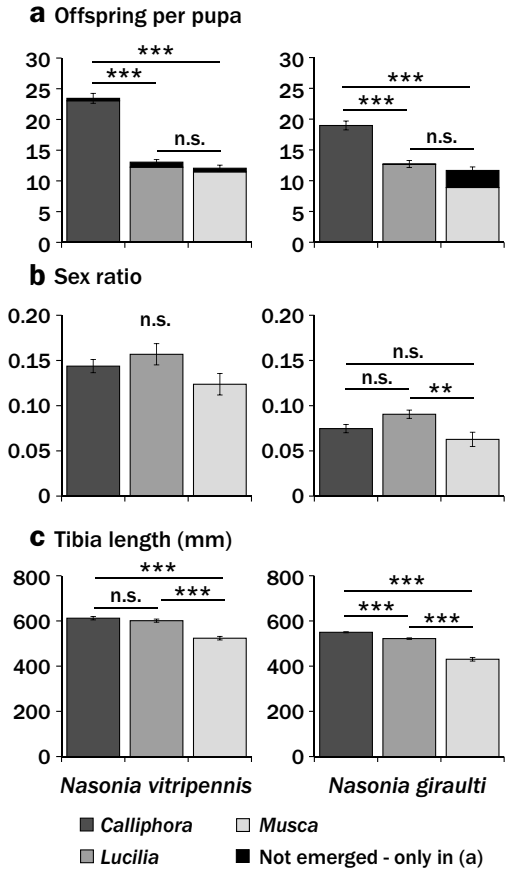

Figure 2: Host value parameters for the three host species. (a) the average offspring per pupa per female. The black sections on the bars indicate the number of offspring that did not emerge (diapausing larvae and dead offspring) (n = 20), (b) the sex ratio of emerged offspring (n = 20, and (c) tibia length of emerged female offspring, which is a measure for body size (n = 30). The left panels show data on *N.vitripennis*, the right panels show data on *N. giraulti*. Asterisks indicate the level of significance (*** P < 0.001, ** < P < 0.01, * 0.01 < P < 0.05, n.s. = not significant).

domestica (0.31 ± 0.08) compared to *L. sericata* (Tukey-HSD, $P = 0.010$). The numbers of average dead wasp offspring per pupa are also very low for *N. girault*i in *C. vomitoria* (0) and *L. sericata* (0.01 ± 0.01), but significantly higher for *M. domestica* (2.77 ± 0.46) (Tukey-HSD, $P < 0.001$); the percentage of dead offspring of the total number of offspring per *N. girault*i female per pupa is 23.7% for *M. domestica*.

The total number of offspring consists of emerged offspring as well as diapausing larvae and offspring that had died during development. Host species has an effect on number of total offspring in both *N. vitripennis* ($F_{2,57} = 109.5$, $P < 0.001$) and *N. girault*i ($F_{2,57} = 40.6$, $P < 0.001$). For both species the total number of offspring was highest for *C. vomitoria* (*N. vitripennis*: 23.45 ± 0.81; *N. girault*i: 18.97 ± 0.72). The number of offspring from *L. sericata* (*N. vitripennis*: 13.06 ± 0.42; *N. girault*i: 12.71 ± 0.56) and *M. domestica* (*N. vitripennis*: 12.07 ± 0.51; *N. girault*i: 11.67 ± 0.56) did not differ significantly from each other (Tukey-HSD, *N. vitripennis*: $P = 0.48$, *N. girault*i: $P = 0.46$).

Host species did not have an effect on sex ratio of emerged *N. vitripennis* offspring ($F_{2,57} = 2.57$, $P = 0.085$; *C. vomitoria*: 0.14 ± 0.01, *L. sericata*: 0.16 ± 0.01, *M. domestica*: 0.12 ± 0.01), but did have an effect on sex ratio of emerged *N. girault*i ($F_{2,57} = 4.82$, $P = 0.012$; *C. vomitoria*: 0.07 ± 0.00, *L. sericata*: 0.09 ± 0.00, *M. domestica*: 0.06 ± 0.01). There was a significant difference in sex ratio between *L. sericata* and *M. domestica* (Tukey-HSD, $P = 0.009$).

The size of female offspring, which was determined by measuring hind tibia length, was affected by host species for both *N. vitripennis* ($F_{2,87} = 42.3$, $P < 0.001$) and *N. girault*i ($F_{2,87} = 137.4$, $P < 0.001$). For *N. vitripennis* the size of female offspring was not significantly different for *C. vomitoria* (612.3 ± 7.4 µm) and *L. sericata* (601.4 ± 6.8 µm), but was decreased by 14.5% in *M. domestica* (523.8 ± 8.0 µm) compared to *C. vomitoria*. For *N. girault*i the size of female offspring was highest for *C. vomitoria* (550.0 ± 2.4 µm), intermediate for *L. sericata* (521.9 ± 3.5 µm; 5.1% decreased compared to *C. vomitoria*), and lowest for *M. domestica* (430.6 ± 8.2 µm; 21.7% decreased compared to *C. vomitoria*).

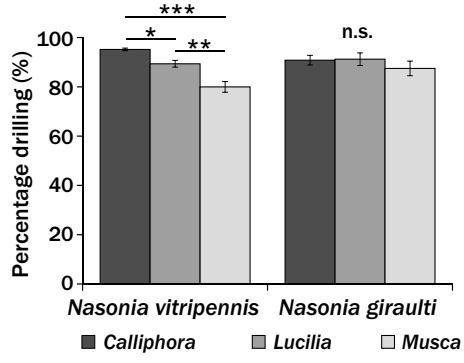

Figure 3: Drilling behavior. Percentage of (a) *N. vitripennis* and (b) *N. girault*i that initiate drilling in a host within 30 minutes after the start of conditioning (n = 5). Asterisks indicate the level of significance (*** $P < 0.001$, ** $< P <$ 0.01, * $0.01 < P < 0.05$, n.s. = not significant).

Drilling percentage

Drilling percentage of *N. vitripennis* is affected by host species ($F_{2,12} = 25.7$, $P <$ 0.001) (Figure 3). The percentage of wasps

that initiate drilling is highest when *C. vomitoria* (95.2 ± 0.53) is offered, intermediate when *L. sericata* (89.4 ± 1.37) is offered, and lowest when *M. domestica* (80.0 ± 2.17) is offered. Drilling percentage of *N. girault*i is not dependent on host species ($F_{2,12}$ = 0.66, *P* = 0.55) (*C. vomitoria*: 90.8 ± 1.96, *L. sericata*: 91.3 ± 2.54, *M. domestica*: 87.5 ± 2.89) (Figure 3).

Memory retention

Memory of *N. vitripennis* was tested 24 and 120 hours after conditioning and memory was present at both time points for all three host species (Figure 4). Memory retention was not affected by the host species that was offered as a reward ($F_{2,56}$ = 0.32, *P* = 0.73). PI decreases over time for all three host species ($F_{1,56}$ = 43.4, *P* < 0.001). Memory of *N. girault*i was tested 24 and 72 hours after conditioning. Again, memory retention was not affected by the host species that was offered as a reward ($F_{2,56}$ = 0.019, *P* = 0.98). Memory was present 24 hours after conditioning, but had decayed after 72 hours (Figure 4). PI decreases over time for all three host species ($F_{1,56}$ = 120.1, *P* < 0.001). These results indicate that the host species that is offered during conditioning does not affect the strength or duration of the conditioned response or memory retention for both *N. vitripennis* and *N. girault*i.

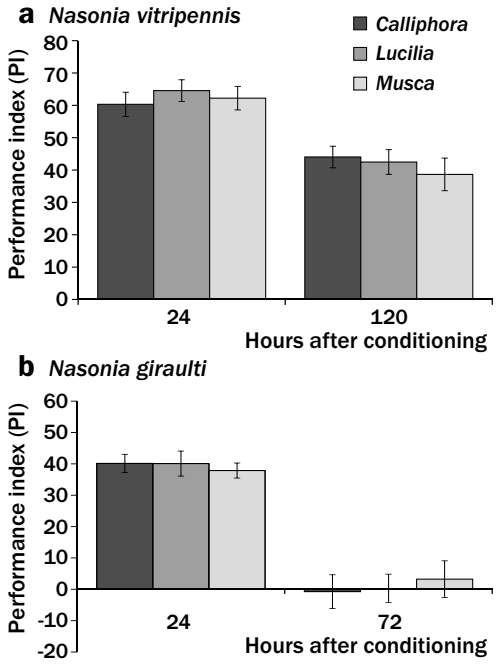

Figure 4: Memory retention. Memory retention (PI) of both (a) *N. vitripennis* and (b) *N. girault*i was determined after a single conditioning trial with *C. vomitoria*, *L. sericata* or *M. domestica* pupae as a reward (n = 10).

DISCUSSION

This study investigated the effect of host value on memory formation in the parasitic wasp species *N. vitripennis* and *N. girault*i. Our results demonstrate that there are significant differences in host value between the three host species for both *Nasonia* species: the number of offspring and offspring size were decreased in the smaller host species. The number of emerged offspring of a female wasp is strongly correlated with Darwinian fitness, but also the size of a wasp has a strong effect on fitness (Rivero & West, 2002): smaller

females typically have lower fat reserves affecting fecundity, longevity and dispersal ability. The total number of offspring, consisting of emerged offspring as well as diapausing larvae and offspring that had died during development shows how many offspring a female has allotted to a host and how she perceives host value. In this study, total numbers of offspring correlate with host value. The numbers of dead offspring are generally low, but were higher in *M. domestica* compared to the other host species, especially for *N. giraulti*. This may indicate a mismatch between perceived and actual host value of *M. domestica*, likely because *Nasonia* is not well adapted to this species (Werren, 1983; Peters & Abraham, 2010). Next to the fitness parameters that we determined, other parameters can affect host value, e.g. specific nutritional characteristics of a host can affect fitness of offspring; the presence of other female wasps or superparasitism can affect offspring numbers, size and sex ratio (Ivens *et al*., 2009; Blaul & Ruther, 2011). These factors were not tested and it cannot be concluded that these will not have an effect on memory retention. However, both *Nasonia* species decrease their total offspring number with decreasing host value. This result strongly indicates that both *N. vitripennis* and *N. giraulti* indeed perceive the differences in host value and also respond to that.

Sex ratio of offspring was determined, because a smaller host size has been hypothesized to result in an increased sex ratio in parasitic wasps (King, 1987). We did not find such a relationship; in fact, there was a trend that sex ratio was lowest in *M. domestica* (although only significant for *N. giraulti*). Other studies have also shown that differences in sex ratio between host species are not necessarily correlated with host size (King, 1996). From this we conclude that sex ratio is not the most informative parameter for host value in this study. We observed that *N. giraulti* overall has a lower sex ratio than *N. vitripennis* (respectively 0.06 ± 0.005 and 0.14 ± 0.007 in *C. vomitoria*). This is considered an adaptation to the mating strategy of *Nasonia giraulti*, which often mates within the host puparium (Drapeau & Werren, 1999).

The difference in host value is not only reflected in offspring numbers and size, but, interestingly, also by drilling behavior of *N. vitripennis*. Possibly, *N. vitripennis* has a search or host acceptance strategy that is adapted to parasitizing larger hosts instead of smaller hosts, as was observed by Desjardins *et al*. (2010). In that study, a higher percentage of *N. vitripennis* would drill in large pupae of *Sarcophaga bullata*. The situation was reversed in *N. giraulti*, which preferred its natural, smaller host species *Protocalliphora* spp. (Desjardins *et al*., 2010). In our study we did not observe a difference in drilling percentage between host species in *N. giraulti*, indicating that this species has a more tolerant host acceptance strategy. This is unexpected as *N. giraulti* is considered a specialist species, but may be explained by the fact that none of the species that we used in our study are the preferred host species of *N. giraulti*.

Both behavioral and fitness parameters clearly indicate that the different host species used in this study differ in host value and the results further indicate that both *N. vitripennis* and *N. giraulti* likely perceive this difference in host value. Surprisingly, host value neither affected the level of the PI nor duration of memory retention in either wasp species. Results on *N. giraulti* should be interpreted with some caution, because the three host species tested were not the preferred host of *N. giraulti*, but both (naïve) *Nasonia* species appear not to use information on host value in memory formation. This is in contrast with a number of other studies in which animals, including other parasitic wasp species, were observed to adjust their conditioned response according to reward value, even when they were inexperienced with the reinforcing stimulus (Diegelmann *et al*., 2006; Wäckers *et al*., 2006; Camacho *et al*., 2009; Burke & Waddell, 2011; Kruidhof *et al*., 2012).

Kruidhof *et al*. (2012) hypothesized that the strength of association in oviposition learning is not only determined by host value, but also by the reliability of the learned association. We have argued that finding a host of a specific quality is likely not a reliably indication for the number and quality of other hosts in the patch for both *N. vitripennis* and *N. giraulti*. Consequently, host value is also not a reliable indication for future reproductive success, which is a potential reason that these species do not adjust memory retention based on host value. This result emphasizes the importance to consider all aspects of 'reward value' in order do understand memory dynamics.

It is possible that *Nasonia* parasitic wasps do not adjust memory retention to hosts of different qualities, because they have not experienced variation in host value. A naïve animal, inexperienced with the reward, can demonstrate a strong conditioned response, even if the value of the reward is low: inexperienced male rats (*Rattus norvegicus*) required less sexual stimulation to learn a cue than experienced rats (Tenk *et al*., 2009). The conditioned response of a parasitic wasp (*Pseudeucoila bochei*) and a hummingbird (*Selasphorus rufus*) was shown to depend on the relative value of a reward compared to previous rewards (van Lenteren & Bakker, 1975; Bateson *et al*., 2003). It is possible that a low host value was sufficiently rewarding for our naïve *Nasonia* wasps, and future studies in which female wasps experience a host of either a higher or lower value before conditioning are needed to assess effects of previous experience on memory retention. Our results on offspring numbers and drilling behavior did indicate, however, that the naïve *Nasonia* wasps were able to estimate host value, without any other host experience.

A difference of our study with the study on the parasitic wasp species *C. glomerata* and *T. evanescens*, is that oviposition took place during conditioning in that study (Kruidhof *et al*., 2012). *Nasonia* parasitic wasps require host feeding for the maturation of eggs and naïve females will mostly perform host feeding during their first period of contact with a host and will initiate oviposition later on (Schurmann *et al*., 2012; Hoedjes & Smid,

2014). The same host can be used for host feeding and oviposition (Rivero & West, 2005). Potentially, the *Nasonia* wasps did not adapt memory retention to host value, because they were not ready to initiate oviposition yet. Host feeding and oviposition will, however, result in similar memory retention in both *N. vitripennis* and *N. giraulti* (Hoedjes & Smid, 2014).

Concluding, this study has shown that the value of the host does not have an effect on memory retention in naïve *N. vitripennis* and *N. giraulti*. This is in contrast to a number of other studies in diverse animal species. We've argued that host value may not be a reliable indication for future reproductive success for *Nasonia*, which could be a reason that these species to not adapt memory retention to host value. Alternatively, previous experience or the occurrence of host feeding versus oviposition may play a role. The results suggest that both the quality of the reinforcer and reliability of the learned association can be important aspects of total reward value. Both aspects should be considered to understand variation in memory formation. Further studies, both on reward value for parasitic wasps as well as other animals, will deepen our knowledge on how a reward affects memory dynamics, which can improve conditioning methods for animals. Comparative studies on parasitic wasps can further elucidate which ecological factors or mechanisms are important for the perception and adaptation of memory dynamics in response to different hosts. This will increase our understanding on the evolution of variation in learning and memory.

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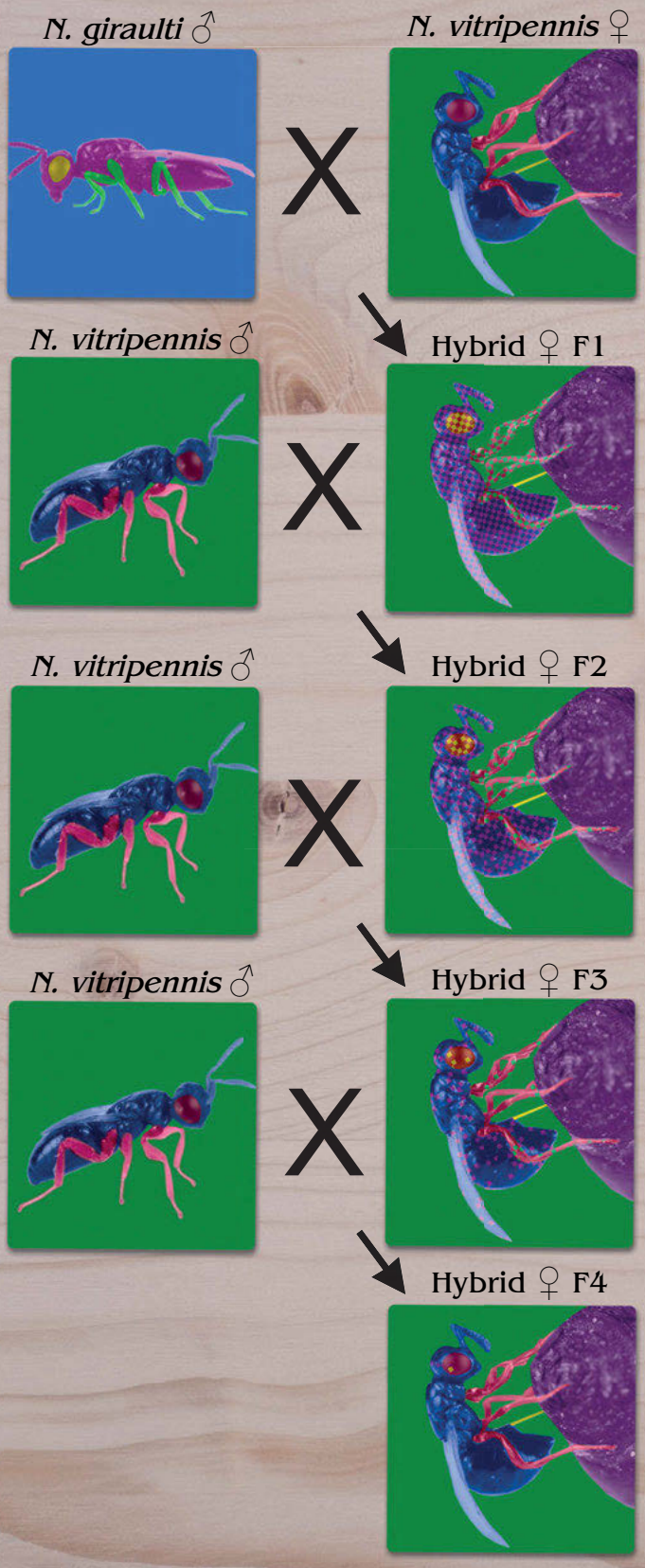
N. girault ♂
N. vitripennis ♀
X
N. vitripennis ♂
Hybrid ♀ F1
X
N. vitripennis ♂
Hybrid ♀ F2
X
N. vitripennis ♂
Hybrid ♀ F3
X
Hybrid ♀ F4

Chapter 6

Introgression study reveals two quantitative trait loci involved in interspecific variation in memory retention among *Nasonia* wasp species

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Under review

ABSTRACT

Genes involved in the process of memory formation have been studied intensively in model organisms, but little is known about the mechanisms that are responsible for natural variation in memory dynamics. There is substantial variation in memory retention among closely related species: the parasitic wasp *Nasonia vitripennis* consolidates long-term memory, which lasts at least 6 days, after a single olfactory conditioning trial. Memory of the closely related species *N. giraulti* is lost within two days after a single conditioning trial. The genetic basis of this inter-specific difference in memory retention was studied in a backcrossing experiment in which the phenotype of *N. giraulti* was selected for in the background of *N. vitripennis* for up to 5 generations. A genotyping microarray analysis revealed 5 candidate regions, which were retained in wasps with decreased memory. Independent introgressions of individual candidate regions were created using linked molecular markers and tested for memory retention. One region on chromosome 1 (spanning ~ 5.8 cM) and another on chromosome 5 (spanning ~ 25.6 cM) resulted in decreased memory after 72 hours, without affecting 24-hour-memory. This phenotype was observed in both heterozygous and homozygous individuals. Transcription factor CCAAT/enhancer-binding protein (C/EBP) and a dopamine receptor, both with a known function in memory formation, are within the regions and are candidates for the regulation of memory retention. Concluding, this study demonstrates a powerful approach to study variation in memory retention and provides a basis for future research on its genetic basis.

INTRODUCTION

The ability to learn and form memory is vital for animal species. Learning will alter innate behaviour with regard to finding food, mates or hosts, which allows species to adapt to variation in their environment. A learning experience can result in the formation of different types of memory, which differ in their characteristics and cellular pathways involved. Immediately after a learning experience, short-term memory (STM) is formed, which is a labile type of memory that typically lasts for minutes up to hours at most (Menzel, 1999; Margulies *et al*., 2005; van den Berg *et al*., 2011). This type of memory can be disrupted by anaesthesia, such as a cold-shock in insects, and is, therefore, also classified as anaesthesia-sensitive memory (ASM). More durable and less-labile types of memory include anaesthesia-resistant memory (ARM), which can last from hours to days, and long-term memory (LTM), which is dependent on protein synthesis and can last up to the entire life-time of an animal (Margulies *et al*., 2005; Eisenhardt, 2006; Smid *et al*., 2007).

Both the process of memory formation and the cellular mechanisms involved are highly conserved among distant animal phyla (Dubnau, 2003). There is, however, inter- and intraspecific natural variation in memory dynamics (Hoedjes *et al*., 2011). A single conditioning trial or multiple massed trials, i.e. trials with short inter-trial intervals, will often result in the formation of STM and/or ARM, which will be lost after hours to days. Many animal species will only form LTM after multiple conditioning trials, which are spaced in time (Margulies *et al*., 2005; Eisenhardt, 2006; Smid *et al*., 2007). However, some species, including a number of parasitic wasps, already consolidate LTM after a single conditioning trial (Krashes & Waddell, 2008; Hoedjes *et al*., 2011). Parasitic wasps lay their eggs in or onto insect hosts, from which their developing larvae will feed. Female wasps can learn cues that are associated with their hosts, so-called oviposition learning (Hoedjes *et al*., 2011). Natural variation in memory retention and the number of conditioning trials required to form LTM can be substantial, even between closely related parasitic wasp species (Smid *et al*., 2007; Hoedjes & Smid, 2014).

Extensive research on genetic model organisms has provided a wealth of knowledge on the genetic and neural mechanisms involved in different aspects of the memory formation process. However, the mechanisms that are responsible for variation in memory dynamics between individuals or species are largely unknown (Hoedjes *et al*., 2011). Few studies have investigated natural variation in memory dynamics in parasitic wasps of the genus *Cotesia* and in fruit flies using an experimental evolution procedure, but did not study the genetic basis of this trait (Mery & Kawecki, 2002; van den Berg *et al*., 2011). Studies using laboratory-generated mutants of *Drosophila melanogaster* have been highly successful in identifying single loci with large effects on memory formation (Margulies *et al*., 2005). Induced expression of a specific splice variant of cAMP-responsive transcription factor

CREB in *Drosophila melanogaster* results in LTM formation after a single conditioning trial, whereas 10 spaced conditioning trials are normally required (Tubon *et al*., 2013). Induced expression of tyrosine phosphatase SHP2 (*corkscrew*), also in *D. melanogaster*, results in LTM formation after 10 massed conditioning trials (Pagani *et al*., 2009). It remains to be investigated if these two specific genes are also involved in natural variation in memory retention, which may depend on more subtle variation in these genes or on completely different genes. The aim of this study is to identify genetic factors that are responsible for interspecific natural variation in memory retention after a single conditioning trial.

Parasitic wasp species of the genus *Nasonia* provide unique opportunities to study variation in memory retention. *Nasonia vitripennis* will form LTM, which will last over 6 days, after a single Pavlovian conditioning trial in which an odor is associated with the reward of finding a host. In contrast, a single conditioning trial results in the formation of ARM in the closely related species *N. giraulti* and this memory will be lost within one to two days after conditioning. Only multiple conditioning trials will result in the formation of long-lasting memory in *N. giraulti* (Hoedjes *et al*., 2012; Hoedjes & Smid, 2014). The genus *Nasonia* has emerged as a model system with powerful genetic tools to study interspecific variation. The combination of a sequenced genome, a high-density genetic map and the possibility to interbreed the four described species of the genus provides excellent opportunities for genetic studies, including QTL mapping (Werren & Loehlin, 2009; Werren *et al*., 2010). Recent research has successfully backcrossed traits of *N. giraulti* into a *N. vitripennis* background in order to study the genetic basis of interspecies differences in wing size and morphology, female host preference behaviour, hybrid incompatibilities and pheromone composition (Niehuis *et al*., 2008; Desjardins *et al*., 2010; Loehlin & Werren, 2012; Niehuis *et al*., 2013). We hypothesized that memory retention can also be introgressed from one *Nasonia* species to another, which facilitates studies on the genetic basis of this trait.

Introgression of memory related phenotypes was successful in a number of studies on mice and rats (Wehner *et al*., 1997; Jarome *et al*., 2010). Compared to these mammalian species, *Nasonia* has a much shorter generation time and is easier to rear and handle. In addition, these hymenopteran species have a haplodiploid mating system which makes them particularly suitable for introgression and genetic dissection (Werren & Loehlin, 2009). These features allow us to study multiple QTLs involved in memory retention and to carry out multiple confirmation experiments in a single study. In addition, this is the first study to map memory retention QTLs by introgression of genes between species, thus exploiting interspecies divergence in memory retention.

We studied memory retention 24 hours after conditioning (likely a form of ARM), which we define as short-lasting memory for the purpose of this study, and after 48 hours or more following conditioning (which can include ARM and LTM), defined here

as long-lasting memory. The aim of this study is to specifically identify genes involved in reduced long-lasting memory (*N. giraulti* phenotype, i.e. short memory retention), without affecting short-lasting memory (observed in both species). Wasps with the desired memory retention are selected for during the genetic introgression process, which was carried out up to the fifth generation. Then, selected introgression lines were genotyped (Desjardins *et al*., 2013). Effects of individual, putative memory retention QTLs were confirmed by testing memory retention of independently created segmental introgression lines for these regions in three confirmation experiments. Two QTLs affecting long-lasting memory, but not short-lasting memory, were identified.

MATERIALS AND METHODS

Nasonia strains and maintenance

Experiments were done with *N. vitripennis* (AsymCx) and *N. giraulti* (RV2x(U)) strains, which are completely homozygous and have a sequenced genome (Werren *et al*., 2010), or on hybrids and introgression lines derived from a cross between these two strains. Wasps were reared on *Calliphora vomitoria* fly pupae as described in Hoedjes *et al*. (2012). Female wasps were collected on the day of emergence, and were then mated, provided honey and water, and kept in a climate cabinet at a temperature of 25°C and a photoperiod of 16L:8D for 1 to 3 days until conditioning.

Olfactory conditioning and memory retention test

Female parasitic wasps were conditioned using a Pavlovian conditioning assay in which an odor (chocolate or vanilla odor, the conditioned stimulus (CS+)) was associated with the reward of access to a host (the unconditioned stimulus (US)), a *C. vomitoria* pupa. The protocol for individual conditioning as described in Hoedjes *et al*. (2012) was adapted to allow conditioning of groups of wasps in order to obtain large numbers of conditioned wasps that were required for the experiments (see Supplementary Information for more details).

Memory retention was tested in a T-maze olfactometer as described in Hoedjes *et al*. (2012). Briefly, the olfactometer consists of two tubular arms, which are connected to a middle tube, with a continuous airflow of 100 ml/min through each arm. Chocolate odor was offered on one side and vanilla odor was offered on the other side. Groups of 10 to 12 wasps were released in the middle of the T-maze, the numbers of wasps in the two arms was recorded after 10 minutes, and the percentage that had chosen each odor was calculated. Immediately afterwards, a reciprocal group of wasps was tested. The difference in odor preference between the two reciprocal groups (group 1 and group 2) is traditionally used

as a measure for memory retention and is represented by the Performance Index (PI) (Tully *et al*., 1994). PI was calculated as follows: group 1 (%CS+) – group 2 (%CS-). A t-test is used to test for significant memory retention, i.e. whether a PI is significantly different from 0 (SPSS version 19; IBM, Armonk, NY, USA)

Initial introgression of memory retention genes from N. giraulti into N. vitripennis background

F1 hybrids were generated by mating *N. vitripennis* females to *N. giraulti* males. This cross was chosen because nuclear-mitochondrial incompatibilities can complicate introgressions in the reciprocal cross (Breeuwer & Werren, 1995). Memory retention of the hybrids was compared to both parental strains using the conditioning procedure and memory retention test as described above. Memory retention was tested 24 (±1) and 48 (±1) hours after conditioning.

A total of 20 F1 hybrid females were mated to *N. vitripennis* males and their female offspring was conditioned and tested for memory retention (Figure 1a). The aim of this experiment was to select wasps that did demonstrate memory retention after 24 hours, but not after 48 hours or later (similar to *N. giraulti*), and to backcross this short memory retention into the background of *N. vitripennis*. Virgin female offspring were mated to *N. vitripennis* males. An entire group of sisters was subsequently conditioned on either vanilla or chocolate odor (CS+) as described above; the aim was to have approximately similar numbers of wasps conditioned on each of the two odors. Wasps were tested 20-24 hours after conditioning and wasps that failed to walk towards the learned odor were removed from the experiment in order to avoid selecting wasps with general defects in the learning and memory pathways. The remaining wasps were tested two times between 60-72 hours after conditioning, which was a more convenient time frame than 48 hours after conditioning. Wasps that chose the 'wrong' odor (CS-) twice were considered to have lost their memory, implying that they did not have long-lasting memory, similar to *N. giraulti*, and these wasps were selected to continue introgression of this memory phenotype. Wasps that chose the learned odor (CS+) twice were considered to be mostly wasps with long-lasting memory, and were selected to create control lines. Control experiments had been done to test effects of multiple memory retention tests on the observed memory behaviour of *N. vitripennis* and *N. giraulti* (see Supplementary Information). Selected females were individually provided 3 hosts in a glass tube closed with a cotton wool plug to generate offspring. Offspring was further backcrossed with *N. vitripennis*, and conditioned and tested as described above up to the 5th generation. Every generation 15 to 25 selected females with decreased long-lasting memory and selected females from the control lines were used to set up a next generation. Offspring from females that were conditioned on vanilla were conditioned on

chocolate the next generation to avoid selection for a specific odor preference. As entire groups of sisters were conditioned on a single odor, an adjusted PI was calculated for this experiment. Approximately 50% of unconditioned wasps will choose the 'learned odor' (as shown in Hoedjes *et al*., 2012) and 'half' of a PI can be calculated by subtracting 50% from the percentage of wasps that choses the learned odor. The PI is obtained by multiplying this number by two: (%CS+ - 50%)*2.

A back-up was created during the 4th generation of introgression in order to ensure continuation of the project during transition from the USA to the Netherlands. Experiments on these back-up lines are described in the Supplementary Information.

Univariate ANOVA was used to test for variation in memory retention and a Tukey-HSD post-hoc test was used when appropriate (SPSS version 19; IBM, Armonk, NY, USA).

Genotyping of memory introgression lines

Four samples of wasps from a sibship of up to 10 wasps with a decreased long-lasting memory (sisters that had chosen the 'wrong' odor twice) and their controls (sisters that had chosen the learned odor twice) were genotyped using the high-density CGH genotyping microarray for *Nasonia*, which contains more than 20,000 markers, and analysed using a bulk-segregant analysis (Desjardins *et al*., 2013). The four samples were composed of groups of sisters that each had been derived from a different female selected in the F2 generation. Two samples were taken from the 4th generation of introgression and two from the 6th generation. In addition, the genotype of individual wasps was confirmed using indel-markers within observed introgressed regions in a polymerase chain reaction (PCR). The genotyping analyses have been described more in detail in the Supplementary Information.

Confirmation of memory retention QTLs by independent introgressions

A total of 5 introgressed regions were observed in wasps with decreased long-lasting memory retention in the microarray experiment. To independently and individually test these candidate regions, each was backcrossed from *N. giraulti* into the background of *N. vitripennis* for a confirmation experiment, using linked molecular markers tracked during introgression by polymerase chain reaction (PCR) genotyping (see Supplementary Information for more details). The experiment is independent from the initial introgression experiment and selection depended on genotype alone and not on phenotype (i.e. memory retention). F1 hybrids were generated as described above and backcrossed to *N. vitripennis* up to the fourth to sixth generation, before the effects of the introgressed regions on memory retention were tested. In each generation, female wasps that were selected based on genotype, were provided 3 hosts individually in a glass vial as described. For each of the

5 regions of interest, multiple primers were used that are located close to the border of the region and/or within the region. We ensured that each of these introgression lines had a single region of interest, and not any of the other 4 regions of interest. The boundaries of the region were not determined. The locations of the regions on the linkage map of Desjardins *et al*. (2013) are given. A single region on chromosome 1 (location: 45.3 - 60.6 cM), a small region on chromosome 4 (location: 86.2 - 90.6 cM) and two regions on chromosome 5 (locations: 0.0 - 2.9 cM and 34.3 - 59.9 cM) were introgressed and tested. Two lines with different parts of the region of interest on chromosome 3 were created (locations: 0.0 - 27.7 cM and 27.7 - 51.8 cM).

The introgressed regions were maintained heterozygously throughout this experiment. Hybrid females were mated to *N. vitripennis*, which, therefore, results in a mix of female offspring with and without the introgressed region. In order to test the effect of an introgressed region on memory retention, sibling females were individually provided 5 hosts in a glass vial as described above. Their female offspring were conditioned and tested for memory retention after 72 (±2) hours; this time point was chosen instead of 48 hours to match the time point of selection in the initial introgression experiment. Memory retention was tested after 4 (chromosome 1 and 3), 5 (chromosome 3 and 5) or 6 (chromosome 4) generations of introgression. Offspring of wasps that were heterozygous for the region of interest were compared to offspring of sister wasps that were homozygous *N. vitripennis* for the region of interest (control group). This approach controls for unlinked regions segregating in the offspring that could affect memory retention. A region was considered to have an effect on memory retention if the PIs of the wasps with the introgressed region differed from those of control wasps.

As a further test, regions which were found to have an effect on PI were investigated by testing memory retention of offspring of males with the introgressed region, which were mated to *N. vitripennis* females to produce isogenic female sibships containing the heterozygous region of interest (Velthuis *et al*., 2005). Wasps were conditioned and memory retention was tested after 24 (±2) and 72 (±2) hours as described earlier. Offspring of males which carried the region of interest were compared to offspring of brothers without the introgressed region (control group).

Experiments were carried out to generate homozygous strains for regions of interest which affected long-lasting memory, in combination with further partitioning of the genomic regions by recombination. We succeeded in generating a strain which was homozygous for a part of the introgressed region on chromosome 1 (location: 54.8 - 60.6 cM). This strain was named 'SIL_LTM1A_gV' and this strain was tested for memory retention after 24 (±2), 72 (±2) and 120 (±2) hours after a single conditioning. Memory retention of this segmental introgression line was compared to *N. vitripennis* and *N. giraulti*. The size of the

introgressed region was determined by PCR as described above.

Univariate ANOVA was used to analyse differences in memory retention and a Tukey-HSD post-hoc test was used when appropriate (SPSS version 19; IBM, Armonk, NY, USA).

RESULTS

Initial introgression of memory retention genes from N. giraulti to N. vitripennis

Memory retention of *N. vitripennis* (NV), *N. giraulti* (NG) and F1 hybrids (*N. vitripennis* females x *N. giraulti* males) was tested in order to determine which of the memory phenotypes was dominant (Figure 1b). There was an effect of genotype on the PI both after 24 and 48 hours (24 hours: $F_{2,27} = 10.93$, $P < 0.001$; 48 hours: $F_{2,27} = 37.65$, $P < 0.001$; n = 10 PIs for each genotype and time point). After 24 hours, memory of *N. vitripennis* and *N. giraulti* differ significantly from each other (Tukey-HSD: NV vs. NG < 0.001) and the PIs of the F1 hybrids were almost intermediate to the parentals (Tukey-HSD: NV vs. F1 = 0.099, NG vs. F1 = 0.045). The finding that F1 hybrids are intermediate suggests the presence of one or more loci affecting short-lasting memory, with at least some co-dominance. By 48 hours, memory of *N. giraulti* has been lost whereas *N. vitripennis* still had significant memory retention. Memory retention of the F1 hybrids is similar to *N. giraulti* (Tukey-HSD: NV vs. F1 < 0.001, NG vs. F1 = 1.000). This demonstrates that the hybrids lose their memory within 48 hours after conditioning, similar to *N. giraulti*.

The dominant *N. giraulti* phenotype was backcrossed into *N. vitripennis* genetic background (Figure 1a). The F2 generation (Figure 1c) is a mixture of different genotypes and memory retention phenotypes, from which introgression lines, with *N. giraulti* phenotype, and control lines were selected. In the F3 generation (Figure 1d), both control and introgression lines have similar PIs at 24 hours after conditioning ($F_{1,40} = 0.44$, $P = 0.835$, introgression: n = 22 PIs, control: n = 20 PIs). After 72 hours the PIs of the introgression lines have decreased in comparison to the control lines ($F_{1,30} = 9.73$, $P = 0.004$, introgression: n = 17 PIs, control: n = 15 PIs). A similar pattern of memory retention is visible in the F4 generation (Figure 1e) (24 hours: $F_{1,50} = 0.15$, $P = 0.699$, introgression: n = 27 PIs, control: n = 25 PIs; 72 hours: $F_{1,42} = 10.25$, $P = 0.003$, introgression: n = 23 PIs, control: n = 21 PIs). In the F5 generation (Figure 1f), the introgression line has higher PIs compared to the control at 24 hours after conditioning ($F_{1,57} = 7.55$, $P = 0.008$, introgression: n = 35 PIs, control: n = 24 PIs), but again a decreased memory retention after 72 hours ($F_{1,46} = 17.36$, $P < 0.001$, introgression: n = 29 PIs, control: n = 19 PIs).

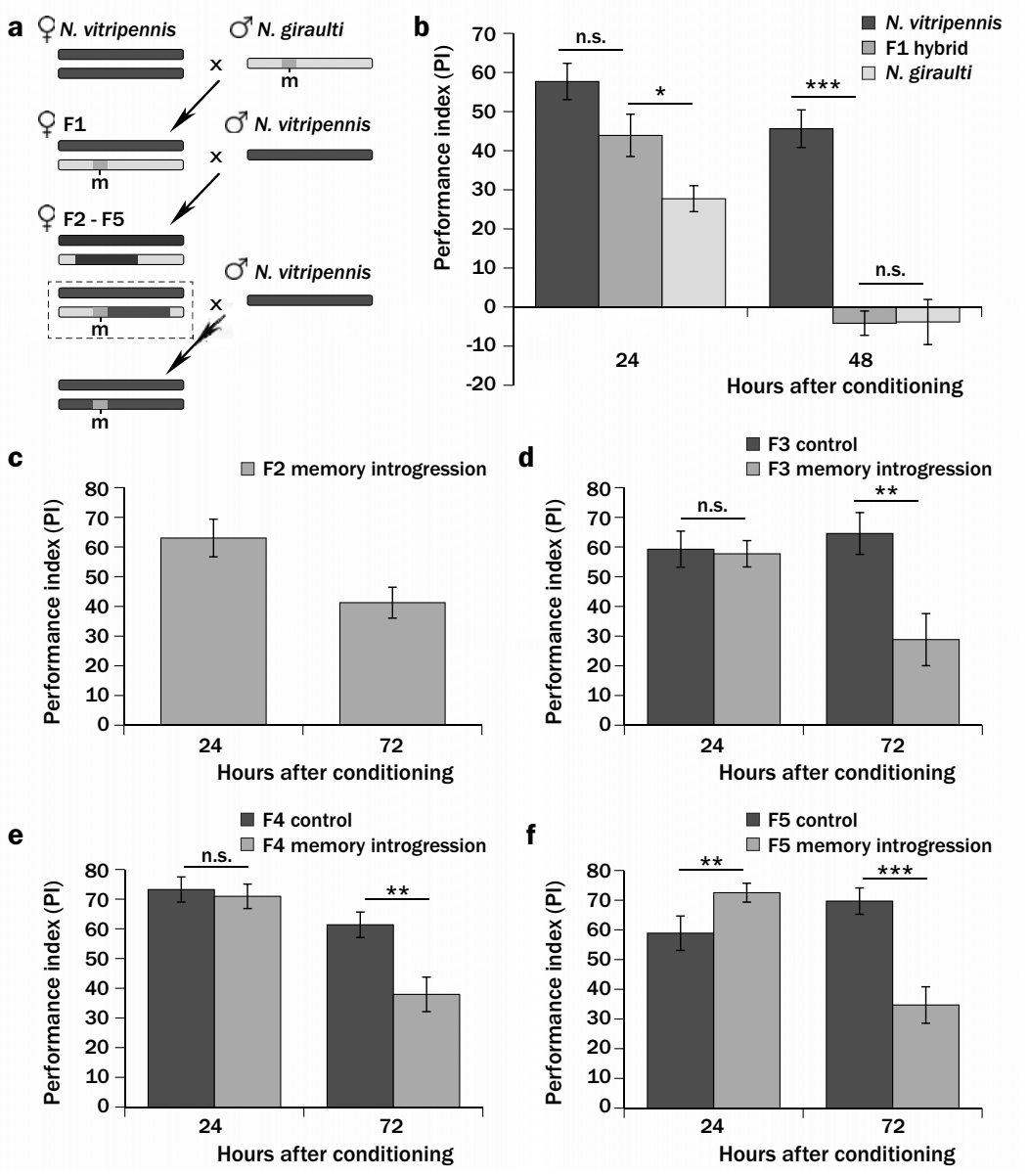

Figure 1: Initial introgression of memory retention genes. (a) Genes that control long-lasting memory ('m') were backcrossed from *N. girault* (<48h memory retention) into the background of *N. vitripennis* (>144h memory retention). (b) Memory retention of F1 hybrids was compared to both parentals. The hybrids have lost their memory after 48 hours, comparable to *N. girault*. (c) Selection for decreased long-lasting memory while backcrossing was started in the F2 generation and resulted in decreased memory scores in the memory introgression lines compared to control lines when tested after 72 hours in the (d) F3, (e) F4, and (f) F5 generations. There was no decrease in memory scores when tested after 24 hours. Level of significance: *** $P < 0.001$, ** $< P < 0.01$, * $0.01 < P < 0.05$, n.s. = not significant.

Genotyping of memory retention introgression lines

A total of 4 selected introgression lines were genotyped using the microarray (Desjardins *et al*. 2013), which revealed a number of regions that were retained during the introgression process (Figure 2a and Table 1). Two samples consisted of wasps from the 4th generation of introgression. The first of these samples (F4A) had retained two introgressed regions. One region was on chromosome 4 in wasps with decreased long-lasting memory (F4A-memory). The same region was, although larger, also retained in the control sample. In addition, almost entire chromosome 5 was retained (F4A-control). The second sample of the F4 generation (F4B) did not contain any detectable *N. giraulti* regions in wasps with decreased long-lasting memory (F4B-memory), whereas the control sample had retained a small region on chromosome 4 (F4B-control).

Two samples consisted of wasps from the 6th generation of introgression. In the first sample (F6A), the wasps with decreased long-lasting memory had retained a region on chromosome 1 and a small region on chromosome 5 (F6A-memory) and the control sample had retained a region on chromosome 3 (F6A-control). The wasps with decreased

Table 1: Potential memory retention QTLs. This table shows the regions that were detected in each of the samples by genotyping microarray analyses and it shows characteristics of these regions: The location of the QTL on the linkage map (cM), the size in cM, and the location of the QTL within the marker clusters on the linkage map of *Nasonia* (Desjardins *et al*. 2013). The QTL size is also estimated in Mb, by adding up scaffold sizes that were mapped to the genetic map by Desjardins *et al*. (2013). The QTL size can be larger than estimated due to gaps between scaffolds and a number of scaffolds that could not be mapped to the genetic map. The table also shows the number of wasps in which the QTL could be confirmed by PCR.

Sample	QTL location	QTL size		Marker clusters	# with QTL
F4A-memory	Chr. 4, 86.2-90.6 cM	~4.4 cM	~2.1 Mb	4.099-4.103	2 of 3 (66.7%)
F4A-control	Chr. 4, 57.7-90.6 cM	~32.9 cM	~6.3 Mb	4.071-4.103	5 of 10 (50.0%)
	Chr. 5, 1.5-73.0 cM	~71.5 cM	~32.4 Mb	5.001-5.091	8 of 10 (80.0%)
F4B-memory	none observed	n.a.	n.a.	n.a.	0 of 4 (0%)
F4B-control	Chr. 4, 4.4-9.5 cM	~5.1 cM	~1.0 Mb	4.005-4.011	7 of 10 (70.0%)
F6A-memory	Chr. 1, 45.3-60.6 cM	~15.3 cM	~44.0 Mb	1.051-1.072	1 of 2 (50.0%)
	Chr. 5, 34.3-59.9 cM	~25.6 cM	~19.4 Mb	5.040-5.075	1 of 2 (50.0%)
F6A-control	Chr. 3, 36.5-51.8 cM	~15.3 cM	~23.9 Mb	3.043-3.064	1 of 3 (33.3%)
F6B-memory	Chr. 3, 0.0-29.2 cM	~29.2 cM	~8.0 Mb	3.000-3.035	7 of 10 (70.0%)
	Chr. 5, 0.0-2.9 cM	~2.9 cM	~1.8 Mb	5.000-5.003	3 of 10 (30.0%)
F6B-control	Chr. 3, 0.0-10.9 cM	~10.9 cM	~4.0 Mb	3.000-3.014	6 of 10 (60.0%)
	Chr. 5, 0.0-1.5 cM	~1.5 cM	~0.8 Mb	5.000-5.001	2 of 10 (20.0%)

long-lasting memory of the second sample (F6B-memory) had retained regions on chromosome 3 and 5. These regions were also retained in the control sample (F6B-control), although both regions were smaller in these wasps. Summarising, a total of 5 regions was found in wasps with decreased long-lasting memory. Two of these regions (on chromosome 1 and part of the region on chromosome 3) were only detected in wasps with decreased long-lasting memory, whereas the other regions were also detected in control samples. None of these 5 regions was detected in multiple samples. Two regions and parts of two other regions were detected only in control samples.

Confirming memory retention QTLs by independent introgressions

Segmental introgression lines for each of the 5 potential memory retention QTLs were created and tested for effects on memory retention (Figure 2b). Offspring of females that were heterozygous for these regions were tested 72 hours after conditioning (Figure 3a - f). The locations of the regions on the linkage map of Desjardins *et al*. (2013) are given. Two out of the 5 regions were observed to result in a decreased 72 hour memory compared to control wasps, although this memory had not completely disappeared as is the case in *N. giraulti*. These two regions are located on chromosome 1 (location: 45.3 - 60.6 cM) ($F_{1,43}$ = 15.22, P < 0.001, QTL: n = 20 PIs, control: n = 25

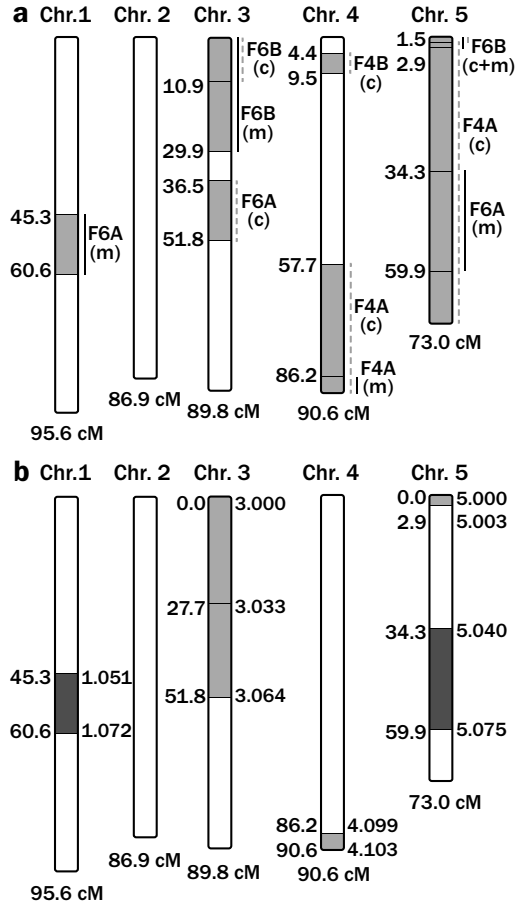

Figure 2: Potential memory retention QTLs. (a) Multiple heterozygous regions were detected by genotyping microarray in the four pairs of samples (F4A, F4B, F6A, F6B). The grey regions show the location on each of the 5 chromosomes of *Nasonia*, which is also given in cM on the left. The lines on the right of each region show the size of the introgressed region in each of the samples (m = decreased long-lasting memory, black lines; c = control, dashed grey lines). (b) A total of 6 new introgression lines were generated to test the effects of individual introgressed regions on memory retention. The grey regions indicate the location of each of these regions on the chromosomes of *Nasonia*, the location is given in cM on the left of each region and the marker cluster on the linkage map of Desjardins *et al*. (2013) is given on the right. The two dark grey regions were found to have an effect on memory retention, whereas the light grey regions did not.

PIs) (Figure 3a) and chromosome 5 (location: 34.4 - 59.9 cM) ($F_{1,25}$ = 10.73, *P* = 0.003, QTL: n = 11 PIs, control: n = 16 PIs) (Figure 3f). No decrease in memory retention was observed for the two tested regions on chromosome 3 (location: 0.0 - 27.7 cM: $F_{1,28}$ = 0.02, *P* = 0.895, QTL: n = 13 PIs, control: n = 17 PIs; location: 27.7 - 51.8 cM: $F_{1,32}$ = 0.65, *P* = 0.426, QTL: n = 15 PIs, control: n = 19 PIs) (Figure 3b and 3c), the region on chromosome 4 (location: 86.2 - 90.6 cM) ($F_{1,31}$ = 0.08, *P* = 0.786, QTL: n = 18 PIs, control: n = 15 PIs) (Figure 3d), and the second tested region on chromosome 5 (location: 0.0 – 2.9 cM) ($F_{1,26}$ = 0.06, *P* = 0.810, QTL: n = 14 PIs, control: n = 14 PIs) (Figure 3e).

Additional confirmation experiments, in which isogenic offspring of males with or without the introgressed region were tested at 24 and 72 hours after conditioning, were carried out for the region on chromosome 1 (location: 45.3 - 60.6 cM) and a smaller subset of this region (location: 54.8 - 60.6 cM), resulting from a recombination event within the introgressed region (Figure 3g), and for the region on chromosome 5 (location: 34.4 - 59.9 cM) (Figure 3h). For the introgressed region on chromosome 1, there was no effect on 24 hour memory retention ($F_{2,46}$ = 1.32, *P* = 0.277, 45.3 - 60.6 cM: n = 11 PIs, 54.8 - 60.6 cM: n = 15 PIs, control: n = 23 PIs), but there was an effect on 72 hour memory retention ($F_{2,49}$ = 15.68, *P* < 0.001, 45.3 - 60.6 cM: n = 11 PIs, 54.8 - 60.6 cM: n = 17 PIs, control: n = 24 PIs). Both the larger introgressed region and the smaller subregion resulted in lower PIs compared to the control, although this decrease was only significant for the smaller subregion, possibly due to the smaller sample size that was available for the larger region (Tukey-HSD; control vs. 45.3 - 60.6 cM: P = 0.103; control vs. 54.8 - 60.6 cM: P < 0.001). The introgressed region on chromosome 5 also does not affect 24 hour memory retention ($F_{1,51}$ = 3.31, *P* = 0.075, 34.4 - 59.9 cM: n = 29 PIs, control: n = 24 PIs), but only 72 hour memory retention ($F_{1,51}$ = 16.69, *P* < 0.001, 34.4 - 59.9 cM: n = 29 PIs, control: n = 24 PIs).

A homozygous introgression line, named "SIL_LTM1A_gV", was created for the region of interest on chromosome 1 (location: 54.8 - 60.6 cM; 913 kb according to *N. vitripennis* genome assembly v1.0). The size of the introgressed region in SIL_LTM1A_gV was characterized (Figure 4a). The region results from one recombination event at ~54.8 cM and a second recombination event between 71.5 and 83.2 cM. The entire introgressed region is located on Scaffold 1 and has a size between 2059 – 4409 kb. Memory retention of SIL_LTM1a_gV (SIL) was compared to memory retention of *N. vitripennis* and *N. giraulti* (Figure 4b). The three genotypes were tested 24 hours after conditioning to observe effects on short-lasting memory retention. At this time the three genotypes differed in memory retention ($F_{2,27}$ = 6.89, *P* = 0.004, n = 10 PIs for each genotype). The PIs of SIL_LTM1a_gV were similar to *N. vitripennis*, but higher than *N. giraulti* (Tukey-HSD: NV vs. SIL = 0.993; NG vs SIL = 0.008). The genotypes were, furthermore, tested after both 72 and 120 hours after conditioning to observe effects on long-lasting memory retention. At both time points the three genotypes

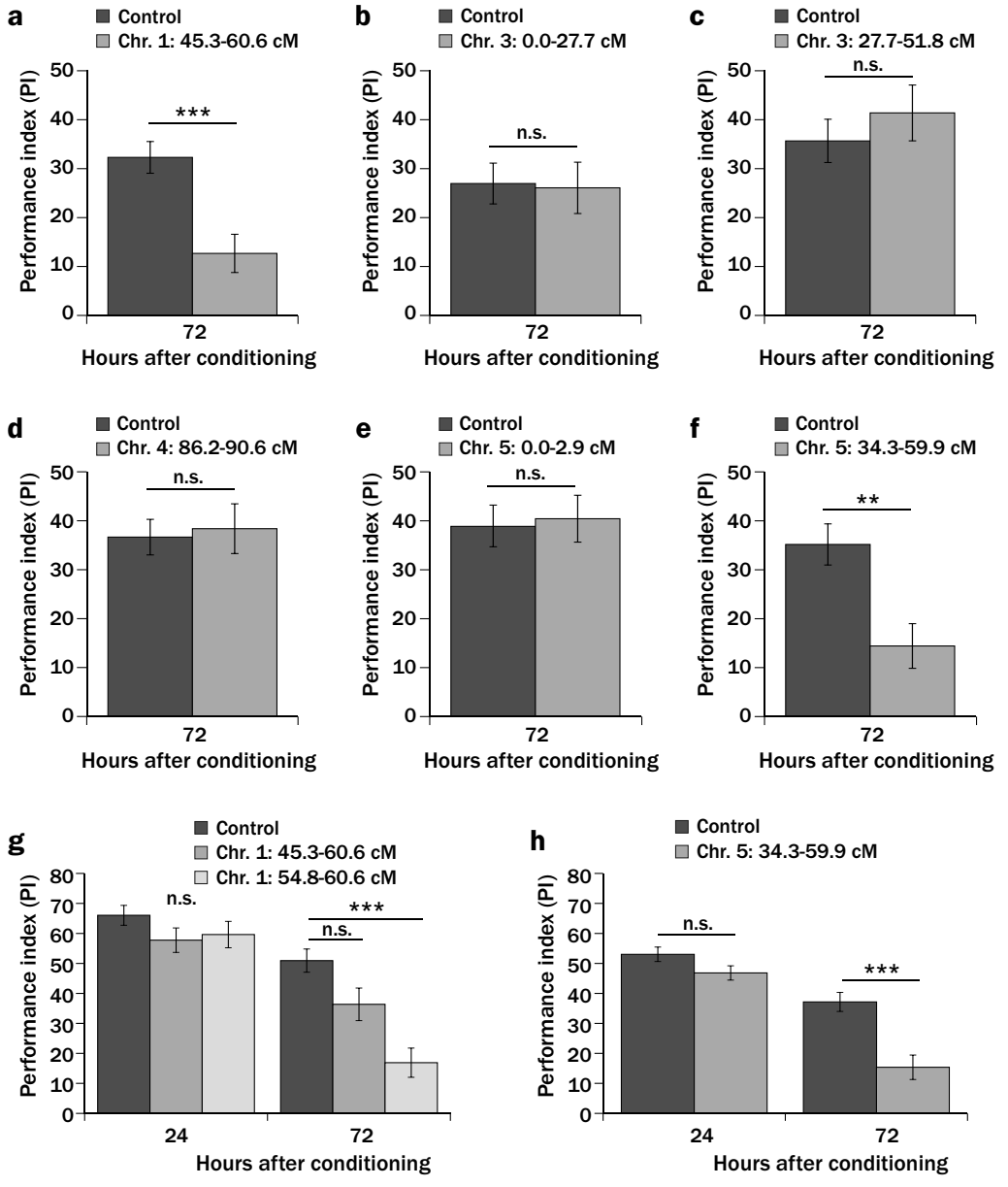

Figure 3: Confirming memory retention QTLs. A total of 6 regions were introgressed and tested for effects on 72 hour memory. (a) The region on chromosome 1 was observed to affect memory. (b and c) Two regions on chromosome 3, (d) a region on chromosome 4 and (e) a small region on chromosome 5 did not appear to affect memory. (f) A second, larger region on chromosome 5 did affect memory. Additional experiments on the regions of interest on (g) chromosome 1 and (h) chromosome 5 confirmed that there was an effect on long-lasting memory (tested 72 hours after conditioning), but not on short-lasting memory (tested 24 hours after conditioning). Level of significance: *** $P < 0.001$, ** $< P < 0.01$, * $0.01 < P < 0.05$, n.s. = not significant.

differed in PI (72 hours: $F_{2,27}$ = 13.34, P < 0.001, n = 10 PIs for each genotype; 120 hours: $F_{2,27}$ = 12.24, P < 0.001, n = 10 PIs for each genotype). At 72 hours, memory retention of SIL_LTM1a_gV has significantly decreased compared to *N. vitripennis*, and is no longer significantly higher than *N. giraulti* (Tukey-HSD: NV vs. SIL = 0.006; NG vs. SIL = 0.220). There is, however, still significant memory retention in SIL_LTM1a_gV (t_9 = 2.79, P = 0.021). After 120 hours, the PI of SIL_LTM1a_gV is no longer significantly different from 0 (t_9 = 1.42, P = 0.189) and not significantly different from *N. giraulti*, whereas *N. vitripennis* still has memory retention (Tukey-HSD: NV vs. SIL = 0.002; NG vs. SIL = 0.628). These results confirm earlier results on the effects of the introgressed region in SIL_LTM1a_gV on long-lasting memory retention (Figures 2a and 2g). The region does not affect 24 hour memory retention, but it does affect memory retention measured after 72 and 120 hours after conditioning. The memory trace appears to be completely lost in SIL_LTM1a_gV when measured after 120 hours.

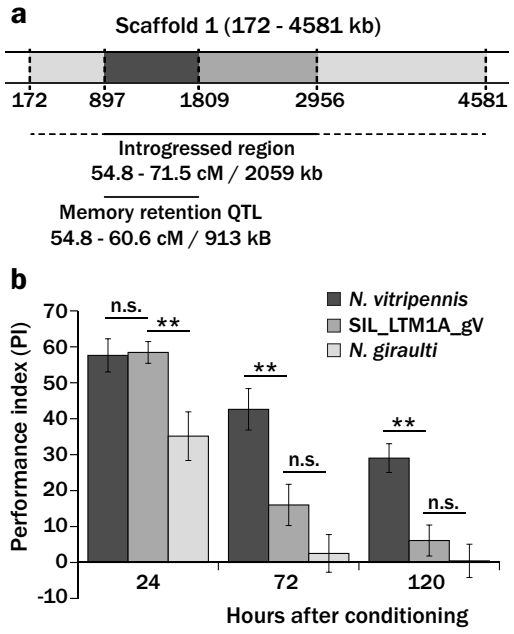

Figure 4: Memory retention of the homozygous introgression strain 'SIL_LTM1A_gV'. A homozygous segmental introgression line was generated (a) which includes the memory retention QTL on chromosome 1, scaffold 1. SIL_LTM1A_gV has an introgressed region of at least 2059 kb (shown in dark grey), between ~ 54.8 – 71.5 cM, which was confirmed by genotyping with PCR. Recombination had occurred between 172 – 897 kb and 2,956 – 4,581 kb (within the light grey areas), which implicates that the introgressed region can have a size of up to 4,409 kb. (b) SIL_LTM1A_gV was observed to have a decreased memory compared to *N. vitripennis* when tested after 72 and 120 hours after conditioning, but not when tested after 24 hours. Level of significance: *** P < 0.001, ** < P < 0.01, * 0.01 < P < 0.05, n.s. = not significant.

DISCUSSION

Introgression of memory retention

The memory retention phenotype of *N. giraulti* was introgressed into the background of *N. vitripennis*. This procedure was chosen, because the phenotype of *N. giraulti* was dominant in F1 hybrids and could be tracked in individuals heterozygous for memory retention QTLs during introgression. Selection for decreased memory retention may, however, raise

concerns, because mechanisms that result in a loss of a trait can potentially be numerous and reduced memory retention is not necessarily related to the process of memory formation. Negative epistatic interactions between nuclear genes of the two species occur in hybrids of *N. vitripennis* and *N. giraulti*, which can cause reduced viability (Breeuwer & Werren, 1995; Gadau *et al*., 1999). Behavioural alterations called 'behaviour sterility' occur in some genotypes of hybrid males (Clark *et al*., 2010). Consequently, hybrid breakdown in learning behaviour may occur in hybrids containing introgressed regions between the species, for example as a result of decreased perception or ability to discriminate odors, disturbed perception or behaviour towards other wasps or hosts, or general defects of cognitive skills. These factors result in a decreased performance during memory retention tests, which can incorrectly be interpreted as decreased memory retention (Tully *et al*., 1994; Mery & Kawecki, 2002). However, our tested memory retention QTLs did not affect memory retention when tested at 24 hours (Figures 3g, 3h and 4b). Also, no reduced response levels were observed. Therefore, we can conclude that these QTLs are not causing general disruption of learning ability, but specifically altered long-lasting memory retention after olfactory conditioning. Throughout most of this study, the introgressed regions were maintained heterozygously in females, thereby further reducing expression of potential hybrid incompatibilities, which have been observed to be mostly recessive (Breeuwer & Werren, 1995). Hybrid breakdown as a result of interspecific introgression appears not to affect the ability to learn and form memories, at least for the regions under study. The initial introgression experiment specifically targeted a decreased long-lasting memory, which allowed further studies on the genetic basis of this trait.

Memory retention QTLs

The genotyping analysis revealed multiple genomic regions that had been retained. A QTL on chromosome 1 (~5.8 cM in size), and a QTL on chromosome 5 (~25,5 cM in size) both reduced memory retention after 72 hours. Long-lasting memory was, however, not completely lost, as is the case for *N. giraulti*. This phenotype was observed both when the QTLs were maintained heterozygously (both QTLs) and homozygously (only tested for the QTL on chromosome 1). This indicates that the effect of both QTLs on memory retention is dominant, but that neither region alone results in the complete *N. giraulti* memory retention phenotype. Considering the complexity of this behavioural phenotype, it can be expected that multiple loci with potential epistatic interactions among them control memory retention. Long-lasting memory (> 48 h) of *N. vitripennis* is known to consist of multiple memory types that may occur in parallel. These include a form of ARM, which is expressed between approximately 72 hours up to 96 hours after a single conditioning trial (Schurmann *et al*., 2009), and LTM, which is observed at 96 hours (Schurmann *et al*., 2012;

Hoedjes & Smid, 2014).

The two QTLs identified in this study may affect a single memory type, which could explain a reduction, but not a complete loss of 72 hour memory. A complete loss of memory after 120 hours was observed for the QTL on chromosome 1, which suggests that this QTL affects LTM formation. The two QTLs may have an additive effect, especially if they affect different memory types: combining both QTLs into a single introgression line may result in *N. giraulti* phenotype. Additionally, the QTLs may interact with the other observed introgressed regions, which did not affect memory retention on their own when tested after 72 hours (Carlborg & Haley, 2004). Further research is required to elucidate epistatic interactions.

Genetic factors that regulate memory retention

The goal of this study was to determine genetic factors that underlie natural variation in (long-lasting) memory retention in the genus *Nasonia*. The observed QTLs provide a basis for further research to determine the exact mechanisms involved. Several genes with a known function in the memory formation process are located on the genomic regions, which contain the two observed QTLs. These include the transcription factor CCAAT/enhancer-binding protein (C/EBP) and a dopamine receptor (El-Ghundi *et al*., 2007; Alberini, 2009). It is, however, preliminary to select such potential candidates for further research, as the introgressed regions with memory retention QTLs contain hundreds of genes. Further partitioning of the genomic regions by recombination, combined with the excellent mapping and molecular tools available in *Nasonia*, will allow fine-scaling of memory retention loci (Werren *et al*., 2010; Desjardins *et al*., 2013).

An alternative approach could be to compare differential gene expression of selected introgression lines with both parental species. A study on *Drosophila melanogaster* compared gene expression between two selected lines differing in geotaxis behaviour, and this was shown to be a successful approach to identify genes involved in this behavioural trait (Toma *et al*., 2002). In addition, RNA interference can effectively be applied in *Nasonia* (Lynch & Desplan, 2006) and so can be implemented to address the effects of specific genes. Future studies can also address epigenetic factors, which have been shown to have a role in memory formation (Barrett & Wood, 2008; Lockett *et al*., 2010).

Correlated memory traits

Another aspect to be tested is if decreased memory retention that is observed in our assay, in which an odor (CS) is paired with a host (US), is also observed when other conditioned and unconditioned stimuli are used, e.g. visual cues (CS), a sucrose reward (US) or a female to mate with (US) in case of conditioning of males. Such correlations were observed in

selection experiments in other insects (Chandra *et al*., 2001; Mery *et al*., 2007b). Correlation experiments can demonstrate if the observed QTLs affect the memory formation process in general or if they are rather involved in the perception and/or processing of a specific cue (e.g. either the odor or the host reward). The availability of alternative conditioning procedures provides opportunities to study correlated memory traits in *Nasonia*, for example male learning in mate finding and color learning (Oliai & King, 2000; Baeder & King, 2004).

Conclusion

Learning and memory formation are universal animal traits, but there is variation in memory retention. We have introgressed the short memory retention of *N. giraulti* into the genetic background of *N. vitripennis* and have identified two QTLs which result in decreased long-lasting memory. Species-specific variation in learning and memory performance may have large implications for host finding behaviour in parasitic wasps, and likely represents an important evolutionary adaptation to changing environmental conditions (Hoedjes *et al*., 2011). Our study with *Nasonia* is the first to provide insight in the genetic mechanisms that regulate natural variation in memory retention. Further studies are required to fine-scale the identified QTLs and to investigate epistatic interaction among QTLs, in order to identify genetic factors that regulate memory retention. The generated introgession lines can be used to study correlations between a decreased olfactory-appetitive memory and other types of memory. The *Nasonia* model system provides excellent possibilities to pursue these experiments. Knowledge of the genetic basis of natural variation in memory retention is important for our understanding of the evolution of this variation, not only in *Nasonia*, but also in other animal species.

ACKNOWLEDGEMENTS

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SUPPLEMENTARY INFORMATION

This supplementary information file contains additional information on (1) the conditioning procedure and memory retention test used in this study, (2) information on the methods used and results from the back-up lines that were generated during the initial introgression experiment, and (3) additional information on the genotyping methodology.

Adaptations to the conditioning and memory retention testing procedures that have been described in Hoedjes et al. (2012)

The conditioning method as described by Hoedjes *et al*. (2012) was adapted for this study to facilitate conditioning of groups of wasps instead of individual wasps. A group of up to 30-40 wasps was conditioned in a Petri dish (diameter: 90 mm; Greiner Bio-One, Alphen aan de Rijn, The Netherlands) in which 30-40 host pupae were present. Immediately before conditioning, 5 µl of vanilla or chocolate extract (Nielsen-Massey Vanillas Intl., Leeuwarden, The Netherlands) was applied to a piece of filter paper (± 2 cm^2) and placed in the Petri dish. Then the female wasps were released inside the Petri dish and were allowed contact with the hosts for 1 hour. Wasps typically initiate drilling into the host pupae during this period; wasps that did not exhibit this behaviour were carefully removed from the experiment after 30 minutes. After an hour, the wasps were gently taken from the hosts and placed in a rearing vial for 15 minutes, next they were exposed to a second odor, respectively 5 µl chocolate or vanilla extract (CS-), applied to a piece of filter paper which is immediately thereafter inserted in the rearing vial with the wasps, without a reward present for another 15 minutes. When conditioning was finished, wasps were transferred to rearing vials with access to honey and water and kept in a climate cabinet (25°C, 16L:8D photoperiod) until testing. Reciprocal groups of wasps (with either vanilla or chocolate as CS+) were conditioned simultaneously.

Control experiments compared memory retention between individually and group conditioned wasps. Research on *Drosophila melanogaster* has shown that the social environment during conditioning and/or testing can affect the memory scores that are observed (Chabaud *et al*., 2009; Foucaud *et al*., 2013). Memory retention of both *N. vitripennis* and *N. giraulti*, which was measured 24 and 48 hours after group conditioning, was comparable to earlier results by Hoedjes *et al*. (2012). In this supplemental experiment we have compared memory retention after group conditioning vs. individual conditioning of *N. vitripennis* 120 (±1) hours after conditioning (Figure S1a). No effect of conditioning procedure ($F_{1,18}$ = 0.97, P = 0.337, n = 10 PIs for both procedures) could be detected. We, therefore, conclude that group conditioning is suitable for this study.

An adaptation was made to the memory retention test during the initial introgression experiment. Memory retention is typically only tested once in each individual, because

memory recall (without a reward present) can affect memory dynamics. Exposing animals to the learned cue without a conditioned stimulus, so-called extinction tests, can result in a decay of memory as was shown in the parasitic wasp *Leptopilina boulardi* and *Drosophila melanogaster* (Kaiser *et al*., 2003; Lagasse *et al*., 2009). Alternatively, memory can be reconsolidated, depending on the number of extinction tests (Lagasse *et al*., 2009). The effects of multiple tests on memory are species-specific. During the initial introgression experiment, *Nasonia* wasps were tested three times in total: once after 24 hours and two times after 72 hours. The aim of this supplementary experiment was to assess if there were any effect of multiple tests on memory retention. Groups of *N. vitripennis* and *N. girault*i were conditioned and tested as described in the Materials and Methods section. One group of wasps was tested after 24 (±1) hours and once again after 72 (±2) hours (2x tested); a second group was tested once after 72 (±2) hours (1x tested). Both groups were then tested a second time after 72 (±2) hours and compared to a third group of wasps that had not been tested before (control). No effects of multiple tests on memory retention were observed for either species (*N. vitripennis*: $F_{2,27}$ = 1.37, P = 0.280, n = 10 PIs for all test procedures; N. girault*i*: $F_{2,27}$ = 0.26, P = 0.775, n = 10 PIs for all test procedures) (Figure S1b). This indicates that testing the wasps 3 times during the initial introgression experiment did not have effects on the expected memory retention and this procedure was suitable for selection on memory retention.

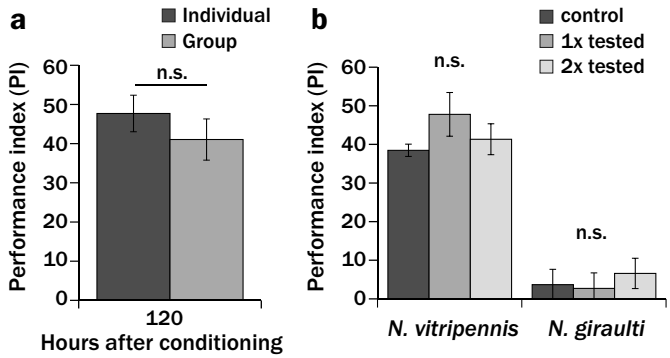

Figure S1: Adaptations to the conditioning procedure and memory retention test. (a) Group conditioning was used in this study instead of individual conditioning. This adaptation did not affect memory retention of *N. vitripennis* when tested 120 hours after conditioning. (b) During the initial introgression experiments, wasps were tested multiple times in order to select for learning rate. Testing for memory retention multiple times was not found to have an effect on memory of *N. vitripennis* and *N. girault*i.

Initial introgression experiment (Backup lines F5-F7)

In addition to the initial introgression experiment as described in this paper, a back-up was created during the 4th generation of introgression in order to ensure continuation of the project during transition from the laboratory in the USA to the Netherlands. Individuals

that had descended from similar females in the F2 were kept together, resulting in 4 lines selected for a low learning rate and three control lines. Sibmating was allowed in these lines in order to maintain genomic regions involved in regulation of memory retention. Selection for short memory retention was continued, but memory retention was only tested twice 60-72 hours after conditioning. Selection continued up to the 7th generation as described earlier, after which diapause was induced. In addition to the control lines that were established in the F2 generation, as described in the materials and methods section, new control lines were established in the 5th and 6th generation by selecting females that chose the learned odour twice from the low learning rate lines (indicated as long memory retention lines). This was done to confirm that the memory retention phenotype of *N. vitripennis* could still be selected for. Univariate ANOVA was used to test for variation in memory retention between control lines and short memory retention lines and a Tukey-HSD post-hoc test was used when appropriate (SPSS version 19; IBM, Armonk, NY, USA).

Memory was decreased when measured after 72 hours in the short memory retention introgression lines compared to the control lines in the 5th generation of introgression ($F_{1,26}$ = 8.14, P = 0.008, short memory: n = 16 PIs, control: n = 12 PIs) (Figure S2a). In the 6th generation short memory retention introgression line was compared to the control (F2) and the newly created long memory retention lines (F5) (Figure S2b). There was significant variation in memory measured after 72 hours among these lines ($F_{2,89}$ = 3.41, P = 0.038, short memory: n = 42 PIs, long memory (F5): n = 32 PIs, control (F2): n = 18 PIs). The short memory retention introgression lines had decreased memory retention compared to the two other lines, although not significantly (Tukey-HSD: short memory vs. control (F2) = 0.067, short memory vs. long memory (F5) = 0.111, control (F2) vs. long memory (F5) = 0.848). In the 7th and final generation of selection, 72-hour-memory also differed among lines ($F_{3,119}$ = 4.17, P = 0.008, short memory: n = 38 PIs, long memory (F6): n = 34 PIs, long

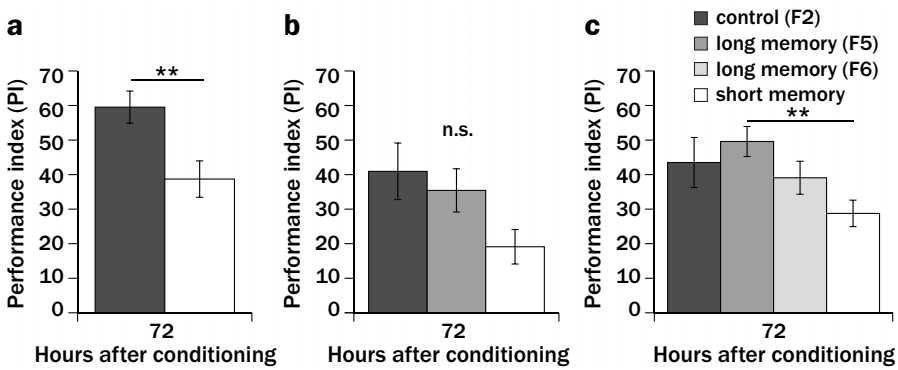

Figure S2: Initial introgression of memory retention: back-up lines. Introgression of memory retention was successfully continued during the (a) 5th, (b) 6th, and (c) 7th generation.

memory (F5): n = 39 PIs, control (F2): n = 12 PIs) (Figure S2c). Pairwise comparisons revealed a significant difference in memory retention between the short memory lines and the long memory lines (F5) (Tukey-HSD: short memory vs. long memory (F5) = 0.004). These results demonstrate that selection for decreased memory retention was successful up to at least 7 generations. Selection for long memory retention, the phenotype of *N. vitripennis*, is still possible from the introgression lines which were selected for decreased memory retention for 4 to 6 generations, suggesting that this phenotype is likely controlled by genetic factors and not only epigenetic factors.

Genotyping using a genotyping microarray and primers surrounding an indel-marker in a PCR

DNA was extracted individually from all wasps using the Gentra Puregene Cell kit (Qiagen, Antwerp, Belgium) following the protocol for a single *Drosophila* fly. When preparing samples for analysis by genotyping microarray, 2 µl of the DNA from each wasp of a sample was mixed and amplified using the GenomiPhi DNA amplification kit (Sigma-Aldrich, Zwijndrecht, The Netherlands) according to instructions of the manufacturer. DNA was then labelled and hybridized according to Roche NimbleGen's User's Guide and a bulk segregant analysis was performed as described by Desjardins *et al*. (2013) in order to determine which genomic regions were heterozygous for *N. vitripennis* and *N. giraulti*. This analysis determines the proportion of *N. vitripennis* DNA in a sample for each marker (a score of 1 represents 100% *N. vitripennis*, a score of 0 represents 100% *N. giraulti*). Two samples of F1 hybrids were analysed to determine the suitability of each marker in this analysis. Markers that scored >0.9 or <0.1 were considered unsuitable for bulk analysis and were therefore removed; 14949 of 15546 markers were considered suitable. The average proportion of *N. vitripennis* DNA was determined per 50 subsequent markers. When this average was lower than 0.8, an introgressed genomic region of *N. giraulti* was considered to be present. The scores of individual markers were then inspected manually to determine the boundaries of the introgressed genomic region. The genotype of individual wasps was confirmed using indel-markers within observed introgressed regions in a polymerase chain reaction (PCR) using GoTaq Flexi polymerase (Promega, Leiden, The Netherlands) and primers that surround an insertion-deletion polymorphism between *N. vitripennis* and *N. giraulti* (Table S1). These primers allow distinguishing between *N. vitripennis* and *N. giraulti* based on the size of the amplicon. This method and these primers were also used to genotype individual wasps during the experiment 'Confirmation of memory retention QTLs by independent introgressions'. Table S1 provides details on the sets of primers that were used. The location of each primer set is given as marker cluster (based on the genetic map by Desjardins *et al*. (2013), the location on the chromosome in centimorgan (cM), and

the scaffold and base pair position of the 5' base of each forward primer in *N. vitripennis* genome assembly v1.0. All primer sets are suitable when using touch-down PCR conditions: 94°C for 3 min., 9 touch-down cycles in which the annealing temperature drops 1°C per cycle (94°C for 15 sec., 63 - 55°C for 30 sec., 68°C for 1 min.), 28 cycles of (94°C for 15 sec., 55°C for 30 sec., 68°C for 1 min)., 68°C for 6 min. Primers set that have an asterisk following their name also work well using a regular PCR protocol: 94°C for 3 min., 35 cycles of (94°C for 15 sec., 60°C for 30 sec., 68°C for 1 min)., 68°C for 6 min. Genotype was determined based on size differences between amplicons as visualized on a 1.5% agarose gel.

Table S1: Genotyping primers.

Name	Forward primer	Reverse primer	Marker cluster	Location (cM)	Location (Scaffold)
P1.3*	CGAAATGAGGCTCTACTCGCGCG	GAGAGTATTTATGCACTCGCGCGTG	1.051	Chr1: 45.3	S12-2216571
P1.1*	CGCTTCTACGAACGCGCGGCT	GTGCTCGGCGCATGCAAAACTCG	1.054	Chr1: 47.5	S36-405485
P1.2*	CTGATGCTCCGCGAGGAAAAATCCG	AGAGCGGCAACAGGTGGCGAC	1.054	Chr1: 47.5	S39-358302
P2.9	TGATGCTTTCGACAACATTTCCCCTATCTG	AGCGCACACGATCGCCCTCG	1.057	Chr1: 49.6	S25-258752
P2.5	GCTTCGCTGGCCCGCTATCAG	GCGCCACCGAAAGCCCTCAAC	1.059	Chr1: 51.5	S58-528284
P2.7	CAGCGTCCTGCTCATGTAGCAGC	GCAGGTGAGGTAATTCGCTTGACCG	1.061	Chr1: 52.6	S33-562435
P2.6	GCTGCGCCGTGTCCTCTGTTG	TCGAACTGTATCACACCGCGCACG	1.064	Chr1: 54.8	S1-172028
P2.10	ATATATAATCGGAATGGTCGGACGAGTCG	GATGTTCTCCGCGGACACGCTG	1.064	Chr1: 54.8	S1-896764
P2.8	CGGCAATCACTCGCGAATTTTCGTCC	TGCCACCAGGTTGCAGCCTCAC	1.068	Chr1: 57.7	S1-1267660
P2.4	CAGTGCCGGGAGCAATAACGCG	TGGCAATGGCACGAGGGACTAACAAG	1.071	Chr1: 59.9	S1-1646291
P2.3*	CGCAAAATATAAGACGGATCGGAAGCTCG	CCGACTTAATTGCTGAGATATAATGCGCGC	1.072	Chr1: 60.6	S1-1809330
P2.11	CCGTTTCTCTTAGGCGCGGTATCG	GGAACTCGCTCTCGAGGACGAAC	1.077	Chr1: 66.4	S1-2498836
P2.12	CGGGAAAATTCGCGCGAGAAACAGAC	GCGCTGAGTACACAGAGACGGC	1.084	Chr1: 71.5	S1-2955842
P2.13	GATGGAGTGGCTCTCGGATGACG	CTTGCCTTTCATATTTCATTTCGGCGTATG	1.096	Chr1: 83.2	S1-4580769
P3.1*	CGAGAGACAAGATTTCACGAATACGCAC	ATCACACGCGTCCAATGCGGATGAC	3.000	Chr3: 0.0	S18-59272
P3.2*	ATAAGCGCGGCGACTCCTTCGC	AACGCGCGTACGCAGCCTCC	3.003	Chr3: 2.2	S18-1286317
P3.3	TCCACGACATCCGGCATCGGGATG	GGATTTCACGCTCCGCATTCCGTTG	3.010	Chr3: 8.0	S18-2476428
P4.4	TGCACCCACCCCCACCAATGCTG	CACGTCCGCCCCACTCCACTTG	3.015	Chr3: 11.7	S42-296160
P4.5*	ACTACTGGCTCGCGCGCATTATATAACG	CGACGGGATGGAAAAAAGGGAAATTCAGC	3.017	Chr3: 13.9	S42-504548
P4.6*	CGAACAACATCGCACGCAGCGGAG	GTATTTCCCCGTCGTCGTCGTCG	3.026	Chr3: 22.6	S6-4050087
P4.3	GTCGGCGCGTTAGTGGCGTC	GTAAACCCAATATCGCGTGCAGCG	3.030	Chr3: 25.6	S6-3238822
P4.2*	AGCTTTTGTGCGACGCTTCCGGG	CACGAGCAAACAGGACGCGAGATC	3.033	Chr3: 27.7	S6-2542054
P4.1*	CGGCCCCGACTTTCACCGGC	TTCGAAAAACCAGCCGCAACAGTCG	3.035	Chr3: 29.2	S6-2203321

P9.2*	GCCTCGCAGCGCATAATTTGCCG	CGACGCTCAAGGCCCAAGGC	3.044	Chr3: 37.2	S44-493547
P9.1*	GTCACGAGCAGTGGGTCCGC	CGTGAGCGCGGAGGAAGATCG	3.045	Chr3: 38.0	S111-184751
P9.4*	GCGCGGGGCACTACGCTTTAGG	TATCGCCGAAATAAGGCCAGGCTGAC	3.056	Chr3: 46.0	S22-1568936
P9.3*	ACGGTATCGGATCTCCGGGCTAG	AACGAGGCTGTTTTGACAAGTGTACGCG	3.064	Chr3: 51.8	S22-2444096
P5.1*	GCGGGCTTCGAGTTCAGCGC	CACGCGCGTCTTTGATCTTCCGC	4.005	Chr4: 4.4	S4-4054019
P6.3*	CTTGCCGGCTCATCCGTCCC	CCGCGGGCAGCTGTGTGGTA	4.101	Chr4: 87.6	S9-630535
P6.1*	GTGCGCGACGACGCTCGCCT	TCTTCCCGGGCAACACCCCAC	4.102	Chr4: 89.1	S9-533095
P6.2*	TAATAATCGCGCACTGTCTTCGCCG	GAGTTTCGGTCCGACGCGCC	4.103	Chr4: 90.6	S9-214756
P7.1*	GGCGAGTCGAGAACGGCGCG	GCGAGGAGCAAAGGGTTACATTAGG	5.001	Chr5: 1.5	S14-646706
P7.4	GTCTCTGCATTTAAATCGCCCATCGAGC	GGCCGACTACGCCAGCGATATAC	5.003	Chr5: 2.9	S14-1001415
P7.3	TCATTGCTAACTTTCTAAAGCCGCGTAAGC	GCGAATTGTTTTGACTCGCGGGATTACG	5.003	Chr5: 2.9	S14-1177137
P8.5*	GTGAACGGATAACATTGATCGCAGCCG	AGATGCGATCGCACACGCGCTG	5.017	Chr5: 16.1	S7-2590432
P8.1*	ATGCTGCTGCTGTCCGTGGTGC	CCTGACTCAGTCGGCGTGCG	5.044	Chr5: 36.5	S27-21720
P8.3*	GACCTTCGCCGCAGCTATGTGC	GATACCGGCCACTTCTCCCCC	5.052	Chr5: 41.6	S10-2050116
P8.4	CCGCCCCGAGTTGCAGCACG	GCTTTTTCCTCAAAACTTCCCCGGC	5.075	Chr5: 59.9	S2-2343256

Chapter 7

Learning-induced gene expression in the brains of two *Nasonia* species that differ in long-term memory formation

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Under review

ABSTRACT

Natural variation in memory between animal species or populations is widely observed, but its genetic basis is poorly understood. Closely related species of *Nasonia* parasitic wasps differ in long-term memory (LTM) formation: *N. vitripennis* will form transcription-dependent LTM after a single conditioning trial, which lasts more than 6 days, whereas *N. giraulti* will form ASM and ARM and has lost its memory within 2 days. RNA was collected from heads of both species before and after conditioning of the wasps. It was sequenced strand-specifically, which allows distinguishing sense and antisense transcripts, on the HiSeq Illumina platform and assembled *de novo*. Substantial differences in differential gene expression were observed between the two species when compared immediately after conditioning and, to a lesser extent, also at 4 and 24 hours after conditioning. Differential expression of various genes with a known role in LTM formation was exclusively observed in *N. vitripennis*. Some LTM genes, including genes involved in dopamine synthesis and in the Ras-MAPK and PI3K signalling pathways, were exclusively differentially expressed in *N. giraulti*, which may indicate an LTM inhibitory mechanism in this species. Antisense transcripts of a number of known memory genes were detected and may have a role in regulation of transcription, alternative splicing, or translation. This study is the first to compare expression patterns, of both coding and non-coding (antisense) transcripts, at different time points after conditioning between two closely related animal species that differ in LTM. Opportunities for further in-depth studies on the regulation of LTM formation are discussed.

INTRODUCTION

The ability to learn and form memory has been demonstrated in most animal species, and there is substantial natural variation in memory formation between species (Brenowitz & Beecher, 2005; Hoedjes *et al*., 2011). The opportunity to acquire new skills or adapt behaviour through learning is an important benefit and can increase animal fitness (Papaj & Vet, 1990; Raine & Chittka, 2008). Memory formation can, however, be maladaptive when unreliable associations are formed (Menzel, 1999). In addition, the process of memory formation is energetically costly, depending also on the type of memory that is formed (Laughlin, 2001; Mery & Kawecki, 2005). Therefore, variation in memory formation is considered to be a species-specific adaptation to the ecology of an animal and depends on the costs and benefits of memory formation for this animal (Menzel, 1999).

Three main types of memory can be distinguished based on temporal expression and cellular pathways involved. Anaesthesia-sensitive memory (ASM) (also known as short-term memory (STM)) typically lasts from minutes up to an hour and is sensitive to disruptive treatments (Xia *et al*., 1999; Margulies *et al*., 2005; Müller, 2012). During the ASM phase the formation of more stable and durable types of memory starts, a process called memory consolidation, and this process can take hours to days to complete (Margulies *et al*., 2005; Smid *et al*., 2007). Two main forms of consolidated memory are distinguished. Anaesthesia-resistant memory (ARM) typically lasts from hours to days and formation of this type of memory is thought to depend on changes in existing proteins (Tully *et al*., 1994). Long-term memory (LTM) can last from days up to the entire lifetime of an animal. LTM formation is dependent on both transcription and translation and it is, therefore, considered the most costly type of memory (Margulies *et al*., 2005; Mery & Kawecki, 2005; Müller, 2012). As a result, many animal species require multiple conditioning trials, which are spaced in time, to induce LTM consolidation. Such repeated learning experiences allow animals to evaluate the information before investing in costly LTM (Menzel, 1999). A single conditioning trial or massed conditioning trials, i.e. multiple trials without or with a very short intertrial interval, typically do not induce LTM formation, but result in the formation of ASM and ARM.

There is variation in the number of trials required to form LTM between species (Hoedjes *et al*., 2011). A number of insect species are known to consolidate LTM after a single conditioning trial (Smid *et al*., 2007; Krashes & Waddell, 2008). Ecological factors concerning the value of the rewarding or punishing stimulus and the reliability of the learned association are considered to determine the number of trials required to form LTM (Kruidhof *et al*., 2012). These factors can vary between species, but also between different stimuli. Very little is currently known about genetic and neural factors that are involved in natural variation in LTM formation.

We have studied the genetic basis of variation in LTM formation in the parasitic wasp *Nasonia vitripennis* and its closely related species *N. giraulti*. *Nasonia vitripennis* will form LTM which lasts at least 6 days after a single conditioning trial in which an odour is associated with the reward of a host to parasitize (Hoedjes *et al*., 2012; Schurmann *et al*., 2012; Hoedjes & Smid, 2014). This type of memory becomes visible 4 days after conditioning, as demonstrated by inhibition through transcription- and translation-inhibitors (Hoedjes & Smid, 2014). *Nasonia giraulti*, on the other hand, forms only ASM and ARM after a single conditioning trial and this memory has disappeared within 2 days. Multiple spaced conditioning trials are required to induce long-lasting memory retention (Hoedjes & Smid, 2014). The genus *Nasonia* has emerged as a powerful model with unique opportunities for genetic studies on inter-species differences (Werren *et al*., 2010). In a recent study, the memory phenotype of *N. giraulti* was backcrossed into the genetic background of *N. vitripennis*, which revealed two quantitative trait loci (QTL) that control memory retention (Hoedjes *et al*., submitted). In the present study, we compare gene expression patterns in the brains of *N. vitripennis* and *N. giraulti* before and after conditioning, to provide additional information on the genetic regulation of LTM formation.

The gene expression profiles of both *N. vitripennis* and *N. giraulti* were analysed using Illumina HiSeq sequencing of RNA extracted from the heads of naïve and conditioned wasps. A strand-specific RNA-sequencing protocol was used to identify both sense transcripts and antisense transcripts, i.e. a class of long non-coding RNA, which align to sense transcripts in the reverse orientation. Sequencing RNA strand-specifically is important considering that genes on different strands can overlap (Vanhee-Brossollet & Vaquero, 1998; Katayama *et al*., 2005). This is, to our knowledge, the first study of insect brain transcriptomes that uses strand-specific HiSeq sequencing, in order to take antisense transcripts into account. Wasps were collected immediately, 4 hours or 24 hours after conditioning to observe temporal patterns in expression of genes that are involved in the early and intermediate phases of LTM formation. Differential gene expression related to oviposition behaviour will likely be observed in heads of both *Nasonia* species; however, transcripts related to LTM formation were expected only in *N. vitripennis* because this species forms transcription-dependent LTM after a single conditioning trial. An alternative hypothesis is that genes that are differentially expressed in *N. giraulti* but not in *N. vitripennis* have a role in inhibition of long-term memory formation We hypothesize that comparing differential gene expression patterns between both species will elucidate genetic pathways responsible for the difference in LTM formation between *N. vitripennis* and *N. giraulti*.

MATERIALS AND METHODS

Insects

Nasonia vitripennis (strain AsymCx) and *N. giraulti* (strain RV2x(U)) were used in the experiments. These strains are completely homozygous and have a sequenced genome (Werren *et al*., 2010). Wasps were reared on *Calliphora vomitoria* pupae as described by Hoedjes *et al*. (2012). Female wasps were collected on the day of emergence, were provided honey and water in a polystyrene rearing vial, and were kept in a climate cabinet at 25°C and a photoperiod of 16:8 (L/D).

Conditioning procedure

Female wasps were conditioned using a Pavlovian conditioning assay in which an odour (chocolate) is associated with the reward of a host (*C. vomitoria* pupa) as described by Hoedjes *et al*. (2012). Briefly, wasps were individually given two host pupae (the unconditioned stimulus, US) in the well of a 12 well-microtiter plate in the presence of chocolate odour (the conditioned stimulus, CS+). Wasps were allowed to drill into the pupae and perform host feeding for 1 hour. Oviposition does not take place during this period. Wasps that did not initiate drilling within 30 minutes (~5-10%) were removed from the experiment. After the 1 hour period, the wasps were gently removed from the hosts and transferred to a clean rearing vial. After a 15-minute resting period, wasps were exposed to vanilla odour (CS-) for another 15 minutes without a rewarding or punishing stimulus present. After this conditioning trial, the wasps were transferred to a rearing vial with access to honey and water, and were kept in a climate cabinet as described above. Both *N. vitripennis* and *N. giraulti* were conditioned using this protocol. Three groups of 30 wasps were (individually) conditioned for each species. This was repeated 5 times on different days.

Sample preparation and RNAseq

Groups of 30 wasps were collected for RNA isolation (1) immediately after conditioning, (2) 4 hours after conditioning or (3) 24 hours after conditioning and (4) (naïve wasps), i.e. wasps of the same age that had not been conditioned. Wasps were frozen in liquid nitrogen, heads were cut off with a scalpel and collected in a 1.5 ml microcentrifuge tube which was stored in liquid nitrogen. RNA was extracted from the heads using the RNeasy Micro Kit (Qiagen, Antwerp, Belgium) according to instructions of the manufacturer. A total of 3 biological replicates was collected for each of the three treatments and naïve controls, resulting in 12 samples per Nasonia species. RNA quantity and integrity was measured using a 2100 Bioanalyzer (Agilent Technologies, Amstelveen, The Netherlands). The RNA concentration ranged from 270 – 650 ng/µl and the RNA integrity number (RIN) was between 9.7 and 10 (Schroeder *et al*., 2006).

One microgram total RNA was used for mRNA isolation and subsequent RNAseq library preparation following TruSeq Stranded mRNA Sample Preparation Protocol (Illumina). In short, mRNA was isolated using oligo dT beads and chemically fragmented prior to first strand cDNA synthesis using random hexamer primers. Strand specificity was achieved by replacing dTTP with dUTP during Second Strand synthesis and the addition of transcription inhibitor Actinomycin D to the First Strand Master Mix. Obtained cDNA fragments were used for 3'adenylation and adapter ligation using 24 different barcoded adapters, one for each library. Adapter-ligated cDNA was amplified using 15 PCR cycles. Quality control of libraries was done using Agilent Bioanalyzer2100 DNA 1000 assays. Quantification was performed using Quant-iT PicoGreen dsDNA reagent (Molecular Probes, Invitrogen) and a fluorescence plate reader system (Tecan XFluor). Equimolar amounts of all 24 libraries were pooled together and were applied on two lanes together with Illumina V3 reagents. Paired-end 100 bp sequencing was performed on a HiSeq2000 instrument. De-multiplexing of obtained sequences was done using CASAVA 1.8.1. software.

Transcriptome assembly

All reads were quality filtered and adapter trimmed using Cutadapt (version 0.9.5), options: -O 10, -n 3, -q 10. Data were then filtered using fastq-mcf, options: -k 5, -q 20, -l 50.

The reads of all *N. vitripennis* samples were pooled to assemble the transcriptome *de novo* using Trinity (version r2013-02-15, option: --SS_lib_type RF) (Grabherr *et al*., 2011). The same was done for *N. giraulti*. The assembled transcripts have names that consist of three parts, for example comp100_c0_seq1, of which the first two parts define the "gene" name. All transcripts from one "gene" were considered to be alternative splice variants, for example comp100_c0_seq1 and comp100_c0_seq2. Transcripts smaller than 200 bp and those that had little read support were removed from the transcriptome. The latter was done by first mapping the unfiltered reads of each sample individually back to the transcriptome using bowtie (version 0.12.7, options: -n 2, -e 99999999, -l 25, -3 0, -a, -m 200, -I 1, -X 1000, --nofw). The mapped reads were quantified using eXpress (version 1.3.1). Using this program, the rounded effective read counts per transcript were extracted. These counts were analysed using R (version 2.15.2) and only transcripts with a read count per million (CPM) > 1 for at least 3 out of the 12 samples were kept.

Annotation

Transcripts were annotated by aligning them to the *N. vitripennis* proteome (Nvit 2.0) or NCBI RefSeq database (sept-01-2013) using blastx (options: -max_target_seqs 1, -word_size 11, e-value 10), which is integrated in the Blast facility of the Centre for BioSystems Genomics (CBSG) and Wageningen University (created by Applied Bioinformatics, Plant

Research International). Because the mRNA was sequenced strand-specifically, the sense or antisense orientation of the aligned transcripts could be deduced.

The transcripts were first aligned to the *N. vitripennis* proteome. Transcripts that scored less than 60% to the *N. vitripennis* proteome (Nvit2.0) were aligned to the NCBI RefSeq database. Protein coding transcripts were defined as sense transcripts if they had more than 60% protein alignment length to the *N. vitripennis* proteome or NCBI RefSeq protein database. The transcripts that did not align to a protein with more than 60% protein alignment length could be of different origin: (1) sense RNA encoding proteins not present in the published proteome databases, (2) sense RNA encoding proteins smaller than 60% of the complete protein, for example unknown small splice variants, (3) antisense RNA, (4) long non-coding RNA, or (5) misassembles. Point (2) was addressed by also defining all transcript variants of a protein-coding (sense) transcript as sense transcripts as well, even if they were smaller than 60% if the complete protein. We defined antisense transcripts (3) as transcripts with an antisense orientation to a protein with more than 50% protein alignment length, or with an antisense orientation to a sense transcript with more than 80% antisense transcript alignment length and 95% sequence identity. Antisense transcripts that do not align to a protein, but only to a sense transcript likely have a hit to an untranslated region of that gene. Transcripts that were not categorized as sense or antisense transcripts but aligned to the *N. vitripennis* genome or NCBI RefSeq nucleotide database with more than 80% alignment length and 95% sequence identity are suggested to be long non-coding RNA (4). Transcripts without sense, antisense or long non-coding label were defined as 'unknown' (5) and may include misassembles, but also (anti)sense transcripts or long non-coding RNA with insufficient alignment length or identity to known sequences. Putative open reading frames (ORFs) were determined for long non-coding and unknown transcripts using the script 'transcripts_to_best_scoring_ORFs.pl' from Trinity (options -m 30 -S). Putative ORFs were defined as an ORF with a 5'start and 3'end and minimally 30 amino acids.

Differential expression analysis

Differentially expressed (DE) transcripts in the *N. vitripennis* and *N. giraulti* transcriptomes compared to naïve expression levels were identified using EdgeR (version 3.0.8). The rounded effective read counts of each sample, extracted from eXpress (version 1.3.1), were analysed using a GLM trended dispersion with Pearson correlation with eight degrees of freedom (12 samples minus four sample types) and P=0.05, and also taking the replica effect into account.

Two complementary analyses were used to analyse the differential gene expression patterns of *N. vitripennis* and *N. giraulti*. (1) Gene Ontology (GO) enrichment analyses were

performed on the transcripts that aligned to the *N. vitripennis* proteome (Nvit2.0) using the Blast2go GUI (using a Fisher's exact test, $P < 0.05$) in order to visualize expression patterns of functional clusters of genes. GO terms were linked to the Nvit2.0 proteome using Blast2GO as described on http://www.hymenopteragenome.org/nasonia/?q=evidential_gene_data. Generic GOSlim categories were used to limit the number of GO-term categories (Gene Ontology Consortium, jan-10-2014). Enriched GO terms were compared

Table 1: Categories of transcripts in the transcriptomes. The number (and percentage of the total number of transcripts) and the average length of transcripts classified as 'protein coding (sense)', 'antisense', 'long non-coding RNA' and 'unknown' are given for the total transcriptome and for the differentially expressed transcripts (compared to naïve expression) of (a) *N. vitripennis* and (b) *N. girault*i. For the 'antisense' transcripts, the number of transcripts with a hit to a protein, a sense transcript or both is also given. The number of transcripts with a putative ORF is given for 'long non-coding RNA' and 'unknown'.

	Total transcriptome			DE transcripts		
(a) *N. vitripennis*	# transcripts		length (bp)	# transcripts		length (bp)
protein coding (sense)	22760	75.3%	4076	2175	88.5%	3819
antisense	1525	5.0%		76	3.1%	
with a hit to a protein	730	2.4%	2337	44	1.8%	3236
with a hit to a sense transcript	596	2.0%	1162	26	1.1%	802
with a hit to both	199	0.7%		6	0.2%	
long non-coding RNA	3245	10.7%	1342	112	4.6%	1512
with a putative ORF	220	0.7%		10	0.4%	
unknown	2693	8.9%	946	95	3.9%	1158
with a putative ORF	81	0.3%		4	0.2%	
Total	**30223**		**3389**	**2458**		**3565**

(b) *N. girault*i	# transcripts		length (bp)	# transcripts		length (bp)
protein coding (sense)	23806	80.3%	4004	2008	90.5%	3336
antisense	719	2.4%		30	1.4%	
with a hit to a protein	154	0.5%	1165	4	0.2%	1254
with a hit to a sense transcript	529	1.8%	846	24	1.1%	561
with a hit to both	36	0.1%		2	0.1%	
long non-coding RNA	2244	7.6%	1381	90	4.1%	1398
with a putative ORF	190	0.6%		12	0.5%	
unknown	2872	9.7%	972	92	4.1%	1138
with a putative ORF	92	0.3%		2	0.1%	
Total	**29641**		**3437**	**2220**		**3126**

between *N. vitripennis* and *N. giraulti* total and DE transcripts, for different time points after conditioning for each individual species, and for antisense transcripts. (2) The gene expression patterns of 44 genes that are known from literature to be involved in (long-term) memory formation were analysed for both species (Supplementary table 5). The *Nasonia* homolog of a 'memory' gene was obtained by aligning the *Drosophila melanogaster* gene sequence to the *N. vitripennis* genome (Nvit 2.0) using blastn or blastp.

RESULTS

Transcriptome assembly and annotation

The results of the transcriptome assembly (both filtered and unfiltered) are presented in Supplementary Table 1. The majority of the genes in the transcriptomes, respectively 74.7% for *N. vitripennis* and 73.0% for *N. giraulti*, has a single transcript. Genes with multiple splice variants ('transcripts') account, however, for 61.7% and 62.0% of all transcripts, respectively.

The transcriptomes of *N. vitripennis* and *N. giraulti* were analysed for their alignment to the publicly available *N. vitripennis* proteome and genome and to the NCBI RefSeq database. Respectively 71.5% and 74.5% of the *N. vitripennis* and *N. giraulti* transcripts aligned to an *N. vitripennis* protein with more than 60% protein alignment length. Most of the remaining protein coding transcripts, which had no alignment to the published *N. vitripennis* proteome (Nvit2.0), did align to transcripts of other Hymenoptera or even *N. vitripennis*, showing that the published Nvit2.0 proteome is incomplete. The percentages and average length of protein-coding (sense) transcripts, antisense transcripts, long non-coding RNA and unknown transcripts are shown in Table 1 and Figure 1 (a-b). A portion of the long non-coding RNA

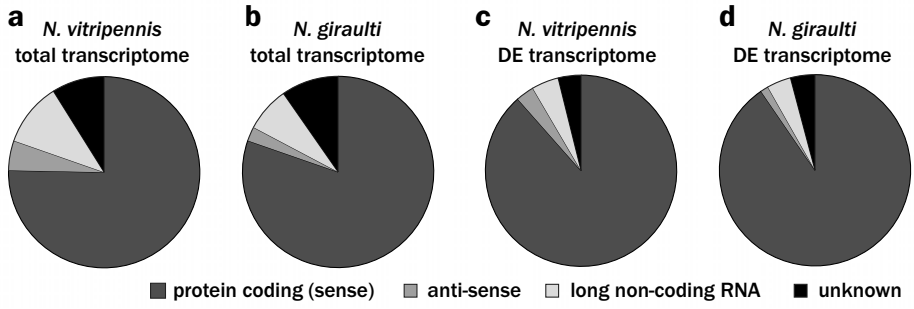


Figure 1: Categories of transcripts in the transcriptomes. The proportion of 'protein coding (sense)', 'antisense', 'long non-coding RNA' and 'unknown' is shown for (a) *N. vitripennis* total transcriptome (30223 transcripts), (b) *N. giraulti* total transcriptome (29641 transcripts), (c) *N. vitripennis* differentially expressed (DE) transcripts (2458 transcripts), and (d) *N. giraulti* DE transcripts (2220 transcripts) (DE compared to naïve expression).

and unknown (i.e. misassembled or misassigned transcripts) contains a putative ORF, suggesting these might be (unknown) protein-coding genes.

Differential gene expression

Differential gene expression was determined compared to naïve expression levels for both species. The percentages and average length of differentially expressed (DE) protein-coding (sense) transcripts, antisense transcripts, long non-coding RNA and unknown transcripts are shown in Table 1 and Figure 1c-d. The proportion of sense transcripts is larger in the DE transcriptomes than in the complete transcriptomes, but a number of antisense transcripts, long non-coding RNA and unknown transcripts are also differentially expressed. An analysis of DE transcripts of each time point after conditioning shows that the majority of the DE transcripts, i.e. 1,759 transcripts of *N. vitripennis* (71.6%) and 1,678 transcripts of *N. giraulti* (75.6%), are differentially expressed at only a single time point (Figure 2), which indicates that gene expression patterns are different when measured immediately, 4 hours or 24 hours after conditioning.

The protein-coding (sense) transcripts of *N. vitripennis* and *N. giraulti* which had a hit to the *N. vitripennis* proteome (Nvit2.0) were compared amongst each other to assess differences in gene expression between the two species. The majority of the transcripts (respectively, 86.1% and 82.9% of the total transcriptome) in the transcriptomes of *N. vitripennis* and *N. giraulti* was observed in both species, which indicates a high level of similarity in transcripts expressed in the brains of both species. However, only 37.8%

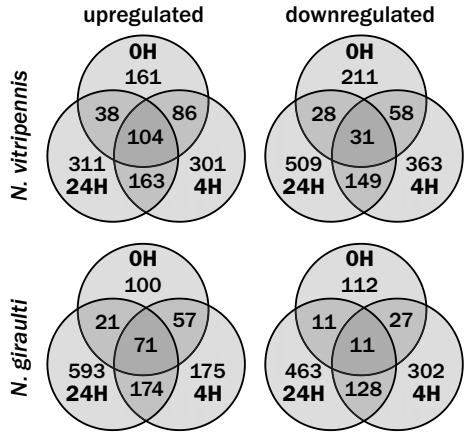


Figure 2: Differentially expressed genes at different time points after conditioning. Venn diagrams show the number of genes differentially expressed (compared to naïve expression) at 0h, 4h and 24h after conditioning and how many of them are differentially expressed at a single time point or at multiple time points. The top row shows results for *N. vitripennis*, the bottom row for *N. giraulti*. Venn diagrams are shown for up- (left column) or downregulated transcripts (right column).

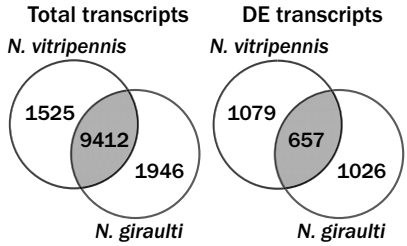


Figure 3: Shared and unique genes observed in the *N. vitripennis* and *N. giraulti* transcriptomes. The number of genes that are observed in the transcriptomes of only *N. vitripennis* or *N. giraulti* and the number of shared genes are shown for the entire transcriptome (left) and differentially expressed transcripts (right).

and 39.0% of the DE transcripts of, respectively, *N. vitripennis* and *N. giraulti* are differentially expressed in both species (Figure 3). This result suggests that there is substantial species-specific differential gene expression between *N. vitripennis* and *N. giraulti* after conditioning.

GO enrichment analysis of differentially expressed genes

Differentially expressed protein-coding transcripts with a hit to the *N. vitripennis* proteome (Nvit2.0) were analysed using GO enrichment analyses to provide insight into functional clusters of genes. Supplementary table 2 shows the complete lists of enriched GO-terms for each of the analyses presented in this paragraph.

As presented earlier, a large number of transcripts are differentially expressed only in *N. vitripennis* or *N. giraulti*. An overrepresentation of GO terms concerning processes involved in reproduction, the response to stimuli and a number of metabolic processes was observed in the transcripts that are unique for *N. vitripennis*. Terms concerning processes involved in cell cycle, cytoskeleton organization, kinase activity and a number of metabolic processes were revealed in transcripts unique to N. giraulti.

Analyses of up- and downregulated transcripts that are differentially expressed immediately (0 hours), 4 hours or 24 hours after conditioning were done for both species separately. The most specific GO terms of the category 'biological process' are presented in Figure 4. When comparing differential expression between the two wasp species immediately after conditioning, it is revealed that there is very little overlap with regard to enriched GO terms. Most of the terms found in *N. vitripennis*, both in up- and downregulated transcripts, indicate processes related to signal transduction or the response to stimuli. In *N. giraulti*, terms concerning processes involved in the regulation of biological processes, structure morphology, cell death and transcription factor activity were observed to be upregulated, whereas actin binding and single-organism process are downregulated.

For both wasp species, the expression patterns observed immediately after conditioning have very little functional overlap with those observed at 4 or 24 hours after conditioning. Both *N. vitripennis* and *N. giraulti* have an overrepresentation of terms that indicate that translation of transcripts is upregulated at both 4 and 24 hours after conditioning. Terms that indicate a number of metabolic processes, including lipid and carbohydrate metabolism, are observed in downregulated transcripts at both time points and in both species. Unique enriched GO terms observed in *N. vitripennis* indicate processes involved in the response to an endogenous signal, which is observed among upregulated transcripts at 4 hours after conditioning, but among downregulated transcripts at 24 hours after conditioning. Enriched terms observed in *N. giraulti* indicate processes involved in cytoskeleton and cell cycle, observed among upregulated transcripts, and homeostasis and

cell recognition, observed among downregulated transcripts. These terms are enriched only 24 hours after conditioning.

Enriched GO terms that are unique for N. vitripennis

We hypothesized that genes that are differentially expressed in *N. vitripennis* but not in *N. girault*i have a role in long-term memory formation. Differences in differential gene expression between the two species are most pronounced immediately after conditioning. Enriched GO-terms concerning processed involved in signal transduction or the response

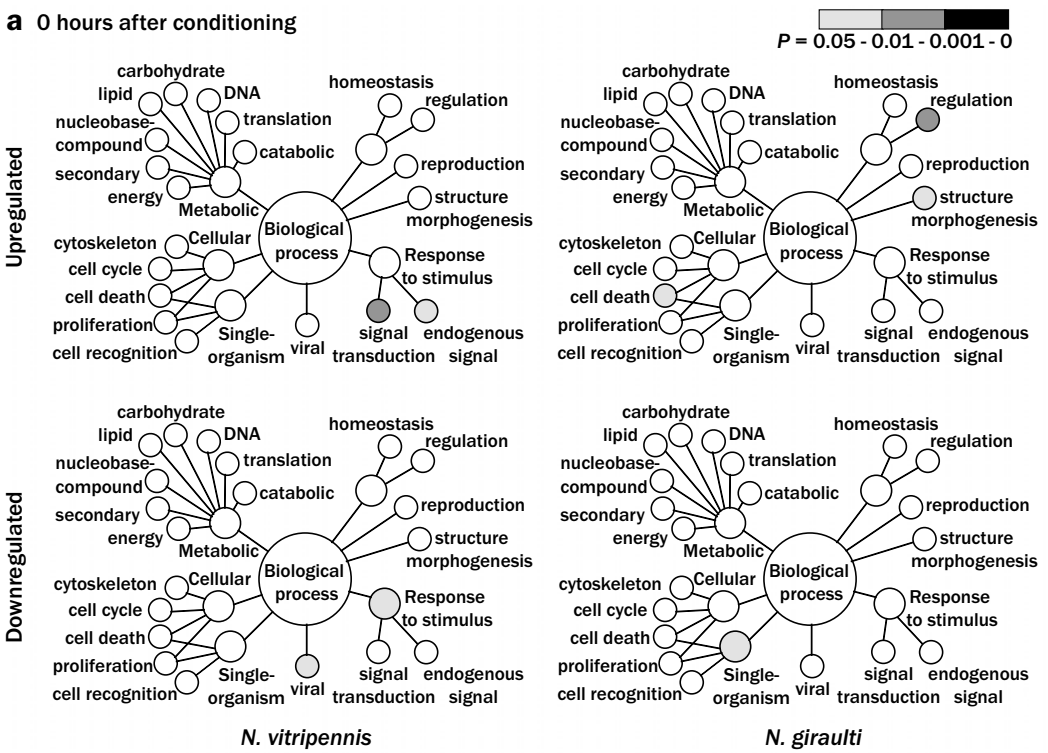
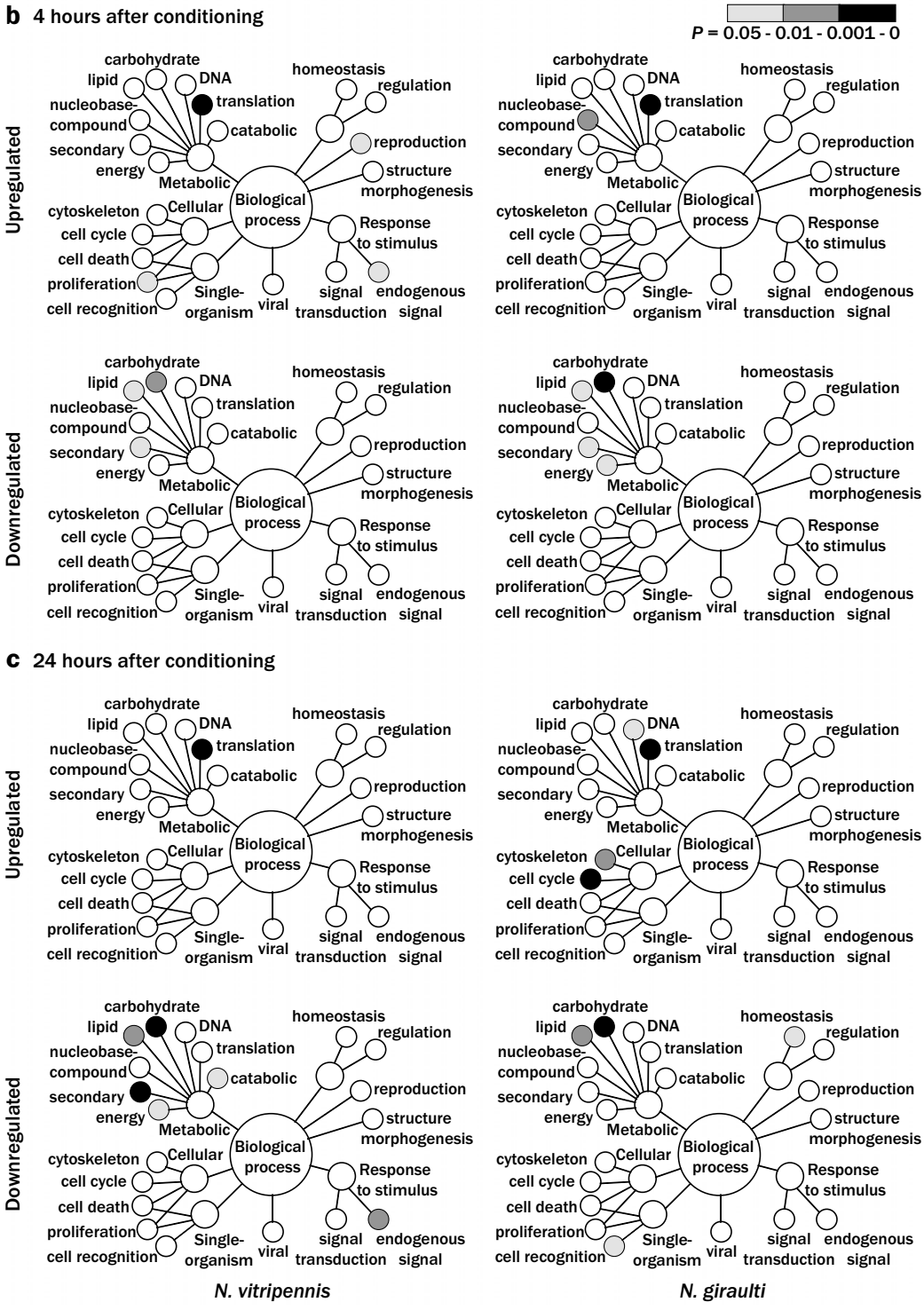


Figure 4: GO-enrichment analyses of differentially expressed genes of different time-points after conditioning compared to naïve wasps for *N. vitripennis* and *N. girault*. The Blast2go GUI (using a Fisher's exact test, $P < 0.05$) was used in order to test expression patterns of functional clusters of genes. Generic GOSlim categories were used to limit the number of GO-term categories. The most specific GO-terms in the category 'Biological process' (as determined using Blast2go) for each time point, respectively (a) 0h, (b) 4h, and (c) 24 h after conditioning, are shown in this network graph. Each node represents a different GO-term. Not all parental GO-terms and interactions between terms are shown in this network graph to simplify the figure. GO-terms at which multiple overrepresented GO-terms converge are shown to demonstrate functional patterns. Uncolored nodes are not overrepresented, but can be the parent of overrepresented GO-terms. Colored nodes are significantly overrepresented ($P < 0.05$), with the shade indicating significance as shown in the color bar.


b 4 hours after conditioning
P = 0.05 - 0.01 - 0.001 - 0
Upregulated
Downregulated
c 24 hours after conditioning
carbohydrate
lipid
DNA
translation
nucleobase-compound
catabolic
secondary
energy
Metabolic
homeostasis
regulation
reproduction
structure morphogenesis
Biological process
cytoskeleton
Cellular
cell cycle
cell death
proliferation
cell recognition
Single-organism
viral
Response to stimulus
signal transduction
endogenous signal
N. vitripennis
N. girault

to stimuli are observed exclusively among DE transcripts of *N. vitripennis*. A literature search was carried out for differentially expressed genes, which were clustered in the enriched GO-terms 'signal transduction' (GO:0007165), 'response to endogenous stimulus' (GO:0009719) and 'response to stimulus' (GO:0050896) immediately after conditioning, to identify specific transcripts known to be involved in memory formation. A total of 71 transcripts (59 genes), were clustered in the GO-terms mentioned (Supplementary table 3). A total of 40 transcripts was differentially expressed in *N. vitripennis*, but not in *N. giraulti*, 23 transcripts were differentially expressed in both species, and 8 transcripts were not observed in the transcriptome of *N. giraulti*. The DE transcripts include members of signalling cascades regulated by members of the Ras small G protein superfamily. Ras is known to activate the mitogen-activated protein kinase (MAPK) signalling pathway and the cAMP signalling cascade, which are both essential for long-term memory formation (Orban *et al*., 1999; Eisenhardt, 2006). A total of 9 different transcripts involved in the Ras signalling cascade are upregulated or downregulated in *N. vitripennis*, but not in *N. giraulti*. Ras-related protein *Rab-32* (Kawasaki *et al*., 1998) is upregulated in *N. vitripennis*, but downregulated in *N. giraulti*. Members of the Rho signalling cascade, a subfamily of the Ras superfamily, are also differentially expressed, i.e. *SLIT-ROBO* Rho GTPase-activating protein, *still life* and *TRIO* (Sone *et al*., 1997). Rho signalling is known to be involved in dendritic remodelling through organization of the actin cytoskeleton and is also essential for long-term memory formation (Threadgill *et al*., 1997; Bailey *et al*., 2004). Other genes with a known role in long-term memory formation include a glutamate receptor (upregulated), a metabotropic glutamate receptor (downregulated) (Riedel *et al*., 2003; Xia *et al*., 2005), and phosphatidylinositol 3-kinase (*PI3KC3*) (Yamada & Nabeshima, 2003), which is upregulated in *N. vitripennis* and downregulated in *N. giraulti*. Epigenetic mechanisms are also known to have an important role in memory formation (e.g. Levenson & Sweatt, 2006; Barrett & Wood, 2008; Lockett *et al*., 2010). The lysine-specific histone demethylase 1A was observed to be upregulated in *N. vitripennis* after conditioning, which may indicate a role in memory formation.

Enriched GO terms that are unique for N. giraulti

We hypothesized that genes that are differentially expressed in *N. giraulti* but not in *N. vitripennis* have a role in inhibition of long-term memory formation. For this reason, enriched GO-terms, which were observed exclusively among DE transcripts of *N. giraulti* were also studied. A literature search was carried out for differentially expressed genes, which were clustered in the enriched GO-terms 'regulation of biological process' (GO:0050789), 'anatomical structure morphogenesis' (GO:0009653), 'cell death' (GO:0008219) and 'single-organism process' (GO:0044699) immediately after conditioning. A total of 90

transcripts (71 genes), were clustered in the GO-terms mentioned (Supplementary table 4). A total of 35 transcripts was differentially expressed in *N. giraulti*, but not in *N. vitripennis*, 31 transcripts were differentially expressed in both species, and 16 transcripts were not observed in the transcriptome of *N. vitripennis*. Transcripts that are DE in *N. giraulti*, but not in *N. vitripennis*, include a number of genes involved in the regulation of transcription (including chromatin complexes subunit *BAP18*) and some genes encoding proteins involved in cytoskeleton organization, i.e. *talin-1*, *slingshot*, *cappuccino*, and γ-tubulin complex component 2. In addition, a few members of Ras/Rho and the MAPK signalling cascades are differentially expressed, i.e. Rho guanine nucleotide exchange factor, Rab5-activating protein 6, and *son-of-sevenless*, although not as many different genes are observed as in *N. vitripennis*. Also in *N. giraulti*, genes with a known role in LTM formation are differentially expressed, i.e. transcription factor *CREB A* is upregulated immediately after conditioning (Margulies *et al*., 2005; Müller, 2012). Two rate-limiting enzymes involved in the synthesis of neurotransmitter dopamine, i.e. GTP cyclohydrolase 1 and tyrosine 3-monooxygenase, are downregulated (Schultz, 2002; Meiser *et al*., 2013).

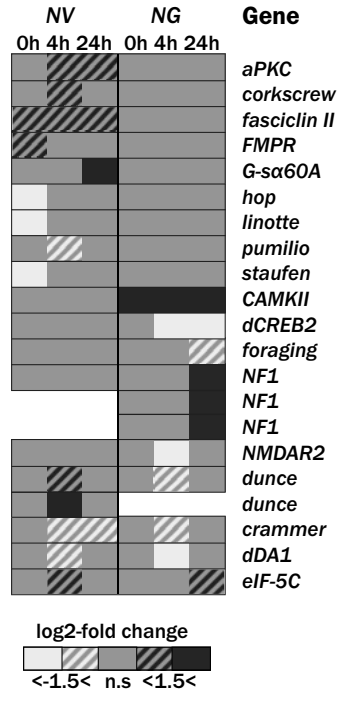


Figure 5: Memory genes that are differentially expressed after conditioning. A total of 18 genes (21 transcripts) of the 44 known 'memory' genes are differentially expressed after conditioning in *N. vitripennis* and/or *N. giraulti*. For most genes, a single transcript is differentially expressed (except *NF1*: 3 transcripts in *N. giraulti*, and *dunce*: 2 transcripts in *N. vitripennis*). The expression level is compared to naïve expression is shown for 0, 4 and 24 hours (n.s. = not significantly DE, log2-fold change shows if a transcript is upregulated (< 1.5 or >1.5) or downregulated (< -1.5 or > -1.5).

Memory gene expression analysis

A total of 44 genes with a known role in (long-term) memory formation were studied and 18 of these genes were observed to be differentially expressed after conditioning in *N. vitripennis* and/or *N. giraulti* (Supplementary table 5 and Figure 5). A total of 9 genes was differentially expressed in *N. vitripennis* only, 5 were differentially expressed in *N. giraulti* only, and 4 genes were differentially expressed in both species. In *N. vitripennis* 7 genes were upregulated and 6 were downregulated, whereas in *N. giraulti* most of the genes were downregulated (6 out of 9 genes). This may implicate that long-term memory formation is actively inhibited in *N. giraulti* by downregulation of certain 'memory' genes.

The cAMP-signalling cascade is an important cascade in the formation of both short-term memory (STM) and long-term memory (LTM) (Margulies *et al*., 2005; Eisenhardt, 2006). Transcription factor *dCREB2* (cAMP response element binding protein), which is essential for LTM formation, is down-regulated in *N. giraulti*. Another gene in this signalling cascade, *dunce* (cAMP phosphodiesterase), which is also involved in STM, was downregulated in *N. giraulti*, but upregulated in *N. vitripennis*. Another gene that was upregulated in *N. vitripennis* is *corkscrew* (SHP2 phosphatase); upregulation of this gene in *D. melanogaster* can shorten the inter-trial interval required for LTM induction and as a result massed conditioning trials, instead of multiple conditioning trials that are spaced in time, were sufficient for LTM induction (Pagani *et al*., 2009). Other genes that were upregulated in *N. vitripennis* are *aPKC* (atypical PKC, known to be involved in ARM), *fasciclin II* (a cell adhesion molecule), *FMRP* (Fragile-X mental retardation protein), and *G-sα60A* (a stimulatory G-protein) (Keene & Waddell, 2007; Banerjee *et al*., 2010; Müller, 2012). Among the downregulated genes in *N. vitripennis* are *pumilio* and *staufen*, both involved in the subcellular localization of mRNA translation (Dubnau *et al*., 2003), *hop* (Janus kinase), and *linotte* (RYK tyrosine kinase) (Keene & Waddell, 2007; Copf *et al*., 2011). Genes that were downregulated only in *N. giraulti* include the NMDA glutamate receptor 2 (Xia *et al*., 2005) and *foraging*, a cGMP-dependent protein kinase that has large effects on various aspects of (learning) behaviour, including STM and LTM formation (Mery *et al*., 2007a). The calcium/calmodulin dependent kinase *CAMKII* (Ashraf *et al*., 2006) and *NF1* (ras GTPase activating protein) (Keene & Waddell, 2007) are upregulated in *N. giraulti* only. Few genes are differentially expressed in both species: *crammer* (trans inhibitors of cathepsins) and *dDA1* (dopamine receptor) are downregulated in both species, *eIF-5C* (involved in translation) was upregulated in both species (Keene & Waddell, 2007).

In conclusion, approximately 40% of the known memory genes that were studied are differentially expressed after conditioning in one or both wasp species. Both up- and down-regulation of genes in a number of signalling pathways is observed in both *N. vitripennis* and *N. giraulti*. This approach yielded additional information when compared to the GO enrichment analysis, showing that both approaches are complementary.

GO enrichment analysis of antisense transcripts

Antisense transcripts can play a role in the regulation of gene expression of their sense transcripts (Pelechano & Steinmetz, 2013). Antisense transcripts with a hit to the *N. vitripennis* proteome (Nvit2.0) were analysed using GO enrichment analyses to provide insight into functional clusters of genes (results shown in Supplementary table 6). The two categories of antisense transcripts were analysed separately: 'antisense2protein' transcripts that have a hit to a *N. vitripennis* protein and 'antisense2sense' transcripts

that have a hit to a sense transcript only (and this sense transcript must have a hit to a *N. vitripennis* protein). GO-enrichment analyses were not carried out for the differentially expressed antisense transcripts with a hit the *N. vitripennis* proteome, because these numbers were too small to provide informative results using this analysis (antisense2protein: 45 from *N. vitripennis*, 3 from *N. giraulti*; antisense2sense: 32 from *N. vitripennis*, 26 from *N. giraulti*).

A diverse group of overrepresented GO terms was observed in antisense transcripts of both wasp species. For *N. vitripennis* these terms concern processes involved in lipid and DNA metabolism and cytoskeleton organisation in antisense2protein transcripts and behaviour in antisense2sense transcripts. For *N. giraulti*, terms concerning processes involved in gene expression were observed in antisense2protein transcripts. Cell-signalling, response to an abiotic stimulus, organelle organization, growth, anatomical structure morphogenesis and symbiosis were observed in antisense2sense transcripts. These results suggest that antisense transcripts of both *N. vitripennis* and *N. giraulti* play a role in diverse processes. The terms 'behaviour', 'cell signalling' and 'response to an abiotic stimulus' can implicate that part of these antisense transcripts are involved in synaptic processes or memory formation.

An analysis of the 44 known 'memory' genes (Supplementary table 5), described in the previous paragraph, revealed that 8 of these genes aligned with an antisense transcript (Table 2). None of these antisense transcripts were differentially expressed

Table 2: Antisense transcripts that align to memory genes. Antisense transcripts that align to known memory genes (see Supplementary table 5) are shown. Antisense transcripts were observed to align to 8 of the 44 memory genes tested. A number of these align to a sense transcript only (shown as $^{(s)}$), others align to a protein and a sense transcript (shown as $^{(s/p)}$).

Gene/ transgene	Gene description	antisense transcript ID	
		N. vitripennis	*N. giraulti*
CAMKII	calcium/calmodulin dependent kinase II	comp45169_c0_seq1$^{(s)}$	n.a.
crammer	trans-inhibitor of cathepsins	comp36751_c0_seq2$^{(s/p)}$	n.a.
dunce	cAMP phosphodiesterase	comp24558_c0_seq1$^{(s)}$	comp281362_c0_seq1$^{(s)}$
leonardo	14-3-3 zeta protein family	n.a.	comp18485_c0_seq1$^{(s/p)}$
NF1	ras GTPase activating protein	comp43282_c0_seq1$^{(s)}$	n.a.
radish	rap GTPase activating protein	n.a.	comp28992_c3_seq1$^{(s)}$
S6KII	ribosomal S6 kinase (RSK)	comp14791_c0_seq1$^{(s)}$	comp130445_c0_seq1$^{(s)}$
tequila	neurotrypsin	comp38985_c0_seq3$^{(s/p)}$	n.a.
		comp38985_c0_seq4$^{(s/p)}$	

after conditioning. These genes are *CAMKII*, *crammer*, *dunce*, *leonardo*, *NF1*, *radish*, *S6KII* and *tequila*. For the majority of these genes, an antisense transcript is detected from only one species (4 from *N. vitripennis* only, 2 from *N. giraulti* only). For only two of the memory genes an antisense transcript of both *N. vitripennis* and *N. giraulti* was observed. All 11 observed antisense transcripts have a hit to a sense transcript, but 4 transcripts have a hit to a protein as well. This result corresponds with results from the GO-enrichment analyses, which indicate that interesting terms concerning 'behaviour', 'cell signalling' and 'response to an abiotic stimulus' were overrepresented in antisense2sense transcripts only. It suggests that antisense transcripts align more often to untranslated regions of known memory genes than to protein-coding regions.

DISCUSSION

Differential gene expression after conditioning

The aim of this study was to identify genetic pathways that are responsible for the difference in LTM formation between the closely related parasitic wasp species *N. vitripennis* and *N. giraulti* by studying differential gene expression after a single conditioning trial. The results show that there are substantial differences in the differentially expressed genes between the two species, especially when compared immediately after conditioning. LTM formation requires at least two waves of transcriptional activity that occur during or shortly after conditioning and several hours after conditioning, respectively (Barzilai *et al.*, 1989; Alberini, 2009). Our results demonstrate that differential gene expression patterns differ substantially between the two *Nasonia* species immediately after conditioning, a procedure that lasts in total 1.5 hour in our experiment. This early differential gene expression is likely important for the initiation, or inhibition, of LTM formation after a single conditioning trial. Differences in differential gene expression between *N. vitripennis* and *N. giraulti* are also observed at 4 and 24 hours after conditioning, which may indicate that processes involved in the initiation or inhibition of LTM formation continue for hours up to at least a day after conditioning.

GO enrichment analyses demonstrated that terms involved in signalling are over-represented in *N. vitripennis*, whereas terms involved in regulation and cell morphology are overrepresented in *N. giraulti*. An analysis of these 'signalling' and 'regulatory/ cell morphology' genes was done in both species. A number of genes, with a known role in LTM formation, were differentially expressed in *N. vitripennis* only and these genes are likely involved in the ongoing process of LTM formation in this species. These genes, which belong to various genetic pathways, may be responsible for the difference in LTM formation between *N. vitripennis* and *N. giraulti*. In addition to differential gene expression

involved in LTM formation, a gene known to be involved in ARM formation, *aPKC* (Müller, 2012), was upregulated in *N. vitripennis*, but not in *N. giraulti*. In *N. vitripennis* two types of ARM are distinguished (Schurmann *et al*., 2012). One type is observed from an hour up to at least a day after conditioning, a type that is likely also formed in *N. giraulti*. A second type of ARM, which can be blocked by ethacrynic acid, is observed at 72 hours after conditioning (Schurmann *et al*., 2009). The observed differential expression of *aPKC* in *N. vitripennis* may be related to the formation of this type of ARM. Both up- and downregulation of 'memory' genes is observed at approximately equal numbers, which may point to activation of positive regulatory mechanisms, as well as the removal of LTM inhibitory mechanisms (Abel & Kandel, 1998). This result differs from a recent transcriptome study in honeybee brains after conditioning, which suggested that downregulation of gene expression is predominant after conditioning (Wang *et al*., 2013). The results from this study are, however, difficult to compare to our study, due to differences in conditioning procedure, timing of sample collection and the fact that the honeybees were tested for memory retention before RNA extraction.

A number of genes with a known role in LTM formation were differentially expressed in *N. giraulti* only. This species does not form LTM after a single conditioning trial and these differentially expressed genes may rather be part of an active inhibitory mechanism of LTM formation in this species. A targeted approach, in which differential gene expression of 44 known memory genes was investigated, confirms that differential expression of memory genes occurs in both species. These memory genes are often, but not always, downregulated in *N. giraulti*. Two genes involved in dopamine neurotransmitter synthesis, i.e. GTP cyclohydrolase 1 and tyrosine 3-monooxygenase (tyrosine hydroxylase), are downregulated immediately after conditioning in *N. giraulti* and an attractive hypothesis is that these genes have a role in the inhibition of LTM in this species. Dopamine has been widely shown to be important for memory formation, but different subsets of dopaminergic neurons with distinct functions have been reported, different dopaminergic receptors have different functions, and inhibition of dopamine signalling has been reported to abolish, but also enhance LTM formation (Berry *et al*., 2012; Klappenbach *et al*., 2013; Waddell, 2013). This example shows that it is difficult to predict effects of differential gene expression in whole brains on (long-term) memory formation.

In this study, differential gene expression patterns after conditioning, compared to naïve expression levels, were compared among two species with a different memory performance. Few other studies have compared gene expression levels between naïve animals that differ in their memory performance. Pravosudov *et al*. (2013) report on two populations of chickadees that differ in spatial memory performance and Armbrecht *et al*. (2014) compared control mice and mice with impaired memory performance. These studies

report differences in gene expression in various genes, including genes in the Ras-MAPK pathway and glutamate receptors in the chickadees, and genes in the Ras-MAPK and PI3K signalling pathways in mice. Differences in the expression patterns of these same genes and genetic pathways were observed between *N. vitripennis* and *N. giraulti*, which could indicate that these genes have an evolutionary conserved role in regulating variation in memory.

There are not only differences in differential gene expression between *N. vitripennis* and *N. giraulti*, but also similarities. A number of genes involved in signalling pathways and memory formation are differentially expressed in both *Nasonia* species. An upregulation of genes involved in translation is observed in both species at 4 and 24 hours after conditioning, whereas various metabolic pathways are then downregulated, including lipid and carbohydrate metabolism. A number of these genes may be involved in STM or ARM formation, which do not depend on transcription during or shortly after conditioning (Margulies *et al*., 2005), but may induce differential gene expression during or after their formation. In addition, genes involved in processes that have been induced by contact with the host during conditioning may be observed in both *Nasonia* species. During conditioning, the wasps will touch, evaluate and typically also feed from the host haemolymph, which induces the formation of eggs that are required for future oviposition. A recent study in *N. vitripennis* females indicated a downregulation of various metabolic processes in ovipositing females compared to resting females (Pannebakker *et al*., 2013). It is, however, difficult to compare our data to this study, because oviposition did not take place during our procedure and we used heads, not whole bodies as Pannebakker *et al*. (2013) did.

Alternative splicing

Alternative splicing is detected in large numbers of multi-exon genes and is known to be important for protein function, especially in neuronal genes (Lipscombe, 2005). In addition, neuronal activity can also induce alternative splicing (Hermey *et al*., 2013). For the transcription factor *CREB*, important for LTM formation, both inhibiting and activating transcript variants have been described and the balance of different transcript variants determines the number of trials required to initiate LTM consolidation in D. melanogaster (Yin *et al*., 1994; Tubon *et al*., 2013). Different splice variants of *FMRP* in *D. melanogaster* are thought to be involved in STM and LTM, respectively (Banerjee *et al*., 2010). Information on splice variants is, therefore, crucial for understanding gene functioning, but reliable and accurate determination of splice variants is challenging due to the small length of HiSeq reads that were analysed in this study. Multiple splice variants were detected for approximately 25% of all genes in the (head) transcriptomes of *N. vitripennis* and *N. giraulti* and for the majority of the studied memory genes (33 out of 44). For many differentially expressed

genes, only a single splice variant is actually differentially expressed. In a number of other differentially expressed genes, one or more transcripts are upregulated, whereas others are downregulated. In addition, temporal expression patterns can be different for different splice variants. These results indicate the importance of precise splice variant information on the interpretation of gene function. Further studies on the function of individual splice variants of interesting genes detected in this study are necessary to interpret differences in memory formation between our species.

Non-coding sequences

Sequences that do not encode proteins have important roles in the regulation of gene expression (Mattick, 2003; Pelechano & Steinmetz, 2013). In this study we focused mainly on antisense RNA (a class of long non-coding RNA, which aligns to a sense transcripts in the reverse orientation), although other long non-coding RNAs (> 200 bp in length) were also distinguished. Smaller non-coding RNAs were mostly lost during RNA isolation and were not studied.

The importance of antisense transcripts for gene regulation has only recently been recognized (Pelechano & Steinmetz, 2013) and novel methods for strand-specific Illumina Hi-Seq sequencing now allow transcriptome-wide studies on the expression of these transcripts. Antisense transcripts regulate transcription of their sense transcripts, but also of neighbouring genes; they regulate alternative splicing and affect mRNA stability and translation efficiency (Pelechano & Steinmetz, 2013). Antisense transcripts can affect chromatin structure and DNA methylation, which are also known to be important for alternative splicing and transcription regulation in the brain and for memory formation specifically (Li-Byarlay *et al*., 2013; Levenson & Sweatt, 2006). A total of 5.0% (1525) and 2.4% (719) of all transcripts were classified as antisense transcripts in *N. vitripennis* and *N. giraulti*, respectively. The actual number of antisense transcripts may be higher, because only transcripts with a high percentage of alignment length and identity were classified as 'antisense transcript' and others were classified as 'long non-coding RNA' or 'unknown'. An interesting observation is that 47.9% (730) and 21.4% (154) of these *N. vitripennis* and *N. giraulti* antisense transcripts, respectively, only align to a known protein, but not a sense transcript in the transcriptome, which may suggest that these sense transcripts have been silenced. The majority of the remaining antisense transcripts (i.e. 75.0% (596) and 93.6% (529), respectively) align to a sense transcript, but not to a protein, which indicates that these transcripts are likely located in the untranslated regions of protein-coding transcripts. GO enrichment analyses of this group of antisense transcripts revealed an overrepresentation of genes involved in 'behaviour' and 'signalling', which hints towards a role in the regulation of memory formation related genes. In addition, antisense transcripts

were observed for 8 out of 44 known memory genes that were studied, but none of these antisense transcripts are differentially expressed after conditioning. Although the significance of these observations remains to be investigated, they also hint towards a role of antisense transcripts in the regulation of long-term memory formation.

Conclusion

Learning and memory formation have an important role in animal and human behaviour and variation in memory formation is believed to reflect adaptations to species-specific ecological constraints. Studies on the genetic basis of natural variation in memory between animal species are necessary to understand the evolution of this variation. Our results demonstrate that there are substantial differences in differential gene expression in the brains of two closely related wasp species after single trial conditioning, which induces transcription-dependent LTM in only one of the two species. Various genes, with a known role in LTM formation, from different genetic pathways are up- or downregulated in *N. vitripennis* and point towards a role in the ongoing process of LTM formation in this species. However, differential expression of known (long-term) memory genes is also observed in *N. giraulti*, which does not form LTM after a single conditioning trial. This may suggest that LTM is actively inhibited in this species by components of known LTM genetic pathways. Alternative splicing is prominent in the *Nasonia* brain transcriptomes, including in known memory genes. Considering that different splice variants can have distinctly different roles in the formation of (long-term) memory, future studies should take the function and relative proportions of splice variants into account in order to understand the significance of differential gene expression patterns. Our study, furthermore, demonstrates that a significant proportion of the transcriptomes consists of non-coding RNA, including antisense transcripts. Antisense transcripts were observed for a number of known memory genes. Our results suggest that antisense transcripts have a role in the regulation of memory genes and may affect transcription, alternative splicing and translation. In conclusion, this study presents an extensive overview of both sense transcripts and non-coding transcripts that can be involved in variation in LTM formation between animal species. Many differentially expressed genes that were observed in this study have a known role in memory formation and future studies on regulation of these genes, including epigenetic regulation, the function of specific splice variants, and spatial expression patterns in the brain will provide insight on how these genes are also involved in variation in memory formation. This is important for understanding the evolution of variation in memory formation, but it can also provide novel insights for studies on (treatments for) neurodegenerative diseases, in which known memory genes are involved as well.

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SUPPLEMENTARY INFORMATION

Supplementary table 1: Transcriptome analysis statistics. The numbers of reads before and after quality filtering and adapter trimming are shown, together with statistics of the de novo transcriptome assemblies for both *N. vitripennis* and *N. giraulti*. These statistics include the number of 'Genes' and the number of 'Transcripts' both before and after filtering (> 1 cpm for at least 3 samples). The definition of 'Genes' and 'Transcripts' has been described in the materials and methods section. The number of 'Genes' with either a single or multiple splice variant ('Transcripts') is also given. The transcriptome size in bp (i.e. the combined length of the 'Genes'), the N50 (the number of transcripts with the largest sizes which together make up for half the transcriptome) and maximum transcript length were determined from the filtered transcripts.

	N. vitripennis	*N. giraulti*
Raw reads	294,289,458	294,210,825
Filtered reads	218,100,037	220,260,984
Unfiltered 'Genes'	92,097	82,351
Unfiltered 'Transcripts'	139,448	129,06
Filtered 'Genes'	15,789	15,453
Single splice variant	11,574	11,274
Multiple splice variants	4,215	4,179
Filtered 'Transcripts'	30,223	29,641
Transcriptome size (bp)	33,232,483	32,353,139
N50	4,949	5,019
Maximum transcript length	48,825	49,445

Supplementary table 2: Enriched GO-terms in differentially expressed protein-coding transcripts. GO-enrichment analyses of differentially expressed genes of different time-points after conditioning compared to naïve wasps for *N. vitripennis* and *N. giraulti*. Blast2go (Fisher's exact test, $P < 0.05$) was used to visualize expression patterns of functional clusters of genes. Generic GOSlim categories were used to limit the number of GO-term categories and the most specific terms were determined using Blast2Go. The enriched GO-terms (GO-ID and Term) that are up- or down-regulated, the category of the GO term (C = cellular component, F = molecular function, P = biological process), their *P*-value and the number of genes in the test-set and the reference-set are given.

Differentially expressed transcripts unique for *N. vitripennis*

GO-ID	Category	Term	*P*-Value	#Test	#Ref
GO:0003824	F	catalytic activity	3,62E-04	280	2071
GO:0005509	F	calcium ion binding	2,29E-03	21	85
GO:0009719	P	response to endogenous stimulus	4,07E-03	11	33
GO:0008289	F	lipid binding	2,25E-02	12	50
GO:0005102	F	receptor binding	2,25E-02	12	50
GO:0000003	P	reproduction	3,57E-02	43	275
GO:0006629	P	lipid metabolic process	3,70E-02	19	101
GO:0019748	P	secondary metabolic process	4,79E-02	5	15

Differentially expressed transcripts unique for *N. giraulti*

GO-ID	Category	Term	*P*-Value	#Test	#Ref
GO:0000166	F	nucleotide binding	1,55E-05	109	731
GO:0006259	P	DNA metabolic process	2,59E-04	25	108
GO:0007049	P	cell cycle	3,81E-04	45	257
GO:0009058	P	biosynthetic process	5,20E-03	70	512
GO:0007010	P	cytoskeleton organization	1,04E-02	33	211
GO:0008135	F	translation factor activity, nucleic acid binding	1,07E-02	13	59
GO:0019538	P	protein metabolic process	1,89E-02	96	785
GO:0030234	F	enzyme regulator activity	3,17E-02	20	124
GO:0003774	F	motor activity	3,32E-02	7	28
GO:0030246	F	carbohydrate binding	3,48E-02	6	22
GO:0016301	F	kinase activity	4,91E-02	45	348

***N. vitripennis* 0H after conditioning - Upregulated**

GO-ID	Category	Term	*P*-Value	#Test	#Ref
GO:0005509	F	calcium ion binding	1,32E-03	10	96
GO:0007165	P	signal transduction	5,32E-03	30	584
GO:0005215	F	transporter activity	2,57E-02	18	344

GO:0005811	C	lipid particle	2,74E-02	6	70
GO:0009719	P	response to endogenous stimulus	4,37E-02	4	40

***N. vitripennis* 0H after conditioning - Downregulated**

GO-ID	Category	Term	*P*-Value	#Test	#Ref
GO:0050896	P	response to stimulus	1,84E-02	33	936
GO:0016032	P	viral process	3,74E-02	2	11
GO:0004871	F	signal transducer activity	4,81E-02	5	78

***N. vitripennis* 4H after conditioning - Upregulated**

GO-ID	Category	Term	*P*-Value	#Test	#Ref
GO:0005840	C	ribosome	1,27E-14	32	78
GO:0006412	P	translation	2,08E-11	42	186
GO:0005198	F	structural molecule activity	1,90E-09	33	142
GO:0003723	F	RNA binding	1,89E-04	28	200
GO:0005730	C	nucleolus	2,49E-03	13	77
GO:0000003	P	reproduction	4,36E-03	31	287
GO:0008283	P	cell proliferation	1,37E-02	13	97
GO:0009719	P	response to endogenous stimulus	1,46E-02	7	37
GO:0005215	F	transporter activity	4,10E-02	30	332

***N. vitripennis* 4H after conditioning - Downregulated**

GO-ID	Category	Term	*P*-Value	#Test	#Ref
GO:0005811	C	lipid particle	7,62E-04	10	66
GO:0016787	F	hydrolase activity	1,30E-03	54	866
GO:0005975	P	carbohydrate metabolic process	2,24E-03	13	120
GO:0005764	C	lysosome	1,98E-02	3	12
GO:0019748	P	secondary metabolic process	4,29E-02	3	17
GO:0006629	P	lipid metabolic process	4,84E-02	9	111

***N. vitripennis* 24H after conditioning - Upregulated**

GO-ID	Category	Term	*P*-Value	#Test	#Ref
GO:0005840	C	ribosome	1,51E-50	63	47
GO:0005198	F	structural molecule activity	6,01E-35	63	112
GO:0006412	P	translation	3,64E-29	65	163
GO:0005811	C	lipid particle	2,37E-07	18	58
GO:0005829	C	cytosol	5,32E-03	17	130

GO:0003723	F	RNA binding	7,22E-03	23	205

***N. vitripennis* 24H after conditioning - Downregulated**

GO-ID	Category	Term	*P*-Value	#Test	#Ref
GO:0005509	F	calcium ion binding	6,26E-06	18	88
GO:0003824	F	catalytic activity	2,03E-05	155	2196
GO:0019748	P	secondary metabolic process	3,98E-05	7	13
GO:0005975	P	carbohydrate metabolic process	4,41E-04	17	116
GO:0005811	C	lipid particle	1,65E-03	11	65
GO:0016209	F	antioxidant activity	2,49E-03	4	8
GO:0006629	P	lipid metabolic process	3,35E-03	14	106
GO:0008289	F	lipid binding	4,21E-03	9	53
GO:0009719	P	response to endogenous stimulus	6,77E-03	7	37
GO:0019825	F	oxygen binding	7,73E-03	2	1
GO:0009056	P	catabolic process	1,06E-02	30	346
GO:0008092	F	cytoskeletal protein binding	1,81E-02	14	132
GO:0006091	P	generation of precursor metabolites and energy	2,57E-02	10	86

***N. girault* 0H after conditioning - Upregulated**

GO-ID	Category	Term	*P*-Value	#Test	#Ref
GO:0003700	F	sequence-specific DNA binding transcription factor activity	4,50E-04	11	166
GO:0003677	F	DNA binding	5,12E-04	16	322
GO:0000166	F	nucleotide binding	5,80E-03	26	814
GO:0050789	P	regulation of biological process	6,89E-03	38	1362
GO:0008219	P	cell death	2,16E-02	8	175
GO:0009653	P	anatomical structure morphogenesis	3,73E-02	18	597
GO:0005215	F	transporter activity	4,77E-02	12	362

***N. girault* 0H after conditioning - Downregulated**

GO-ID	Category	Term	*P*-Value	#Test	#Ref
GO:0003779	F	actin binding	2,34E-02	4	84
GO:0044699	P	single-organism process	4,89E-02	31	1897

***N. girault* 4H after conditioning - Upregulated**

GO-ID	Category	Term	*P*-Value	#Test	#Ref
GO:0005730	C	nucleolus	1,29E-06	16	80

GO:0008135	F	translation factor activity, nucleic acid binding	1,40E-04	11	61
GO:0000166	F	nucleotide binding	1,57E-04	55	785
GO:0006412	P	translation	3,24E-04	21	205
GO:0006139	P	nucleobase-containing compound metabolic process	5,31E-03	37	556

***N. girault*i 4H after conditioning - Downregulated**

GO-ID	Category	Term	*P*-Value	#Test	#Ref
GO:0005975	P	carbohydrate metabolic process	7,16E-06	16	117
GO:0006629	P	lipid metabolic process	1,58E-02	9	108
GO:0006091	P	generation of precursor metabolites and energy	2,11E-02	8	95
GO:0019748	P	secondary metabolic process	3,13E-02	3	18
GO:0005811	C	lipid particle	4,28E-02	6	71
GO:0016787	F	hydrolase activity	4,74E-02	40	889

***N. girault*i 24H after conditioning - Upregulated**

GO-ID		Term	*P*-Value	#Test	#Ref
GO:0005840	C	ribosome	4,44E-47	65	45
GO:0006412	P	translation	1,74E-42	86	140
GO:0005198	F	structural molecule activity	6,01E-33	68	113
GO:0007049	P	cell cycle	7,24E-07	46	256
GO:0008135	F	translation factor activity, nucleic acid binding	1,01E-05	17	55
GO:0005829	C	cytosol	1,36E-05	26	120
GO:0005811	C	lipid particle	9,83E-05	16	61
GO:0000166	F	nucleotide binding	4,63E-03	80	760
GO:0005694	C	chromosome	8,43E-03	17	108
GO:0007010	P	cytoskeleton organization	9,75E-03	28	216
GO:0006259	P	DNA metabolic process	1,52E-02	17	116
GO:0009055	F	electron carrier activity	2,65E-02	5	19
GO:0005730	C	nucleolus	4,40E-02	12	84

***N. girault*i 24H after conditioning - Downregulated**

GO-ID	Category	Term	*P*-Value	#Test	#Ref
GO:0005509	F	calcium ion binding	2,07E-04	13	86
GO:0005975	P	carbohydrate metabolic process	3,78E-04	15	118

GO:0003774	F	motor activity	2,89E-03	6	29
GO:0006629	P	lipid metabolic process	9,15E-03	11	106
GO:0005811	C	lipid particle	1,42E-02	8	69
GO:0005215	F	transporter activity	2,16E-02	24	350
GO:0052689	F	carboxylic ester hydrolase activity	3,16E-02	2	5
GO:0019725	P	cellular homeostasis	3,26E-02	6	52
GO:0008037	P	cell recognition	3,49E-02	5	39

Supplementary table 3: DE transcripts involved in signalling in *N. vitripennis*. Differentially expressed genes involved in signalling are overrepresented in *N. vitripennis* (NV), but not in *N. giraulti* (NG) when compared immediately after conditioning. These genes are shown in this table. The log2- FC (fold change), log2-CPM (count per million, a measure for abundance of the transcript) and the level of significance of the DE transcript are given for the time point '0 hours after conditioning'. In addition, the DE pattern of a gene is shown for 4 and 24 hours after conditioning (↓ = downregulated, ↑ = upregulated, n.a. = transcript not observed in the transcriptome). (a) Genes that are up- or downregulated in *N. vitripennis*, but not in *N. giraulti* are shown. (b) Genes that are up- or downregulated in both *N. vitripennis* and *N. giraulti* are shown. (c) Differentially expressed genes in *N. vitripennis* that are not found in the *N. giraulti* transcriptome are shown.

a. Signalling genes that are differentially expressed in *N. vitripennis* and not differentially expressed in *N. giraulti*

OGS2.0 ID	Transcript ID	Gene description	Log2 FC	Log2 CPM	*P*-value	DE pattern (NV)	DE pattern (NG)
Nasvi2EG013237t1	comp39228_c0_seq3	still life	-10,14	0,02	3,37E-08	↓0h,24h	not DE
Nasvi2EG008626t2	comp483118_c0_seq1	unknown	8,2	-0,35	9,96E-05	↑0h,4h,24h	not DE
Nasvi2EG013237t2	comp29402_c0_seq1	still life	7,71	-0,77	2,30E-04	↑0h,4h,24h	not DE
Nasvi2EG023096t1	comp34611_c0_seq2	lysine-specific histone demethylase 1A	5,43	-0,47	6,12E-05	↑0h	not DE
Nasvi2EG017531t1	comp38970_c2_seq11	TGF-beta receptor type-1	-4,5	0,69	1,19E-05	↓0h	not DE
Nasvi2EG019393t1	comp39881_c3_seq1	Glutamate receptor subunit 1	4,2	-0,44	5,82E-04	↑0h	not DE
Nasvi2EG003595t2	comp27330_c0_seq1	SLIT-ROBO Rho GTPase-activating protein 1	3,92	0,92	1,07E-06	↑0h,4h,24h	not DE
Nasvi2EG009337t1	comp36228_c0_seq7	Tyrosine-protein kinase receptor	3,82	1,21	3,21E-07	↑0h	not DE
Nasvi2EG003595t2	comp27330_c0_seq4	SLIT-ROBO Rho GTPase-activating protein 1	3,72	1,99	4,72E-08	↑0h,4h,24h	not DE
Nasvi2EG009337t1	comp36228_c0_seq6	Tyrosine-protein kinase receptor	3,68	0,31	1,41E-04	↑0h	not DE
Nasvi2EG007191t1	comp13537_c0_seq1	Death related ced-3/Nedd2 protein	-3,39	0,46	8,47E-05	↓0h	not DE
Nasvi2EG006407t3	comp35269_c1_seq1	Tyrosine-protein kinase Src64B	-3,39	0,16	8,61E-04	↓0h	not DE
Nasvi2EG004379t4	comp37393_c0_seq4	E3 ubiquitin-protein ligase UBR2	-2,73	1,16	9,18E-05	↓0h	not DE
Nasvi2EG014516t1	comp39309_c2_seq19	ras-related protein Rab-6A	-2,52	2,39	6,00E-06	↓0h	not DE
Nasvi2EG002305t1	comp32573_c0_seq3	GTP-binding protein Di-Ras2, putative	2,51	0,72	3,76E-04	↑0h	not DE
Nasvi2EG014393t1	comp43869_c0_seq1	unknown	2,35	1,96	5,24E-05	↑0h,4h	not DE

Nasvi2EG005268t1	comp34705_c0_seq5	crumbs protein	2,27	3,11	6,31E-08	↑0h,4h,24h	not DE
Nasvi2EG005229t1	comp27827_c0_seq2	Signal transducing adapter molecule 1	-2,25	2,75	8,24E-06	↓0h	not DE
Nasvi2EG019874t3	comp38852_c1_seq15	Triple functional domain protein	2,25	1,87	1,17E-04	↑0h	not DE
Nasvi2EG015607t1	comp36072_c0_seq2	deoxyribodipyrimidine photo-lyase	-2,16	1,86	3,48E-04	↓0h	not DE
Nasvi2EG010744t1	comp39149_c0_seq12	metabotropic glutamate receptor (mangetout)	-2,14	1,04	1,12E-03	↓0h,4h,24h	not DE
Nasvi2EG022273t2	comp36648_c0_seq8	GTP-binding protein Rheb homolog	-1,97	4,08	8,19E-10	↓0h	not DE
Nasvi2EG015809t1	comp33742_c0_seq1	innexin inx1, putative	1,87	3,41	5,32E-07	↑0h,4h	not DE
Nasvi2EG009786t4	comp38864_c1_seq5	Guanine nucleotide-releasing factor 2	-1,83	3,75	1,86E-07	↓0h	not DE
Nasvi2EG008107t4	comp27284_c0_seq3	OTU domain-containing protein 7B, putative	1,78	3,27	2,37E-06	↑0h,4h	not DE
Nasvi2EG007375t3	comp33212_c0_seq3	E3 ubiquitin-protein ligase suppressor of deltex	-1,65	2,98	1,31E-04	↓0h	not DE
Nasvi2EG009786t4	comp38864_c1_seq7	Guanine nucleotide-releasing factor 2	-1,58	3,26	9,43E-05	↓0h	not DE
Nasvi2EG003715t2	comp37447_c0_seq28	Afadin	-1,58	4,66	4,92E-09	↓0h	not DE
Nasvi2EG009786t4	comp38864_c1_seq6	Guanine nucleotide-releasing factor 2	1,48	2,92	6,04E-04	↑0h,4h,24h	not DE
Nasvi2EG008626t2	comp38080_c0_seq26	Unknown	1,43	4,34	6,29E-07	↑0h ↓24h	not DE
Nasvi2EG001535t1	comp34948_c0_seq2	Serine/threonine-protein kinase SNF1 kinase 2	1,4	2,97	7,75E-04	↑0h,24h	not DE
Nasvi2EG009786t4	comp38864_c1_seq3	Guanine nucleotide-releasing factor 2	1,2	3,45	5,82E-04	↑0h,4h,24h	not DE
Nasvi2EG002993t3	comp39642_c22_seq8	serine/threonine-protein phosphatase 2A	0,84	5,68	8,05E-08	↑0h	not DE
Nasvi2EG007441t4	comp38606_c0_seq1	1-phosphatidylinositol-4,5-bisphos-phate phosphodiesterase classes I and II	-0,8	5,04	1,18E-04	↓0h	not DE
Nasvi2EG013237t2	comp39228_c0_seq8	still life, protein	-0,73	5,07	3,44E-04	↓0h	not DE
Nasvi2EG013710t2	comp37252_c0_seq1	ADIPOR receptor CG5315-like, putative	0,58	6,91	7,06E-06	↑0h,4h,24h	not DE
Nasvi2EG010166t1	comp14378_c0_seq1	Transforming protein Ski	0,53	6,74	5,05E-05	↑0h	not DE

Nasvi2EG009605t2	comp32432_c0_seq1	tyrosine-protein kinase Src42A, putative	-0,51	5,82	7,88E-04	↓0h	not DE
Nasvi2EG007947t2	comp39607_c4_seq2	guanine nucleotide-binding protein G(q)	0,51	6,04	3,73E-04	↑0h	not DE
Nasvi2EG006840t1	comp40159_c0_seq1	Nuclear protein 1	-0,38	10,11	1,01E-06	↓0h,4h	not DE
							not DE

b. Signalling genes that are differentially expressed in *N. vitripennis* and *N. giraulti*

OGS2.0 ID	Transcript ID	Gene description	Log2 FC	Log2 CPM	*P*-value	DE pattern (NV)	DE pattern (NG)
Nasvi2EG037341t1	comp37043_c0_seq6	Ras-related protein Rab-32	4,3	-0,16	4,54E-04	↑0h	↓4h,24h
Nasvi2EG023114t1	comp39910_c1_seq23	phosphatidylinositol 3-kinase catalytic subunit type 3	3,8	-0,13	3,27E-04	↑0h	↓0h
Nasvi2EG013660t1	comp33709_c0_seq3	biotin--protein ligase	-2,89	0,5	4,04E-04	↓0h	↓4h
Nasvi2EG015011t1	comp37930_c2_seq4	parathyroid hormone-related peptide receptor	-2,42	1,23	1,47E-04	↓0h	↓24h
Nasvi2EG015580t1	comp35764_c0_seq4	armadillo segment polarity protein	-2,3	5,31	5,21E-28	↓0h,4h,24h	↓24h
Nasvi2EG000868t1	comp38978_c0_seq7	tachykinin peptides receptor 99D-like, putative	2,01	1,21	9,61E-04	↑0h	↑4h,24h
Nasvi2EG002499t2	comp39261_c0_seq105	retinal degeneration B protein	1,72	2,72	1,17E-03	↑0h,4h,24h	↓24h
Nasvi2EG015580t1	comp35764_c0_seq5	armadillo segment polarity protein	1,62	4,88	1,38E-12	↑0h,4h,24h	↓24h
Nasvi2EG037341t1	comp37043_c0_seq1	Ras-related protein Rab-32	-1,39	3,46	4,55E-05	↓0h,4h,24h	↓4h,24h
Nasvi2EG003084t2	comp26839_c0_seq1	Reticulon-1 (Fragment)	-1,2	7,63	1,33E-24	↓0h,4h,24h	↓0h,24h
Nasvi2EG037152t1	comp31826_c2_seq2	Ecdysone-induced protein 75B	1,17	7,2	1,75E-21	↑0h,4h,24h	↑0h,4h,24h
Nasvi2EG025129t1	comp34836_c0_seq1	B-cell lymphoma 3-encoded protein	-1,13	4,29	8,02E-05	↓0h	↑0h,4h
Nasvi2EG007441t6	comp38606_c0_seq20	1-phosphatidylinositol-4,5-bisphos-phate phosphodiesterase classes I + II	-1,12	4,56	1,64E-05	↓0h	↑24h
Nasvi2EG011878t2	comp35527_c5_seq2	roadkill, protein	0,89	7,21	3,60E-13	↑0h,4h	↑0h
Nasvi2EG005342t1	comp42369_c0_seq1	Raf protein serine/threonine-protein kinase dRAF-1	0,85	5,78	1,84E-08	↑0h	↑0h

Nasvi2EG010931t1	comp14073_c0_seq2	mob as tumor suppressor	-0,81	5,62	1,07E-06	↓0h ↑4h,24h	↓0h,4h
Nasvi2EG008393t1	comp40145_c0_seq1	Antimicrobial peptide Def1-1	-0,6	7,57	2,01E-07	↓0h	↑4h,24h
Nasvi2EG002888t3	comp13915_c1_seq1	spinster protein, putative	-0,57	5,46	6,96E-04	↓0h	↓4h
Nasvi2EG014068t1	comp41689_c0_seq1	Transcription factor AP-1	0,57	5,86	1,39E-04	↑0h	↑24h
Nasvi2EG008392t1	comp40158_c0_seq1	Antimicrobial peptide Def1-1	-0,56	5,97	1,45E-04	↓0h,24h	↑24h
Nasvi2EG033203t1	comp13782_c0_seq1	cGMP-dependent 3',5'-cyclic phos-phodiesterase	0,5	5,96	6,74E-04	↑0h	↑0h
Nasvi2EG003084t2	comp26839_c0_seq2	Reticulon-1 (Fragment)	0,42	8,42	3,79E-05	↑0h	↓0h,24h
Nasvi2EG003943t4	comp27087_c0_seq1	Myosin heavy chain, muscle	0,27	11,41	7,00E-06	↑0h ↓24h	↓24h

c. Signalling genes that are differentially expressed in *N. vitripennis*, but that are not observed in the transcriptome of *N. giraulti*

OGS2.0 ID	Transcript ID	Gene description	Log2 FC	Log2 CPM	*P*-value	DE pattern (NV)	DE pattern (NG)
Nasvi2EG006681t8	comp39113_c5_seq5	Arginine-glutamic acid dipeptide repeats protein	-1,14	4,12	2,65E-04	↓0h	n.a
Nasvi2EG014089t3	comp37425_c0_seq5	TBC1 domain family member CG11727	-1,95	4,41	5,77E-10	↓0h,24h	n.a
Nasvi2EG011643t3	comp26279_c0_seq1	DNA repair endonuclease XPF	2,26	1,3	4,00E-04	↑0h	n.a
Nasvi2EG014089t3	comp37425_c0_seq17	TBC1 domain family member CG11727	2,86	1,65	1,75E-05	↑0h	n.a
Nasvi2EG008869t3	comp39661_c11_seq7	unknown	-3,46	0,13	3,28E-04	↓0h	n.a
Nasvi2EG004424t1	comp39301_c4_seq16	dystrophin, isoforms A/C/F/G/H, putative	-3,81	-0,06	5,77E-04	↓0h	n.a
Nasvi2EG014089t3	comp37425_c0_seq2	TBC1 domain family member CG11727	4,01	1,99	1,27E-08	↑0h,4h,24h	n.a
Nasvi2EG001811t1	comp38721_c1_seq2	unknown	-5,9	0,36	4,58E-07	↓0h	n.a

Supplementary table 4: DE transcripts involved in regulation in *N. giraulti*. Differentially expressed genes involved in regulation, anatomical structure and single-organism processes are overrepresented in *N. giraulti* (NG), but not in *N. vitripennis* (NV) when compared immediately after conditioning. These genes are shown in this table. The log2-FC (fold change), log2-CPM (count per million, a measure for abundance of the transcript) and the level of significance of the DE transcript are given for the time point '0 hours after conditioning'. In addition, the DE pattern of a gene is shown for 4 and 24 hours after conditioning (↓ = downregulated, ↑ = upregulated, n.a. = transcript not observed in the transcriptome). (a) Genes that are up- or downregulated in *N. giraulti*, but not in *N. vitripennis* are shown. (b) Genes that are up- or downregulated in both *N. giraulti* and *N. vitripennis* are shown. (c) Differentially expressed genes in *N. giraulti* that are not found in the *N. vitripennis* transcriptome are shown.

a. regulatory genes that are differentially expressed in *N. giraulti* and not differentially expressed in *N. vitripennis*

OGS2.0 ID	Transcript ID	Gene description	Log2-FC	Log2-CPM	*P*-value	DE pattern (NG)	DE pattern (NV)
Nasvi2EG011988t1	comp34712_c0_seq13	JmjC domain-containing protein 5	9.22	0.86	1.68E-08	↑0h,4h,24h	not DE
Nasvi2EG005239t5	comp33563_c2_seq9	Unknown	8.94	-0.37	1.59E-06	↑0h,4h,24h	not DE
Nasvi2EG009943t1	comp33078_c4_seq2	Replication factor C subunit 4	-8.80	-1.08	1.65E-05	↓0h	not DE
Nasvi2EG010411t2	comp30397_c0_seq1	Coiled-coil and C2 domain-containing protein 1	8.18	-0.74	4.71E-04	↑0h,4h,24h	not DE
Nasvi2EG022901t2	comp35283_c1_seq7	Tyrosine 3-monooxygenase	-7.97	-0.14	2.27E-05	↓0h	not DE
Nasvi2EG013810t2	comp33359_c5_seq1	son of sevenless protein	-7.89	1.41	1.44E-05	↓0h ↑24h	not DE
Nasvi2EG001205t1	comp30979_c0_seq1	chromatin complexes subunit BAP18	6.31	0.45	1.63E-09	↑0h	not DE
Nasvi2EG002134t1	comp35449_c1_seq1	Talin-1	-5.33	-0.97	1.85E-04	↓0h	not DE
Nasvi2EG001388t1	comp31944_c2_seq6	one cut domain family member 2	4.43	-0.09	2.83E-05	↑0h,4h,24h	not DE
Nasvi2EG013985t2	comp32252_c0_seq10	Solute carrier organic anion transporter family member 4A1	-3.56	0.61	3.47E-05	↓0h	not DE
Nasvi2EG011110t2	comp34468_c0_seq26	multidrug resistance-associated protein 1, putative	-3.47	-0.05	2.20E-04	↓0h	not DE
Nasvi2EG005116t4	comp34192_c0_seq8	PITSLRE serine/threonine-protein kinase CDC2L1	3.31	0.44	2.34E-04	↑0h,4h,24h	not DE
Nasvi2EG013161t1	comp33029_c0_seq7	Metal response element-binding transcription factor-1	3.28	1.50	7.74E-07	↑0h,4h,24h	not DE

Nasvi2EG008358t1	comp34966_c1_seq66	voltage-dependent calcium channel type D subunit alpha-1	3.25	0.18	4.95E-04	↑0h,4h,24h	not DE
Nasvi2EG002818t3	comp34386_c7_seq14	phosphatase Slingshot	2.98	1.66	2.32E-06	↑0h,4h,24h	not DE
Nasvi2EG007798t3	comp31488_c0_seq5	GTPase-activating protein and VPS9 domain-containing protein 1	2.88	0.64	1.03E-04	↑0h,24h	not DE
Nasvi2EG009129t2	comp20701_c0_seq2	Gamma-tubulin complex component 2	-2.76	2.85	8.93E-08	↓0h	not DE
Nasvi2EG005811t7	comp34771_c1_seq46	sodium/hydrogen exchanger 7	-2.55	0.69	4.11E-04	↓0h	not DE
Nasvi2EG014349t1	comp28785_c0_seq3	mucosa-associated lymphoid tissue lymphoma translocation protein 1	-2.39	2.35	1.90E-05	↑0h,24h	not DE
Nasvi2EG036938t2	comp34059_c0_seq4	Tyrosine-protein kinase PR2	2.21	1.90	3.64E-04	↑0h	not DE
Nasvi2EG021057t2	comp34884_c0_seq1	ESF1	-2.11	2.60	1.30E-04	↓0h,4h	not DE
Nasvi2EG022638t2	comp35034_c0_seq13	Formin 1,2/cappuccino	-2.10	1.81	2.72E-04	↓0h,4h	not DE
Nasvi2EG002463t3	comp33061_c0_seq6	arf-GAP with SH3 domain, ANK repeat and PH domain protein 2	-1.74	2.79	4.54E-04	↓0h	not DE
Nasvi2EG007284t2	comp30468_c1_seq8	Phosphatidylinositol-4-phosphate 3-kinase C2	-1.59	3.41	1.21E-04	↓0h	not DE
Nasvi2EG001023t1	comp32180_c0_seq6	Rho guanine nucleotide exchange factor	1.57	3.76	1.36E-05	↑0h,24h	not DE
Nasvi2EG037014t1	comp33596_c0_seq4	eyes shut protein	1.49	4.31	2.12E-05	↑0h,4h,24h	not DE
Nasvi2EG019970t1	comp33072_c0_seq5	slowpoke-binding protein	-1.41	3.91	6.07E-05	↓0h,4h	not DE
Nasvi2EG002014t7	comp34902_c10_seq25	liprin-alpha-2, putative	1.37	3.41	5.34E-04	↑0h	not DE
Nasvi2EG004277t1	comp31840_c0_seq2	GTP cyclohydrolase 1	-1.30	3.85	3.77E-04	↓0h	not DE
Nasvi2EG002499t4	comp35017_c0_seq60	retinal degeneration B protein	1.06	5.73	1.19E-07	↑0h,4h	not DE
Nasvi2EG018583t3	comp31305_c0_seq1	trafficking kinesin-binding protein milt	-0.90	5.97	3.38E-06	↓0h	not DE
Nasvi2EG009270t2	comp21911_c1_seq1	Cyclic AMP response element-binding protein A	0.77	5.45	2.46E-04	↑0h	not DE
Nasvi2EG009732t1	comp36934_c0_seq1	CTP synthase, putative	0.74	6.16	4.64E-05	↑0h	not DE
Nasvi2EG005065t2	comp34219_c6_seq1	Activating transcription factor of chaperone	0.58	7.25	3.08E-04	↑0h	not DE

Nasvi2EG011931t2	comp16316_c0_seq1	Battenin	-0.57	6.84	6.57E-04	↓0h	not DE

b. regulatory genes that are differentially expressed in *N. giraulti* and *N. vitripennis*

OGS2.0 ID	Transcript ID	Gene description	Log2 FC	Log2 CPM	P-value	DE pattern (NG)	DE pattern (NV)
Nasvi2EG002835t1	comp30530_c0_seq1	myosin-IA	-10.71	0.40	5.15E-11	↓0h,24h	↓4h,24h
Nasvi2EG017709t1	comp34998_c2_seq96	potassium channel subfamily T member 1	-10.24	-1.05	1.28E-06	↓0h,4h	↑4h
Nasvi2EG023365t1	comp33689_c0_seq4	HIV Tat-specific factor 1 protein	-8.24	0.88	1.70E-06	↓0h ↑24h	↑24h
Nasvi2EG008896t1	comp33991_c0_seq5	inwardly rectifying potassium channel	7.45	0.19	2.71E-10	↑0h,4h	↑4h
Nasvi2EG008896t1	comp33991_c0_seq18	inwardly rectifying potassium channel	4.95	-0.39	1.85E-05	↑0h	↑4h
Nasvi2EG000786t1	comp347886_c0_seq1	Cold-shock DNA-binding family protein	4.92	-0.09	2.24E-05	↑0h,4h,24h	↑4h,24h
Nasvi2EG023114t1	comp32330_c0_seq5	phosphatidylinositol 3-kinase catalytic subunit type 3	-4.64	-0.14	1.51E-04	↓0h	↑0h
Nasvi2EG029478t1	comp35393_c1_seq30	Nuclear pore complex protein Nup98-Nup96	-4.14	2.05	2.66E-06	↓0h ↑24h	↓24h
Nasvi2EG011177t1	comp32070_c5_seq8	Unknown	-4.02	0.31	4.34E-06	↓0h,4h,24h	↑4h ↓4h
Nasvi2EG037320t1	comp33443_c0_seq6	nitric oxide synthase	3.16	0.62	1.15E-04	↑0h	↑0h,4h,24h
Nasvi2EG002548t1	comp38074_c0_seq1	Patched domain-containing protein 3	2.58	6.26	1.26E-41	↑0h,4h,24h	↑0h,4h,24h
Nasvi2EG003411t3	comp30646_c1_seq11	glycogenin-1	2.57	2.50	8.85E-06	↑0h,24h	↑0h,4h ↓0h
Nasvi2EG003549t1	comp21690_c2_seq1	distal antenna, protein	2.38	2.30	1.50E-05	↑0h	↑0h
Nasvi2EG003411t3	comp30646_c1_seq9	glycogenin-1	2.35	3.02	1.43E-06	↑0h,4h,24h	↑0h,4h ↓0h
Nasvi2EG001213t1	comp27928_c0_seq3	diacylglycerol kinase 1	-2.32	2.05	8.76E-05	↓0h	↓24h
Nasvi2EG033203t1	comp31299_c0_seq3	cGMP-dependent 3',5'-cyclic phospho-diesterase	2.23	2.83	4.67E-06	↑0h	↑0h
Nasvi2EG020539t3	comp34396_c0_seq1	STE20 serine/threonine-protein kinase	2.20	3.88	4.11E-09	↑0h,4h,24h	↑4h,24h ↓24h

Nasvi2EG033203t1	comp31299_c0_seq1	cGMP-dependent 3',5'-cyclic phosphodiesterase	2.18	3.39	9.29E-08	↑0h	↑0h
Nasvi2EG011177t1	comp32070_c5_seq2	Unknown	-2.14	3.44	5.63E-08	↓0h,4h	↑4h ↓4h
Nasvi2EG001000t1	comp34598_c0_seq50	Serine/threonine-protein kinase Doa	2.07	2.90	1.15E-05	↑0h	↑24h
Nasvi2EG030445t1	comp19061_c0_seq1	inosine-5'-monophosphate dehydrogenase	1.75	4.13	2.40E-07	↑0h,4h,24h	↑4h,24h
Nasvi2EG008896t1	comp33991_c0_seq4	inwardly rectifying potassium channel	1.66	3.77	2.42E-06	↑0h	↑4h
Nasvi2EG021226t1	comp31848_c2_seq1	Transcription factor HES-4	1.65	3.95	1.08E-06	↑0h	↑0h
Nasvi2EG037152t1	comp27324_c0_seq2	Ecdysone-induced protein 75B	1.55	6.26	1.97E-17	↑0h,4h,24h	↑0h,4h,24h
Nasvi2EG001311t2	comp20587_c0_seq1	Unknown	1.45	4.19	5.78E-06	↑0h,4h	↑0h,4h
Nasvi2EG025129t1	comp27762_c0_seq1	B-cell lymphoma 3-encoded protein protein	1.27	4.17	1.04E-04	↑0h,4h	↓0h
Nasvi2EG010980t1	comp30623_c0_seq1	Doublesex female isoform	1.14	6.94	9.76E-12	↑0h,4h	↑0h,4h
Nasvi2EG010980t1	comp30623_c0_seq2	Doublesex female isoform	1.02	6.09	3.49E-08	↑0h,4h	↑0h,4h
Nasvi2EG016792t2	comp32992_c1_seq1	Ankyrin repeat domain-containing protein	-1.00	5.42	5.55E-06	↓0h	↓4h
Nasvi2EG017709t1	comp34998_c2_seq37	potassium channel subfamily T member 1, putative	1.00	4.76	3.80E-04	↑0h	↑4h
Nasvi2EG001221t1	comp17334_c0_seq1	Unknown	0.98	5.82	4.07E-07	↑0h	↑0h
Nasvi2EG010485t2	comp33564_c2_seq2	Zinc finger protein	0.96	5.52	3.43E-06	↑0h	↑0h,4h
Nasvi2EG011761t1	comp36912_c0_seq1	Max-binding protein MNT	0.89	5.67	7.85E-06	↑0h	↑0h
Nasvi2EG005342t1	comp38059_c1_seq1	Raf protein serine/threonine-protein kinase dRAF-1	0.82	5.77	2.61E-05	↑0h	↑0h
Nasvi2EG003411t3	comp30646_c1_seq2	glycogenin-1	-0.76	5.37	5.14E-04	↓0h ↑24h	↑0h,4h ↓0h
Nasvi2EG011878t2	comp26678_c0_seq1	roadkill, protein	0.65	7.41	5.65E-05	↑0h	↑0h,4h
Nasvi2EG010931t1	comp17536_c0_seq2	mob as tumor suppressor	-0.65	6.60	1.66E-04	↓0h,4h	↓0h ↑4h,24h
Nasvi2EG009148t1	comp32819_c0_seq2	Unknown	0.64	6.35	4.05E-04	↑0h,4h	↓24h

Nasvi2EG002737t1	comp27959_c0_seq3	Choline-phosphate cytidylyltrans-ferase B	0.63	7.03	1.25E-04	↑0h	↑0h,4h,24h

c. regulatory genes that are differentially expressed in *N. girault*i, but that are not observed in the transcriptome of *N. vitripennis*

OGS2.0 ID	Transcript ID	Gene description	Log2 FC	Log2 CPM	*P*-value	DE pattern (NG)	DE pattern (NV)
Nasvi2EG015484t1	comp30877_c0_seq1	Segmentation protein cap'n'collar	11.71	2.51	1.55E-19	↑0h,4h,24h	n.a.
Nasvi2EG001072t1	comp569819_c0_seq1	Histone H2A.J	9.23	-1.06	1.20E-05	↑0h,4h	n.a.
Nasvi2EG012128t3	comp30957_c0_seq3	Ras-related protein Ral-A	-4.96	0.84	2.05E-08	↓0h	n.a.
Nasvi2EG001072t1	comp67785_c0_seq1	Histone H2A.J	4.17	0.55	7.24E-07	↑0h,4h	n.a.
Nasvi2EG001072t1	comp60918_c0_seq1	Histone H2A.J	2.97	0.36	3.83E-04	↑0h	n.a.
Nasvi2EG012128t3	comp28773_c0_seq1	Ras-related protein Ral-A	2.85	2.92	8.16E-09	↑0h	n.a.
Nasvi2EG001072t1	comp424968_c0_seq1	Histone H2A.J	2.75	1.31	2.89E-05	↑0h,4h,24h	n.a.
Nasvi2EG001072t1	comp42972_c0_seq1	Histone H2A.J	2.48	3.59	9.61E-11	↑0h,4h,24h	n.a.
Nasvi2EG001072t1	comp44221_c0_seq1	Histone H2A.J	-2.43	1.73	6.05E-04	↓0h,4h	n.a.
Nasvi2EG001072t1	comp42982_c0_seq1	Histone H2A.J	2.35	3.79	9.94E-11	↑0h,4h	n.a.
Nasvi2EG001072t1	comp45800_c0_seq1	Histone H2A.J	2.34	3.78	1.22E-10	↑0h,4h	n.a.
Nasvi2EG012917t4	comp34507_c0_seq24	diaphanous protein	-1.73	2.74	5.20E-04	↓0h	n.a.
Nasvi2EG005471t1	comp17653_c0_seq1	Membrane-bound O-acyltransferase domain-containing protein 2	-1.56	3.47	5.95E-05	↓0h	n.a.
Nasvi2EG001072t1	comp41580_c0_seq1	Histone H2A.J	1.47	4.63	2.13E-07	↑0h,4h,24h	n.a.
Nasvi2EG001072t1	comp46753_c0_seq1	Histone H2A.J	1.37	4.86	6.06E-07	↑0h,4h	n.a.
Nasvi2EG001072t1	comp42743_c0_seq1	Histone H2A.J	-1.04	4.37	6.21E-04	↓0h,4h	n.a.

Supplementary table 5: Differential expression of known memory genes after conditioning. (a) The gene expression patterns of 44 genes that are known from literature to be involved in (long-term) memory formation were analysed for both species (Keene & Waddell, 2007) [(a)], (Alberini, 2009) [(b)], (Pagani *et al*., 2009) [(c)], (Hirano *et al*., 2013) [(d)], (Banerjee *et al*., 2010) [(e)], (Mery *et al*., 2007a) [(f)], (Copf *et al*., 2011) [(g)], Shuai *et al*., 2010 [(h)]). The *N. vitripennis* (OGS2.0) homolog of each memory gene was determined by aligning the *D. melanogaster* gene sequence to the *N. vitripennis* genome (Nvit 2.0) using blastn or blastp. The number of transcripts that was observed in the transcriptome is given for each gene. Also, the differential expression pattern after conditioning is given, compared to naïve expression levels (not DE = not differentially expressed, n.a. = not observed in the transcriptome, ↑ = upregulated, ↓ = downregulated). (b) 18 genes were differentially expressed in *N. vitripennis* (NV) and/or *N. giraulti* (NG) after conditioning. For each DE transcript, the log2-CPM (count per million, a measure for abundance of the transcript), log2-fold change (FC), and the level of significance (*P*) of the DE transcript are given for all time points after conditioning'. This information is not given for transcripts of the same gene that are not differentially expressed.

a. Genes known to be involved in (long-term) memory formation

Gene/ transgene	Gene description	OGS2.0 ID	# isoforms NV	# isoforms NG	DE pattern NV	DE pattern NG
aPKC [(a)]	atypical protein kinase C	Nasvi2EG010162	5	3	↑4h,24h	not DE
CAMKII [(b)]	calcium/calmodulin dependent kinase II	Nasvi2EG036901	16	15	not DE	↑0h,4h,24h
Corkscrew [(c)]	SHP2 phosphatase	Nasvi2EG012844	2	2	↑4h	not DE
Crammer [(b)]	trans-inhibitor of cathepsins	Nasvi2EG034880	2	2	↓4h,24h	↓4h
dCREB2 [(b)]	cAMP response element binding protein	Nasvi2EG004930	11	9	not DE	↓4h,24h
dDA1 [(a)]	dopamine receptor	Nasvi2EG001652	8	5	↓4h	↓4h
Dunce [(a)]	cAMP phosphodiesterase	Nasvi2EG011498	10	7	↑4h	↓4h
eIF-5C [(a)]	elongation initiation factor-5C	Nasvi2EG002237	1	1	↑4h	↑24h
fasciclin II [(a)]	neural cell adhesion molecule	Nasvi2EG000400	6	6	↑0h,4h,24h	not DE
FMRP [(e)]	fragile X mental retardation protein	Nasvi2EG013697	8	9	↑0h	not DE
foraging [(f)]	cGMP dependent protein kinase	Nasvi2EG004831	2	2	not DE	↓24h
G-sα60A [(a)]	stimulatory G protein	Nasvi2EG010676	2	1	↑24h	not DE
hop [(g)]	Janus tyrosine kinase	Nasvi2EG014300	2	2	↓0h	not DE
linotte [(a)]	RYK tyrosine kinase receptor	Nasvi2EG007570	2	1	↓0h	not DE
NF1 [(a)]	ras GTPase activating protein	Nasvi2EG011800	4	7	not DE	↑24h
NMDAR2 [(a)]	glutamate receptor subunit	Nasvi2EG012232	6	5	not DE	↓4h

pumilio [a]	RNA binding protein	Nasvi2EG006115	5	4	↓4h	not DE
staufen [a]	mRNA translocation	Nasvi2EG013994	3	2	↓0h	not DE
armitage [a]	SDE3 helicase	Nasvi2EG003373	2	1	not DE	not DE
C/EBP [b]	CCAAT-enhancer binding protein	Nasvi2EG010468	0	1	n.a.	not DE
CBP/p300 [b]	CREB binding protein/ p300	Nasvi2EG004485	15	5	not DE	not DE
CRTC [d]	cAMP-regulated transcriptional cofactor	Nasvi2EG020434	8	15	not DE	not DE
damb [a]	dopamine receptor	Nasvi2EG017876	2	6	not DE	not DE
DC0 [a]	PKA catalytic subunit	Nasvi2EG014242	1	1	not DE	not DE
ERK-A [b]	extracellular signal-related kinase	Nasvi2EG002789	1	2	not DE	not DE
latheo [a]	origin recognition complex	Nasvi2EG008683	1	1	not DE	not DE
leonardo [a]	14-3-3 zeta protein family	Nasvi2EG004640	1	1	not DE	not DE
nalyot [a]	adf1 transcription factor	Nasvi2EG008127	0	0	n.a.	n.a.
nebula [a]	calcineurin inhibitor	Nasvi2EG014610	5	5	not DE	not DE
NMDAR1 [a]	glutamate receptor subunit	Nasvi2EG000002	4	4	not DE	not DE
Notch [a]	Notch receptor	Nasvi2EG009801	3	5	not DE	not DE
oamb [a]	octopamine receptor	Nasvi2EG003445	1	4	not DE	not DE
oskar [a]	translation control	Nasvi2EG013571	1	1	not DE	not DE
p38 MAPK [b]	mitogen activated protein kinase	Nasvi2EG000447	8	8	not DE	not DE
PKA-RI [a]	cAMP dependent protein kinase 1	Nasvi2EG005068	2	2	not DE	not DE
Rac1 [h]	Rho family GTPase	Nasvi2EG000948	1	1	not DE	not DE
radish [a]	Rap GTPase activating protein	Nasvi2EG000376	2	2	not DE	not DE
rutabaga [a]	adenylyl cyclase (type 1)	Nasvi2EG013346	2	5	not DE	not DE
S6KII [a]	ribosomal S6 kinase (RSK)	Nasvi2EG013142	1	1	not DE	not DE
Stat92E [g]	STAT transcription factor	Nasvi2EG007751	2	2	not DE	not DE
synapsin [a]	presynaptic vesicle protein	Nasvi2EG017400	4	4	not DE	not DE
Tbh [a]	tyramine beta- hydroxilase	Nasvi2EG012708	1	1	not DE	not DE
tequila [a]	neurotrypsin	Nasvi2EG010908	3	4	not DE	not DE
volado [a]	α-integrin	Nasvi2EG012335	1	1	not DE	not DE

b. Differentially expressed memory genes

Gene/	*N. vitripennis*	NV	NV 0h		NV 4h		NV 24h	
transgene	Transcript ID	CPM	FC	*P*	FC	*P*	FC	*P*
aPKC	comp35553_c1_seq4	3,88	0,95	3,17E-03	1,07	9,73E-04	0,99	2,09E-03
corkscrew	comp32621_c0_seq1	7,17	0,27	2,96E-02	0,49	5,03E-05	0,23	6,05E-02
fasciclin II	comp34025_c1_seq3	5,59	0,63	1,03E-04	0,68	2,39E-05	0,62	1,15E-04
FMRP	comp34913_c0_seq6	5,12	0,67	6,65E-04	0,13	5,12E-01	0,06	7,57E-01
G-sα60A	comp35901_c1_seq2	0,05	-6,88	1,83E-03	1,87	3,97E-02	3,62	2,05E-04
hop	comp25200_c0_seq2	2,3	-2,92	7,40E-07	-1,06	5,52E-02	-1,13	3,79E-02
linotte	comp33720_c1_seq2	1,24	-5,95	2,78E-07	0,82	3,44E-01	1,25	8,49E-02
pumilio	comp38467_c2_seq2	6,06	0,1	4,94E-01	-0,6	3,05E-05	-0,25	7,46E-02
staufen	comp32934_c0_seq2	4,19	-1,51	3,38E-07	-0,44	1,23E-01	-0,73	1,10E-02
dunce	comp32729_c1_seq5	5,71	0,23	1,39E-01	0,64	3,83E-05	0,38	1,47E-02
	comp34929_c0_seq3	0,55	2,1	8,10E-03	3,49	1,53E-05	1,82	2,04E-02
crammer	comp32552_c0_seq1	8,44	-0,17	9,97E-02	-0,81	2,09E-15	-0,57	1,72E-08
dDA1	comp37078_c0_seq2	2,89	-0,18	6,69E-01	-1,38	1,79E-03	0,41	3,19E-01
eIF-5C	comp40716_c0_seq1	7,66	-0,01	9,34E-01	0,4	3,71E-04	0,03	8,00E-01

Gene/	*N. girault*	NG	NG 0h		NG 4h		NG 24h	
Transgene	Transcript ID	CPM	FC	*P*	FC	*P*	FC	*P*
CAMKII	comp34936_c1_seq35	-0,78	7,56	4,91E-04	9,81	1,09E-06	7,62	3,86E-04
dCREB2	comp32151_c0_seq1	2,27	-1,15	1,60E-01	-3,67	1,24E-05	-6,19	8,08E-14
foraging	comp22710_c0_seq2	4,03	-0,56	8,65E-02	-0,33	3,16E-01	-1,05	1,72E-03
NF1	comp33011_c0_seq3	3,83	0,22	5,45E-01	-0,33	3,65E-01	1,78	7,03E-06
	comp33011_c0_seq5	3,03	1,28	4,62E-03	0,65	1,39E-01	2,15	2,41E-06
	comp33011_c0_seq6	5,08	0,13	5,91E-01	0,37	1,33E-01	1,99	7,32E-15
NMDAR2	comp30975_c0_seq3	0,74	0,55	4,51E-01	-2,31	9,22E-04	0,94	1,93E-01
dunce	comp34739_c4_seq3	5,36	-0,33	1,21E-01	-0,76	4,53E-04	-0,64	2,93E-03
crammer	comp21791_c0_seq1	7,15	-0,33	4,23E-02	-0,71	1,48E-05	-0,41	1,09E-02
dDA1	comp32875_c0_seq2	2,44	-0,1	8,47E-01	-4,38	8,59E-12	-0,34	5,34E-01

Supplementary table 6: Enriched GO-terms in antisense transcripts. GO-enrichment analyses of antisense transcripts for *N. vitripennis* and *N. girault*i. The two categories of antisense transcripts were analysed separately: 'antisense2protein' transcripts that have a hit to a *N. vitripennis* protein and 'antisense2sense' transcripts that have a hit to a sense transcript only. The Blast2go GUI (Fisher's exact test, $P < 0.05$) was used to visualize expression patterns of functional clusters of genes. Generic GOSlim categories were used to limit the number of GO-term categories and the most specific terms were determined using Blast2Go. The enriched GO-terms (GO-ID and Term) that are up- or down-regulated, the category of the GO term (C = cellular component, F = molecular function, P = biological process), their *P*-value and the number of genes in the test-set and the reference-set are given.

***N. vitripennis* antisense2protein transcripts**

GO-ID	Category	Term	*P*-Value	#Test	#Ref
GO:0005929	C	cilium	6,12E-03	3	13
GO:0006629	P	lipid metabolic process	8,13E-03	8	112
GO:0005578	C	proteinaceous extracellular matrix	2,29E-02	2	8
GO:0007010	P	cytoskeleton organization	2,96E-02	11	229
GO:0004518	F	nuclease activity	3,48E-02	3	27
GO:0005777	C	peroxisome	3,79E-02	2	11
GO:0006259	P	DNA metabolic process	3,98E-02	7	125

***N. vitripennis* antisense2sense transcripts**

GO-ID	Category	Term	*P*-Value	#Test	#Ref
GO:0005929	C	cilium	1,85E-02	4	12
GO:0005578	C	proteinaceous extracellular matrix	2,47E-02	3	7
GO:0007610	P	behavior	3,33E-02	24	220
GO:0005886	C	plasma membrane	4,98E-02	34	350

***N. girault*i antisense2protein transcripts**

GO-ID	Category	Term	*P*-Value	#Test	#Ref
GO:0003824	F	catalytic activity	2,31E-02	11	2348
GO:0097159	F	organic cyclic compound binding	2,50E-02	8	1403
GO:1901363	F	heterocyclic compound binding	2,50E-02	8	1403
GO:0010467	P	gene expression	4,03E-02	3	274
GO:0005694	C	chromosome	4,74E-02	2	123

***N. girault*i antisense2sense transcripts**

GO-ID	Category	Term	*P*-Value	#Test	#Ref
GO:0007267	P	cell-cell signaling	4,18E-03	19	216
GO:0009628	P	response to abiotic stimulus	1,35E-02	11	112

GO:0005102	F	receptor binding	1,55E-02	7	56
GO:0006996	P	organelle organization	1,90E-02	32	495
GO:0040007	P	growth	2,17E-02	12	137
GO:0009653	P	anatomical structure morphogenesis	3,46E-02	35	580
GO:0005829	C	cytosol	4,16E-02	11	135
GO:0005794	C	Golgi apparatus	4,64E-02	9	104
GO:0044403	P	symbiosis, encompassing mutualism through parasitism	4,88E-02	3	17

Chapter 8

General discussion

Katja M. Hoedjes

VARIATION IN LEARNING AND MEMORY FORMATION: THE GENUS *NASONIA* AS A NEW MODEL FOR MULTIDISCIPLINARY STUDIES

Learning and memory formation are cognitive traits. Cognition refers to processes by which animals respond to information from their environment, which includes acquisition, processing, storage and use of information (Shettleworth, 2010). Next to learning and memory formation, processes such as perception, attention, use of language and decision-making are also considered cognitive processes (Shettleworth, 2010). The research field of cognitive ecology studies the ecology and evolution of cognition with the aim of understanding differences in cognition between animal species (Dukas & Ratcliffe, 2009). Learning and memory formation have an important role in animal cognition and behaviour, and the ability to learn and form memory is widespread among the Animal Kingdom. Species-specific differences in learning and memory are observed. This variation reflects differences in ecology: species are thought to have evolved learning abilities and memory dynamics that suit their needs (Dukas & Ratcliffe, 2009; Shettleworth, 2010).

The aim of this thesis is to elucidate both ecological factors that can explain species-specific differences in learning and memory formation, as well as the genetic mechanisms that underlie variation in these traits. In this thesis, I argue that parasitic wasps are excellent model organisms for multidisciplinary studies on natural variation in learning and memory formation for a number of reasons: (1) substantial differences in memory dynamics have been observed among closely related species, (2) there are clear-cut differences in the ecology of closely related species, which allows studies on the effect of ecological factors on learning and memory, and (3) recent advances in genetic and genomics tools now allow studies on genetic aspects of variation in memory formation in parasitic wasp species **(Chapter 2)**.

I present parasitic wasps of the genus *Nasonia* as a novel model for multidisciplinary studies on interspecific variation in memory formation. A prerequisite for studies on learning and memory formation in any animal species is the availability of methods for both conditioning and for testing memory retention. A high-throughput method was developed in which female wasps associated an odour with the reward of a host. This method can be used for all *Nasonia* species, which is essential for comparative studies, and allows conditioning of large numbers of wasps. A high-throughput device for testing memory retention, a T-maze olfactometer, is used to test large numbers of wasps **(Chapter 3)**. Significant differences in olfactory/oviposition learning were detected between the homozygous and sequenced strains of *N. vitripennis* (AsymCx), *N. longicornis* (IV7(U)) and *N. giraulti* (RV2x(U)). The differences between *N. vitripennis* and *N. giraulti* were most pronounced and a further characterization of the memory dynamics of these two species was carried out **(Chapter 4)**. The two species differ in their memory formation after a single

conditioning trial: *N. vitripennis* will form transcription-dependent long-term memory (LTM), which lasts more than 6 days, whereas *N. giraulti* likely forms anaesthesia-resistant memory (ARM) and loses its memory after 1 to 2 days after conditioning. *Nasonia giraulti* will only form long-lasting memory (lasting >5 days) after multiple conditioning trials, which are spaced in time. The results from these chapters demonstrate that memory dynamics of *N. vitripennis* (AsymCx) are comparable to those of a German field population of *N. vitripennis* (Schurmann *et al*., 2009; Schurmann *et al*., 2012). The use of homozygous and sequenced strains of *Nasonia* species has, however, important advantages for genetic studies. In the remaining chapters of this thesis, I have studied ecological factors **(Chapters 4 & 5)** and genetic factors **(Chapters 6 & 7)** that are responsible for this difference in LTM formation between *N. vitripennis* and *N. giraulti*.

Variation in learning and memory formation in the genus *Nasonia* is, however, not restricted to the strains of *N. vitripennis* and *N. giraulti* that were used throughout this thesis. *Nasonia longicornis* was found to form a long-lasting memory (lasting >5 days) after a single conditioning trial, similar to *N. vitripennis*. The observed performance index (PI) was, however, much lower compared to *N. vitripennis*. A weaker behavioural response to learned information could have large implications for host finding behaviour (Papaj & Vet, 1990; Raine & Chittka, 2008) and it presents another interesting variation in memory formation in the genus *Nasonia*. In addition to inter-specific variation in learning and memory formation, also intra-specific variation may be present in the genus *Nasonia*. Intra-specific variation in learning abilities, due to variation in the ecology of subpopulations, has been observed in bees, birds, parasitic wasps, squirrels and snails (Ings *et al*., 2009; Roth *et al*., 2012; Thiel *et al*., 2013; Bruck & Mateo, 2010; Orr *et al*., 2009). Studies on German populations of *N. vitripennis* have suggested that two ecotypes exist that utilize either fly pupae on carrion or in birds' nests, which are two distinctly different ecological niches (Schröder & Abraham, 1997; Schröder, 1999). Populations of *N. vitripennis* have, furthermore, been collected from all over the world and are being maintained by different research groups of the *Nasonia* community, providing another source of potential variation in learning and memory formation. For example, different populations of *N. vitripennis* have been collected from different locations along a latitudinal cline in Europe (Paolucci *et al*., 2013). A genetically diverse population of *N. vitripennis*, collected in the Netherlands, was recently constructed and its genetic composition was characterized (van de Zande *et al*., 2014). These resources provide additional possibilities to study ultimate and proximate factors involved in inter- or intraspecific variation in learning and memory formation.

ULTIMATE FACTORS RESPONSIBLE FOR NATURAL VARIATION IN LEARNING AND MEMORY FORMATION

Variation in learning abilities and memory formation are believed to reflect species- or population-specific differences in ecological factors. For many species it has been hypothesized why differences in memory are adaptive, e.g. food-caching birds require a better spatial memory than birds that do not cache food (Roth *et al*., 2012; Clayton & Dickinson, 1998), paper wasps that nest in groups have an increased ability to learn faces compared to solitary wasps, because it allows them to determine a dominance hierarchy (Sheehan & Tibbetts, 2011). Parasitic wasps can learn environmental cues that guide them towards food or hosts to lay their eggs in, so-called oviposition learning. Ecological factors such as the variability of the environment, the value of reward (i.e. the quality of the host for offspring development), and reliability of the learned association have all been proposed to affect learning and memory formation **(Chapter 2)**. In this thesis, I focus on factors that were hypothesized to affect LTM formation in oviposition learning of parasitic wasps by changing the rewarding stimulus (i.e. the host) during conditioning.

First, I investigated the role of two aspects of the rewarding host stimulus, i.e. host feeding and oviposition, in memory formation **(Chapter 4)**. Parasitic wasps are known to experience multiple stimuli during oviposition, i.e. contact with host traces or haemolymph, host feeding and oviposition. Oviposition was observed to induce increased memory retention, compared to contact with host traces alone, in the parasitic wasp species *Microplitis croceipes* (Takasu & Lewis, 2003). For this reason, I hypothesized that oviposition was a more rewarding stimulus than host feeding alone. However, memory retention of both *N. vitripennis* and *N. giraulti* was not affected by the rewarding host stimulus. Second, I investigated the effect of three host species, with different qualities as a host, on memory retention of *N. vitripennis* and *N. giraulti* **(Chapter 5)**. Reward quality had already been shown to be important for memory dynamics in several animal species. For example, a reward of higher quality or intensity resulted in increased memory retention in male rats that received different intensities of sexual stimulation (Camacho *et al*., 2009) and in several insect species that received food of varying nutritional value (Wäckers *et al*., 2006; Burke & Waddell, 2011). A recent study on two parasitic wasp species, *Cotesia glomerata* and *Trichogramma evanescens*, had shown that LTM was formed after conditioning on one host species, whereas only ARM was formed after conditioning on another host species, with a lower quality (Kruidhof *et al*., 2012). A combination of both reward quality (of the host) and reliability of the learned association was hypothesized to be responsible for these differences in memory dynamics. I observed that the three hosts that I used in **Chapter 5** had profound differences in host quality, but this did not affect memory retention in either *N. vitripennis* or *N. giraulti*.

The results from both experiments demonstrate that *N. vitripennis* and *N. giraulti* respond differently to variation in the rewarding stimulus than a number of other parasitic wasp species (as discussed in **Chapters 4 & 5**). Variation in LTM formation between closely related parasitic wasp species has evolved independently in multiple parasitic wasp lineages, e.g. in the genus *Nasonia* (Hymenoptera: Pteromalidae) and the genus *Cotesia* (Hymenoptera: Braconidae) (Smid *et al.*, 2007; van den Berg *et al.*, 2011; **Chapters 3 & 4**). Species of these genera have profoundly different ecologies and have co-evolved with different host species. Consequently, different parasitic wasp species perceive and respond to different host cues, which constitute the rewarding stimulus in oviposition learning, and underlying mechanisms are likely different between species (Wajnberg & Colazza, 2013). In addition, learning and memory formation may play a different role in foraging behaviour of different species. As a result, ecological factors that control LTM formation can be different between species of distant families, even though comparable variation in LTM formation is observed in these distant families. Some parasitic wasp species may have evolved mechanisms that allow them to adapt their memory dynamics to host quality, whereas these mechanisms may not be beneficial for other species.

PROXIMATE FACTORS RESPONSIBLE FOR NATURAL VARIATION IN LEARNING AND MEMORY FORMATION

The process of long-term memory formation involves perception of sensory information by an animal, the induction of activity in neurons that encode and integrate these stimuli and structural changes to specific neurons that represent a memory. The result is a change in response of the nervous system to specific sensory information, which can be observed as an altered behavioural response (Davis, 2011). Manipulation of specific neurons and a large number of different genes can affect LTM formation (Davis, 2011; Müller, 2012), which indicates that the number of mechanisms that can be involved in variation in LTM formation is potentially large. Which mechanisms are responsible for natural variation in LTM formation is, however, poorly understood.

A unique opportunity for genetic studies in the genus *Nasonia* is provided by the possibility to interbreed the closely related species. This characteristic makes it possible to backcross a phenotypic trait of one species into the genetic background of another species (Werren *et al.*, 2010; Werren & Loehlin, 2009). I have backcrossed the memory phenotype of *N. giraulti*, i.e. no LTM formation after a single conditioning trial, into the genetic background of *N. vitripennis* and I identified two quantitative trait loci (QTLs) that reduce long-lasting memory retention (**Chapter 6**). These results are an important step towards the identification of genomic factors that are responsible for the difference in

LTM formation between *N. vitripennis* and *N. giraulti*. Fine-scale mapping of the memory retention QTLs is a logical next step to determine these regulatory factors. In addition to the introgression study, I compared gene expression levels in the heads of *N. vitripennis* and *N. giraulti* after conditioning (**Chapter 7**). This analysis revealed substantial differences in differential gene expression between the two species. Various genes, with a known role in LTM formation, were uniquely differentially expressed in *N. vitripennis*. These genes may be involved in the ongoing formation of LTM in this species. A number of other genes, also with a known role in LTM formation, were uniquely differentially expressed in *N. giraulti*. These genes may be part of an LTM inhibitory process in this species. In addition, the results point toward the involvement of epigenetic regulatory mechanisms. Genes identified in **Chapter 7** are promising candidates for further research on variation in LTM formation in the genus *Nasonia*.

The combination of two complementary and innovative approaches to determine the genetic basis of variation in LTM formation is the main strength of this thesis and has increased our understanding of the regulation of LTM formation in the genus *Nasonia*. Future studies are needed to confirm genes involved in variation in LTM formation and can greatly benefit from tools available to manipulate genes in *Nasonia* species, including RNAi (Lynch & Desplan, 2006; Werren *et al*., 2009). Techniques to transform *Nasonia* species are currently being developed and are expected to become available in the near future (J.H. Werren, personal communication). In addition, spatial expression patterns of identified candidate genes can and should be studied. The brain is a highly heterogeneous tissue and processes in small numbers of neurons from specific brain regions can play an important role in (long-term) memory formation (Davis, 2011; Davis & Giurfa, 2012; Galizia & Menzel, 2000; Hourcade *et al*., 2009; Müller, 2012). To interpret differences in genomes or brain-wide gene expression between *N. vitripennis* and *N. giraulti*, it is necessary to know where in the brains these differences are expressed. Detailed information on brain morphology and neural networks of these species are instrumental for such studies. Currently, brain atlases of the neuropils of both *Nasonia* species are being constructed and also the neural networks of octopaminergic and dopaminergic neurons, two neurotransmitters which are important for memory formation (e.g. Burke *et al*., 2012), have been mapped (J. Groothuis, A. Haverkamp & H.M. Smid, unpublished results). This information is valuable for studies on spatial expression of genes, but may also reveal structural differences between brains of *N. vitripennis* and *N. giraulti* that are potentially associated with their difference in memory formation.

An interesting question that remains is whether mechanisms responsible for variation in learning and memory are conserved among distant animal phyla. Results from studies on *Nasonia* need to be compared to results from other animal species to answer

this question, e.g. to other parasitic wasp species that differ in LTM formation, but also to distant animal phyla in which variation in memory formation is observed between closely related species. Next-generation sequencing techniques allow gene expression profiling in non-model species that demonstrate variation in learning and memory. Pravosudov *et al*. (2013) compared two populations of chickadees that differ in spatial memory performance, whereas Armbrecht *et al*. (2014) compared control mice and mice with impaired memory performance. These two studies reported variation in a number of genes that I identified in **Chapter 7** as well. An ongoing transcriptome analysis of brains of *Cotesia glomerata* and *C. rubecula*, before and after conditioning (J.J.F.A. van Vugt *et al*., unpublished results), will provide an additional source of information that can be compared to the *Nasonia* species. An advantage of this study is that it is very similar with regard to the observed variation in LTM and experimental set-up compared to **Chapter 7**.

Introgression or QTL mapping can be used to identify genomic regions or genes responsible for variation in learning and memory. Inter-specific introgression of differences in LTM formation is a unique opportunity for the genus *Nasonia* (**Chapter 6**), but intraspecific variation in LTM formation could be studied using introgression as well, both in the genus *Nasonia* and potentially in other parasitic wasp species or other insect species as well. The ability to create homozygous lines, which can be genetically characterized, is a prerequisite for this approach. No other parasitic wasp species currently meet these prerequisites, but species from genera with known variation in memory formation and from which isofemale lines can be generated, e.g. *Trichogramma evanescens* (Huigens *et al*., 2009) or *Leptopilina boulardi* (Carton *et al*., 1989; Kaiser *et al*., 2009) are potential candidate species for future introgression studies. Experimental evolution is another option for identifying genomic factors that are responsible for variation in learning and memory. Experimental evolution experiments, which resulted in intraspecific variation in LTM formation between the selected strains, have been reported in *Drosophila melanogaster* (Mery & Kawecki, 2002) and the parasitic wasp species *Cotesia glomerata* (van den Berg *et al*., 2011). However, neither of these studies investigated genomic differences between selected strains. A genetically diverse population of *N. vitripennis*, which was recently constructed and genetically characterized (van de Zande *et al*., 2013) also provides opportunities for experimental evolution.

NATURAL VARIATION IN LEARNING AND MEMORY: CORRELATED TRAITS

Understanding both ultimate and proximate factors that are responsible for variation in learning and memory will increase our knowledge on how and why this variation has evolved. This variation is considered an adaptation to species-specific differences in

ecology, but this hypothesis has not been tested. Not only ultimate and proximate factors that regulate learning and memory are important in this regard, but also correlated traits need to be considered to understand the effects on the fitness of an animal.

Traits can be correlated if genetic regulatory pathways between two traits are shared; the consequence is that genetic changes that result in variation in one trait will also affect other traits (Schlichting & Pigliucci, 1998). As a result, correlated traits may not evolve independently from each other (Beldade *et al*., 2002; Allen *et al*., 2008; Ellers & Driessen, 2011). Variation in a specific memory trait can affect other memory related traits, as was observed in a number of studies in insects. Honeybees with higher olfactory memory performance were observed to be better at visual learning as well (Brandes & Menzel, 1990). Increased memory performance of fruit flies in an assay that paired a flavour of the oviposition medium with a bitter taste (Mery & Kawecki, 2002) generalized to olfactory-aversive memory performance (using mechanical shocks) (Mery *et al*., 2007b). These correlated memory traits suggest a genetic basis for variation in memory formation that is independent from specific sensory stimuli that were experienced during conditioning. This thesis focused on LTM formation after olfactory/oviposition learning, but the genus *Nasonia* offers ample opportunities to study correlated memory traits as well. Memory performance of the introgression lines with reduced memory retention after olfactory-oviposition learning (**Chapter 6**), can be tested using a visual conditioning procedure (Oliai & King, 2000), or using a different rewarding stimulus such as sucrose or honey (Oliai & King, 2000) or a female to mate with, in case of male wasps (Baeder & King, 2004).

Correlated traits can also be unrelated to learning and memory formation. The gene *foraging*, encoding cGMP-dependent kinase, has been observed to affect STM and LTM formation (Mery *et al*., 2007a), but also foraging behaviour (Osborne *et al*., 1997), the extent to which personal or public information is used (Foucaud *et al*., 2013), phototaxis behaviour (Ben-Shahar *et al*., 2003) and a number of other traits. Increased learning or memory performance can be negatively correlated with other traits. An example is increased LTM performance in *D. melanogaster*, which is correlated with decreased larval competitive ability (Mery & Kawecki, 2003) and longevity (Burger *et al*., 2008). In the butterfly *Pieris rapae*, higher learning ability is correlated with decreased fecundity (Snell-Rood *et al*., 2011). These trade-offs may reflect resource allocation towards neural structures necessary for the increased memory performance at the cost of other life-history traits, which could have a negative impact on fitness.

These studies show that the costs and benefits of variation in learning and memory go beyond just these traits, and can include various other (life-history) traits that are correlated. Correlated traits need to be considered to fully understand the evolution of variation in learning and memory.

BEYOND LONG-TERM MEMORY FORMATION

This thesis has focused on variation in the number of trials required to form LTM between animal species. Memory was investigated in wasps that received a single, or two similar, learning experiences. However, many animals will likely perceive multiple information sources about their environment and may form more than a single memory during their lifetime. In addition, new information can conflict with learned information. This aspect was already discussed briefly in two chapters of this thesis.

Variation in the rate of LTM consolidation was discussed in **Chapter 4**. *Nasonia vitripennis* was observed to express LTM 4 days after a single conditioning trial, which was shown using transcription- and translation-inhibitors, whereas a number of other parasitic wasp species have been shown to consolidate LTM much faster, in the range of hours up to a day after conditioning (Smid *et al*., 2007; Huigens *et al*., 2009; Collatz *et al*., 2006). This variation has been hypothesized to determine the time window of an animal to re-evaluate learned information before it is consolidated as LTM (van den Berg *et al*., 2011; Schurmann *et al*., 2012; **Chapter 4**). The sensitivity of different types of memory to disturbances needs to be assessed to test this hypothesis; disturbances can be conflicting information or unrewarding experiences (Schurmann *et al*., 2012). I briefly touch on this topic in **Chapter 6**, in which I investigate the effect of testing *N. vitripennis* and *N. giraulti* multiple times, so-called extinction trials, on the observed memory performance. These experiences can be regarded as unrewarding experiences, because the wasps detect the learned odour, but they do not experience contact with a host afterwards. Extinction trials can induce reconsolidation of memory or extinction depending on the number of trials in the fruit fly and the honeybee (Stollhoff *et al*., 2005; Lagasse *et al*., 2012). In addition, the number of trials that result in extinction of memory differs among species (Kaiser *et al*., 2003; Stollhoff *et al*., 2005; Lagasse *et al*., 2009). I performed one extinction trial after 24 hours, when ARM is present in both *Nasonia* species, and another after 72 hours, when *N. vitripennis* has formed a second type of ARM. No effect on memory retention was observed (**Chapter 6**). This experiment was not set-up for testing effects of unrewarding experiences on memory retention extensively, as was the case in the studies mentioned, but it does provide first information of this aspect of memory dynamics in the genus *Nasonia*.

Both the rate of LTM consolidation and memory extinction can be considered as processes that have a role in regulating memory of animals when they experience information that conflicts with learned information. The probability of experiencing conflicting information likely depends on ecological factors, such as variability of the environment or the number of lifetime learning experiences (see **Chapter 2** for a discussion of these factors). For this reason, substantial variation in memory processes that deal with conflicting information is expected, especially among parasitic wasp species which

have highly diverse ecologies. Retroactive interference, i.e. when a new memory inhibits the retrieval of older memories (Bouton, 1993), is considered an important memory process that can help animals deal with conflicting information (Anderson, 2003; Reaume *et al*., 2011). Variation in this trait has been reported between and within insect species (Cheng & Wignall, 2006; Reaume *et al*., 2011). LTM consolidation rate, memory extinction and retroactive interference are promising memory processes to further investigate in parasitic wasp species. Variation in these processes may indicate how species deal with multiple, potentially conflicting, sources of information.

EPILOGUE

To remember or to forget, that's the question that was studied in this thesis. I studied this question from both an ecological as well as a genetic point of view by comparing two closely related species of *Nasonia* parasitic wasps that differ in memory retention.

Parasitic wasp species have already been studied for decades to unravel ecological factors that are responsible for observed differences in memory dynamics. Results from this thesis on *N. vitripennis* and *N. giraulti* provide new insights in how ecological factors may affect LTM formation. These results are not only valuable for the research field of cognitive ecology. Behaviour of parasitic wasps has also been studied, because of the application of parasitic wasps in biological control programs. Learning has an important role in host finding and oviposition behaviour of many species, and knowledge on factors that regulate this behaviour may, therefore, improve their success as biological control agents (Tumlinson *et al*., 1993).

Valuable insights were also obtained from the genetic studies on the genus *Nasonia*. This thesis provides a solid basis for the identification of genomic factors that are responsible for the difference in LTM formation between *N. vitripennis* and *N. giraulti*. In addition, the consequences of these genomic factors on gene expression patterns in the brains of these two species have been characterized using a transcriptome analysis. The genetic basis of learning and memory formation are highly conserved among distant animal species and insights from this thesis are likely applicable to other animal species and humans as well. My results are, therefore, interesting for the field of medical neurosciences, which aims to understand and treat neurodegenerative diseases or to improve memory in animals or humans. A substantial number of genes that have been identified in this thesis have a known role in pathology or are already being considered for potential treatments. Information on how these genes are involved in naturally occurring differences in LTM formation can provide novel insights for medical applications.

I combine ecology and genetics in this thesis, which is important because the

interaction between genetic and environmental factors is responsible for the evolution of variation in learning and memory. *Nasonia* parasitic wasp species offer unrivalled opportunities for multidisciplinary studies on variation in LTM formation, as shown in this thesis. I have exploited these excellent opportunities to investigate ecological and genetic factors. This thesis demonstrates how state-of-the-art techniques provide opportunities for genetic studies in non-model species. The multidisciplinary approach, as I demonstrate in this thesis, can be used in other animal species as well to study variation in learning and memory formation, but also to study variation in other cognitive traits, behaviour or completely different traits. Altogether, this thesis is a successful example of how genetics and ecology can be connected to achieve understanding of differences in learning and memory formation.

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Summary

The ability to learn and form memory has been demonstrated in various animal species, ranging from relatively simple invertebrates, such as snails and insects, to more complex vertebrate species, including birds and mammals. The opportunity to acquire new skills or to adapt behaviour through learning is an obvious benefit. However, memory formation is also costly: it can be maladaptive when unreliable associations are formed and the process of memory formation can be energetically costly. The balance between costs and benefits determines if learning and memory formation are beneficial to an animal or not. Variation in learning abilities and memory formation between species is thought to reflect species-specific differences in ecology.

This thesis focused on variation in the number of trials required to form long-term memory (LTM). LTM is considered the most stable and durable type of memory, but also the most costly, because it requires protein synthesis. Many animal species require multiple learning experiences, which are spaced in time, to form LTM. This allows re-evaluation of information before an animal invests in costly LTM. There is, however, variation in the number of trials that animal species require to induce LTM formation. A number of insect species, including a number of parasitic wasp species, form LTM after only a single learning experience. Parasitic wasps can learn odours that guide them towards suitable hosts for their offspring, so-called oviposition learning. Substantial differences in LTM formation are observed among closely related species of parasitic wasps, which provides excellent opportunities for comparative studies. Both ecological and genetic factors involved in variation in LTM formation have been studied in this project. A multidisciplinary approach is essential to understand the evolution of variation in LTM formation, because the interaction between genes and environment shapes learning and memory formation.

LTM formation was studied in closely related species of the genus *Nasonia*. These small parasitic wasps (~2 mm in length) lay their eggs in various species of fly pupae and differences in the ecology of the four known species of this genus have been described. A high-throughput method for olfactory conditioning was developed in which the wasps associated an odour, either chocolate or vanilla, with the reward of a host. A T-maze olfactometer was designed for high-throughput testing of memory retention. Using these methods, variation in memory retention was observed between three *Nasonia* species. Both *N. vitripennis* and *N. longicornis* form a long-lasting memory after a single conditioning trial, which lasts at least 5 days. *Nasonia giraulti*, on the other hand, lost its memory after 1 to 2 days after a single conditioning trial. Further studies focused on the difference between *N. vitripennis* and *N. giraulti*, which was most pronounced. By inhibiting LTM with transcription and translation inhibitors, it was confirmed that *N. vitripennis* forms this type of memory

after a single conditioning trial. LTM is visible 4 days after conditioning in *N. vitripennis*. *Nasonia girault*i does not form LTM after a single conditioning trial. Long-lasting memory is only formed after two trials, with a 4-hour interval between them. This difference in LTM formation makes *N. vitripennis* and *N. girault*i excellent model species to study both ecological and genetic factors involved in this difference.

Ecological factors such as the value of the reward and the reliability of the learned association have been shown to affect memory formation in a number of animal species. A recent study on oviposition learning in two parasitic wasp species demonstrated that LTM formation depends on the host species, i.e. the reward offered during conditioning. LTM was formed when a host with a higher quality was offered, but not when a host of lower quality was offered. The effect of host quality on memory retention of *N. vitripennis* and *N. girault*i was tested. Either a large host, *Calliphora vomitoria*, a medium-sized host, *Lucilia sericata*, or a small host, *Musca domestica*, was offered during conditioning. These hosts were observed to differ significantly in their quality, i.e. in the number of parasitoid offspring that emerged and the size of the offspring. There was, however, no effect of host species on memory retention in either *Nasonia* species. These results suggest that host quality is not important for LTM formation in *N. vitripennis* and *N. girault*i. This observation shows that ecological factors that are important for memory formation in one species may not be important for another species.

The genetic basis of memory formation is highly conserved among distant animal phyla. A large number of genes involved in LTM formation have been identified in genetic model organisms, including fruit flies, honeybees, the California sea hare, mice and rats, and the zebra finch. Genetic factors responsible for natural variation in LTM formation between species are currently unknown, however. Two approaches were used to study genetic factors responsible for the difference in LTM formation between *N. vitripennis* and *N. girault*i. The first approach took advantage of the unique possibility to interbreed *Nasonia* species. Hybrid offspring of *N. vitripennis* and *N. girault*i did not form LTM after a single conditioning trial, similar to *N. girault*i. The dominant LTM phenotype of *N. girault*i was then backcrossed into the genetic background of *N. vitripennis* for up to 5 generations. Using a genotyping microarray analysis and subsequent confirmation experiments, we detected two genomic regions (quantitative trait loci – QTLs) that both reduce long-lasting memory, but not completely remove this memory. These results indicate that multiple QTLs regulate the difference in LTM formation between the two *Nasonia* species. Concluding, our approach has provided insights in the genomic basis of a naturally occurring difference in LTM formation between two species. Excellent opportunities for fine-scale QTL mapping

are available for the genus *Nasonia*. This will allow identification of decisive regulatory mechanisms involved in LTM formation that are located in the two genomic regions detected in this study.

The second approach took advantage of next-generation sequencing techniques that allow transcriptome-wide studies of gene expression levels. RNA from heads of *N. vitripennis* and *N. giraulti* was collected before conditioning and immediately, 4 hours, or 24 hours after conditioning. This RNA was sequenced strand-specifically using HiSeq technology, which allows detection of sense and antisense transcripts. Various genes, from a number of different signalling pathways known to be involved in LTM formation, were uniquely differentially expressed after conditioning in *N. vitripennis*. These genes are likely involved in the ongoing process of LTM formation in this species. A number of other genes with a known role in LTM formation, including genes involved in dopamine synthesis and in the Ras-MAPK and PI3K signalling pathways, were uniquely differentially expressed in *N. giraulti*. These genes may have a role in a LTM inhibitory mechanism in this species. Antisense transcripts were detected for a number of known memory genes, which may indicate a role in regulation of transcription, alternative splicing, or translation. This study is the first to compare gene expression patterns after conditioning between two species that differ in LTM formation. The results provide promising candidate genes for future studies in which the regulation of these genes, the function of specific splice variants, and spatial expression patterns in the brain should be studied to understand how these genes are involved in the regulation of LTM formation.

Learning and memory formation have an important role in animal and human behaviour. Novel and valuable insights on both ecological and genetic factors responsible for variation in LTM formation have been revealed by the research presented in this thesis. Integrating ecological factors and genetic factors is essential, as genes are the level on which ecological factors can drive the evolution of variation in learning and memory formation. The genus *Nasonia* has offered excellent opportunities for ecological research as well as unique opportunities for studies on genomic and genetic factors, which were addressed by comparing closely related species that differ in memory formation. This thesis provides the basis for the identification of genomic differences responsible for the difference in memory formation between *Nasonia* species, but it also characterized the consequences of these genomic differences on gene expression. The genetic basis of learning and memory formation is highly conserved among distant animal species and insights from this thesis are likely applicable to other animal species and humans, as well. Altogether, these small parasitic wasps allow us to understand and value differences in memory formation.

Samenvatting

Het vermogen om te leren en geheugen te vormen is aangetoond in diverse diersoorten, zowel in betrekkelijk eenvoudige invertebraten, zoals slakken en insecten, als in meer complexe diersoorten, inclusief vogels en zoogdieren. De mogelijkheid om nieuwe vaardigheden te verkrijgen of om het gedrag aan te passen door middel van leren heeft een duidelijk voordeel. Aan de andere kant zijn er ook kosten verbonden aan geheugenvorming: het kan nadelig zijn om onbetrouwbare associaties te vormen en het proces van geheugenvorming kost daarnaast energie. De balans tussen voor- en nadelen bepaalt of het voordelig is voor een dier om te leren en geheugen te vormen en deze balans verschilt per diersoort. Verschillen in de ecologie tussen soorten zijn waarschijnlijk verantwoordelijk voor variatie in leervermogen en geheugenvorming.

In dit project is onderzoek gedaan aan variatie in het aantal leerervaringen dat nodig is om langetermijn geheugen ('long-term memory': LTM) te vormen. LTM is de meest stabiele en langst durende vorm van geheugen, maar het is daarnaast de meest kostbare vorm, omdat er eiwitsynthese nodig is voor de vorming van LTM. Veel diersoorten vormen dan ook alleen LTM na meerdere leerervaringen die ze met tussenpozen ontvangen. Dit zorgt ervoor dat een dier de geleerde informatie kan evalueren voordat er wordt geïnvesteerd in kostbaar LTM. Het aantal leerervaringen dat vereist is om LTM-vorming te induceren verschilt echter per soort. Sommige insecten, inclusief een aantal soorten sluipwespen, vormen al LTM na één enkele leerervaring. Sluipwespen kunnen geuren leren die hen helpen om geschikte gastheren voor hun nageslacht te vinden. Deze vorm van leren, waarbij de beloning een geschikte gastheer is om eieren in te leggen, wordt ovipositieleren genoemd. Tussen verwante soorten sluipwespen kunnen grote verschillen bestaan in LTM-vorming, waardoor deze soorten ideaal zijn voor vergelijkende studies naar deze vorm van geheugen. In dit project zijn zowel ecologische als genetische factoren die betrokken zijn bij verschillen in LTM-vorming bestudeerd. Een multidisciplinaire aanpak is essentieel om de evolutie van verschillen in LTM-vorming te begrijpen, aangezien de interactie tussen genen en de omgeving van een dier verantwoordelijk is voor het tot stand komen van leervermogen en geheugenvorming.

De vorming van LTM is bestudeerd in sterk verwante sluipwespsoorten van het genus *Nasonia*. Deze kleine sluipwespen (~ 2 mm lang) leggen hun eieren in de poppen van diverse soorten vliegen. De vier beschreven soorten van dit genus verschillen in bepaalde aspecten van hun ecologie. Binnen dit project is er een methode ontwikkeld om snel grote aantallen sluipwespen te kunnen conditioneren, waarbij wespen een associatie vormen tussen een geur (vanille of chocolade geur) en de gastheer, wat in deze methode de beloning is. Ook is er een T-maze olfactometer ontworpen waardoor het mogelijk is om bij grote aantallen

wespen te testen of er geheugen voor de geleerde geuren aanwezig is. Met behulp van deze methodes zijn er verschillen in geheugenvorming aangetoond tussen drie *Nasonia* soorten. Zowel *N. vitripennis* als *N. longicornis* vormen een langdurig geheugen dat minstens 5 dagen aanwezig is na één enkele leerervaring. *Nasonia giraulti*, daarentegen, verliest haar geheugen binnen één tot twee dagen na één enkele leerervaring. Verdere studies binnen dit project hebben zich vervolgens gericht op het verschil tussen *N. vitripennis* en *N. giraulti*, omdat deze twee soorten het meest van elkaar verschillen wat betreft geheugenvorming. Er werd aangetoond dat het langdurend geheugen van *N. vitripennis* inderdaad LTM is door de vorming van dit type geheugen te blokkeren door middel van toediening van transcriptie- en translatieremmers. LTM is zichtbaar in *N. vitripennis* vanaf 4 dagen na het conditioneren. *Nasonia giraulti* vormt geen LTM na één enkele training. Langdurig geheugen wordt wel gevormd na twee trainingen met een tussenpoze van 4 uur. Dit verschil in LTM-vorming maakt *N. vitripennis* en *N. giraulti* ideale modelsoorten voor het bestuderen van zowel ecologische als genetische factoren die verantwoordelijk zijn voor dit verschil.

Het is bekend dat geheugenvorming wordt beïnvloed door een aantal ecologische factoren, zoals de waarde van de beloning en de betrouwbaarheid van de geleerde associatie. Een recente studie waarbij ovipositieleren is onderzocht in twee sluipwespsoorten laat zien dat LTM-vorming afhankelijk is van de soort gastheer die als beloning wordt gebruikt tijdens het conditioneren. LTM werd wel gevormd wanneer een gastheer met een hoge kwaliteit werd gebruikt, maar niet wanneer een gastheer van lagere kwaliteit werd gebruikt. In dit project is het effect van gastheerkwaliteit op geheugenvorming bij *N. vitripennis* en *N. giraulti* onderzocht. Een grote gastheer (*Calliphora vomitoria*), een gastheer van gemiddeld formaat (*Lucilia sericata*) of een kleine gastheer (*Musca domestica*) werden gebruikt tijdens het conditioneren. Deze drie gastheren verschillen significant in kwaliteit als gastheer, die bepaald wordt door het aantal en de grootte van het nageslacht dat zich ontwikkelt in één gastheer. Desondanks werd er geen effect van gastheersoort op geheugenvorming gevonden in zowel *N. vitripennis* als *N. giraulti*. Dit resultaat suggereert dat gastheerkwaliteit niet belangrijk is voor LTM-vorming in beide *Nasonia* soorten. Het laat verder zien dat ecologische factoren die belangrijk zijn voor geheugenvorming in bepaalde soorten niet belangrijk hoeven te zijn voor andere soorten.

Genen die betrokken zijn bij geheugenvorming zijn evolutionair geconserveerd en er bestaan hierdoor grote overeenkomsten in de genetisch basis van LTM-vorming tussen zeer verschillende diersoorten. Dankzij onderzoek aan genetische modelsoorten, zoals de fruitvlieg, de honingbij, de Californische zeehaas, de zebravink, de muis en de rat, zijn er veel genen bekend die een rol in LTM-vorming spelen. Het is echter onbekend welke

genen verantwoordelijk zijn voor natuurlijke variatie in LTM-vorming. In dit project zijn er twee methodes gebruikt om genen te bestuderen die het verschil in LTM-vorming tussen *N. vitripennis* en *N. giraulti* kunnen verklaren. De eerste aanpak maakt gebruik van de unieke mogelijkheid om verschillende *Nasonia* soorten met elkaar te kruisen. Er is gevonden dat hybride nageslacht van *N. vitripennis* en *N. giraulti* geen LTM vormt na één enkele leerervaring, net als *N. giraulti*. Het dominante LTM fenotype van *N. giraulti* is vervolgens 5 generaties teruggekruist in de genetische achtergrond van *N. vitripennis*, waardoor zogenaamde introgressielijnen werden gecreëerd. Met behulp van een microarray analyse is het genotype van deze introgressielijnen bepaald en voor een aantal gevonden genomische regio's zijn de effecten op geheugenvorming verder onderzocht. Er zijn twee genomische regio's gedetecteerd, zogenaamde 'quantitative trait loci' (QTLs), die het langdurig geheugen (vanaf 72 uur na training) verminderen, maar niet geheel laten verdwijnen. Het korte-termijn geheugen (24 uur na training) werd niet beïnvloed. Deze resultaten laten zien dat meerdere QTLs betrokken zijn bij het verschil in LTM-vorming tussen de twee *Nasonia* soorten. Concluderend heeft onze aanpak nieuwe inzichten opgeleverd met betrekking tot de genetische basis van verschillen in LTM-vorming die van nature voorkomen. Er zijn binnen het genus *Nasonia* uitstekende mogelijkheden voor verder onderzoek aan de twee gevonden QTLs, waardoor er nauwkeuriger bepaald kan worden welke genen binnen deze regio's betrokken zijn bij het verschil in LTM-vorming tussen *N. vitripennis* en *N. giraulti*.

De tweede aanpak om de genetische basis van het verschil in LTM-vorming te onderzoeken maakt gebruik van 'next-generation sequencing' technieken, waardoor genexpressie niveau's van het gehele transcriptoom bepaald kunnen worden. Beide *Nasonia* soorten werden één keer geconditioneerd en direct, 4 uur of 24 uur later is er vervolgens RNA geïsoleerd uit de koppen van deze wespen. Ook is er RNA geïsoleerd uit koppen van wespen die niet getraind werden. Het RNA is vervolgens streng-specifiek gesequenced met behulp van HiSeq technologie, waardoor het mogelijk is om 'sense' transcripten, oftewel eiwit-coderende transcripten, en 'antisense' transcripten van elkaar te onderscheiden. Er zijn diverse genen gedetecteerd, waarvan het bekend is dat ze betrokken zijn LTM-vorming, die een veranderd expressie niveau hebben in *N. vitripennis* na het conditioneren, maar niet in *N. giraulti*. Deze genen, die behoren tot een aantal verschillende genetische signaalroutes, zijn waarschijnlijk betrokken bij de vorming van LTM in *N. vitripennis*, wat kort na het conditioneren al begint. Voor een aantal andere genen, waarvan ook bekend is dat ze een rol spelen bij LTM-vorming, is alleen in *N. giraulti* een veranderd expressie niveau gevonden. Deze groep bevat onder andere genen die betrokken zijn bij dopamine synthese en genen die behoren tot de Ras-MAPK en PI3K signaalroutes. Het is mogelijk dat deze groep genen een

rol speelt bij het blokkeren van LTM-vorming in *N. giraulti*. Daarnaast zijn er voor een aantal bekende geheugengenen antisense transcripten gevonden en deze transcripten spelen mogelijk een rol in het reguleren van de transcriptie, alternatieve splicing of translatie van de betreffende geheugengenen. Dit is de eerste studie die genexpressiepatronen vergelijkt tussen twee soorten die verschillen in LTM-vorming na conditioneren. De resultaten wijzen veelbelovende genen aan voor vervolgonderzoek waarin de regulatie van deze genen, de functie van splice varianten en ruimtelijke expressiepatronen in de hersenen onderzocht moeten worden.

Leren en geheugenvorming spelen een belangrijke rol in het gedrag van mensen en dieren. Het onderzoek dat gepresenteerd is in dit proefschrift levert nieuwe en waardevolle inzichten op in zowel ecologische als genetische factoren die verantwoordelijk zijn voor verschillen in LTM-vorming. Het integreren van ecologische en genetische factoren is essentieel, omdat ecologische factoren de evolutie van variatie in leren en geheugenvorming aansturen via genen. Het genus *Nasonia* heeft uitstekende en unieke mogelijkheden geboden voor zowel ecologisch onderzoek en onderzoek aan genetische en genomische factoren. Deze factoren zijn onderzocht door het vergelijken van sterk verwante soorten die verschillen in geheugenvorming. Dit proefschrift vormt het fundament om verschillen in het genoom tussen *N. vitripennis* en *N. giraulti* te identificeren die verantwoordelijk zijn voor het verschil in geheugen tussen de twee soorten. Daarnaast zijn de gevolgen van deze verschillen in het genoom op genexpressiepatronen in de hersenen in kaart gebracht. Gezien het feit dat de genetische basis van geheugenvorming evolutionair geconserveerd is, is het waarschijnlijk dat inzichten van dit onderzoek ook van toepassing zullen zijn op andere diersoorten en zelfs mensen. Alles bij elkaar hebben de kleine sluipwespen van het genus *Nasonia* er dus voor gezorgd dat we de verschillen in geheugenvorming beter kunnen begrijpen en op waarde kunnen schatten.

Curriculum vitae

Katja M. Hoedjes was born on August 1st, 1985 in Zeist, The Netherlands. After secondary school, she moved to Wageningen in 2003 to study Biology at Wageningen University. During her BSc studies she specialized in ecology with a minor in genomics and she graduated in 2006 *with distinction*. Katja specialized in molecular biology and behaviour during her MSc studies, at the same university, and has a minor in plant diseases. She conducted projects on the genetic basis of larval mobility of parasitic wasps (in collaboration with Prof. Louise E.M. Vet & Dr. Hans M. Smid, Wageningen University), the occurrence of *Iris Yellow Spot Virus* in the Netherlands and its transmission by thrips (in collaboration with Prof. Rob Goldbach & Dr. Dick Peters, Wageningen University), and the role of miRNAs in memory formation in *Drosophila melanogaster* (in collaboration with Prof. Sam Kunes, Harvard University). She graduated in 2009 *with distinction*. After graduation, she worked as a visiting scientist for 6 months in the group of Prof. Marla B. Sokolowski at the University of Toronto. Her project focused on protein expression in the brain of *D. melanogaster* and its role in regulation of behaviour.

Katja started her PhD project on variation in memory formation among parasitic wasp species in November 2009 at the Laboratory of Entomology, Wageningen University. This project was supported financially by the Earth and Life Sciences Council of the Netherlands Organization for Scientific Research (NWO-ALW). She acquired additional funding from the Dr. J. L. Dobberke Foundation to initiate collaboration with and work in the group of Prof. John H. Werren, Rochester University, on genetic aspects of variation in memory formation in *Nasonia* parasitic wasps. Katja has published or submitted all chapters of her thesis and she has presented her work at several national and international conferences. In addition, she co-organized the international *Nasonia* meeting in 2013, which took place in Wageningen. Furthermore, she has supervised and trained three MSc students and two BSc students.

Katja is currently working on a grant proposal to pursue a scientific career in the field of behavioural genetics.

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List of publications

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PE&RC Training and Education Statement

With the training and education activities listed below the PhD candidate has complied with the 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 (6 ECTS)
Natural variation in learning rate and memory dynamics in parasitoid wasps: opportunities for converging ecology and neuroscience

Post-graduate courses (5.1 ECTS)
- The ongoing evolution of qPCR; BioMCC, Vienna (2010)
- De novo assembly; NBIC (2013)
- Python scripting; NBIC (2013)
- Introduction to R for statistical analysis; PE&RC (2013)

Laboratory training and working visits (4.5 ECTS)
- Genetic and genomic tools of the *Nasonia* model system (in collaboration with Prof. Jack Werren); University Rochester, USA (2011)
- PhD-excursion: University of Neuchâtel, University of Lausanne & University of Basel, Switserland (2013)

Invited review of (unpublished) journal manuscript (2 ECTS)
- Animal Behaviour: learning and memory in a parasitic wasp species (2013)
- Ecological Entomology: behavioural ecology of parasitic wasp species (2013)

Deficiency, refresh, brush-up courses (2.1 ECTS)
- Basic statistics; PE&RC (2010)

Competence strengthening / skills courses (2.6 ECTS)
- PhD Competence assessment; WGS (2011)
- Writing grant proposals; WGS (2013)
- Career assessment; WGS (2013)

PE&RC Annual meetings, seminars and the PE&RC weekend (3.3 ECTS)
- PE&RC Day (2009-2011)
- Netherlands Annual Ecology Meeting (2010, 2012)
- PE&RC Weekend (2010, 2013)

Discussion groups / local seminars / other scientific meetings (7.5 ECTS)
- Insect-Plant interactions discussion group (2009-2011)
- Wageningen Evolutionary Ecology Symposium (2009-2013)
- Annual symposium of the Netherlands Entomological Society (2009-2013)
- Brains & Behaviour meeting (2011-2013)

International symposia, workshops and conferences (6.7 ECTS)
- *Nasonia* meeting; Cologne, Germany (2012)
- 'New horizons in zoology' (iBeetle conference); Gottingen, Germany (2013)
- 7th Annual Arthropod Genomics Symposium; South Bend, USA (2013)
- *Nasonia* meeting; presentation + organization of the meeting; Wageningen, the Netherlands (2013)

Lecturing / supervision of practical's / tutorials (3 ECTS)
- Molecular aspects of bio-interactions (2009-2010)
- Behavioural ecology (2011-2012)
- Molecular and evolutionary ecology (2012)

Supervision of MSc students (9 ECTS)
- Selection lines on learning rate in *Nasonia vitripennis*
- Host-dependent memory consolidation in *Nasonia* parasitoid wasps
- Memory of the mini wasps: genetic basis of the difference in learning rate between two hymenopteran parasitoids, *Nasonia vitripennis* and *Nasonia giraulti*

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