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Modularity and Plasticity of olfactory learning and memory in *Drosophila*

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Abstract

Cognition refers to the mechanisms by which animals acquire, store, process and act on information from the environment and this includes perception, learning, memory and decision making. Animals have their own perceptual world and adaptation seems to be crucial in order to survive by developing specialized ability in regard of the relevance of each sensory information. The process of storage is another mechanism important for adaptation because learned information can be retained from one occasion to the next. The underlying mechanisms of behavioral adaptation are based on learning and phenotypic plasticity. How this plasticity induces the formation of these adaptive specialized modules still remains unsolved.

The general aim of this PhD hold on the modularity and plasticity of olfactory learning and memory ability in *Drosophila melanogaster*. Drosophila is always confronted to complex environments with generally more than one stimulus that need to be associated with positive or negative reinforcements. In laboratory, it is possible to reproduce that kind of behavior in various protocols of associative learning. I tested adaptation processes at different levels of information processing. I demonstrate in this manuscript that adaptation occurs at each level: perception of complex stimuli, storage of relevant information and also update of memory trace not relevant anymore. Complex cognitive processes revealed the existence of adaptive modules more or less specialized that allows the animal to adapt to its specific environment. Moreover, artificial selection on specific memory ability demonstrates the implication of evolution in the modularity of animal cognition.

Résumé

La cognition se réfère aux mécanismes par lequel l'animal perçoit, apprend, mémorise et agit selon les informations auxquelles il est confronté dans son environnement. Les animaux ont chacun leur propre monde sensoriel et il est primordial qu'ils s'y adaptent en développant des compétences spécialisées en fonction des informations sensorielles qui lui sont les plus utiles. Il en est de même des informations qu'il est utile de stocker afin de pouvoir les utiliser ultérieurement. Les mécanismes sous-jacents à ces processus d'adaptation comportementale sont liés à la plasticité du système. Comment cette plasticité permet la mise en place de modules adaptatifs reste actuellement une question sans complète explication.

Le thème de cette thèse porte sur la plasticité et la modularité des capacités d'apprentissage et de mémoire olfactive chez *Drosophila melanogaster*. Dans la nature, la drosophile est confrontée à des environnements sensoriels complexes comprenant plusieurs stimuli sensoriels qu'elle doit associer à des renforcements négatifs ou positifs selon les conditions. En laboratoire il est possible de reproduire ce genre de comportement et j'ai ainsi pu tester le niveau d'adaptation des drosophiles à différent niveaux de traitement de l'information. Je démontre dans ce manuscrit que l'adaptation se produit à différents niveaux que ce soit la perception de l'information, les mécanismes de stockage des informations pertinentes et aussi la mise à jour de mémoires qui ne sont plus utiles. Ces processus ont révélé l'existence de modules cognitifs plus ou moins spécialisés qui permettent à l'animal de s'adapter spécifiquement à son milieu. De plus, la réalisation d'une sélection artificielle sur les compétences à stocker les informations révèle l'implication de l'évolution dans la mise en place de ces modules.

Introduction

Cognition refers to the mechanisms by which animals acquire, store, process and act on information from the environment and this includes perception, learning, memory and decisions making. The main question about behavior and cognition is to understand what information animals and even humans use to create behavior and what conditions control this behavior. Since the Antiquity, the actual idea about such process is guided by two opposed theories. The idealism view states that already established information specific to the organism is responsible for individual behavior whereas the empiricism view proposes that information provides from the interaction of the organism with the surrounding environment. These two points of view have provided the basis for two different approaches. Ethology focused on the preexisting information inherited from the evolution of species. This domain of research is based on study of comparative cognition in the field and attempts to understand and explain species-specific behavior. Unlike ethology, behaviorism, usually represented by psychologists, attempts to collect information through perception and action using laboratory-based experiments. They focus their experiments on learned behavior and elude the implication of evolution on the animal behavioral state. Separately, ethology and behaviorism cannot elucidate the rules that govern behavior. It is now crucial to link the neurophysiological basis to their effect on behavior. More recently, cognitive neuroscience and other subfields started to offer level of integration between the behavioral science and the neuroscience. Moreover the genetic, ecology and psychology of these traits have also to be included in the study of behavior to give a global view of evolution and plasticity of learning and memory processes.

From stimulus perception to adaptive learning processes:

Ethology is first based on the idea that each animal has its own perceptual world. Animals have to specialize their perception system in order to respond to their environment. One impressive example is animals living at night. Many bats species are able to detect preys in the dark by using sonar detection (Kalko

and Schnitzler 1993). Vision also provides many examples of perception specialization. For instance, what an animal can see is not always the same as what a human is able to see because color resides not in the objects but in the observer's perception of wavelength differences or similarities. Some animals use different wavelengths than human for foraging or mating. Some birds use UV in order to visualize plumage ornamentation wavelength (Cuthill *et al* 1999, Sheldon *et al* 1999). Bees also are able to see UV light to detect difference of reflectance on flowers (Chittka *et al* 1994). Even within species, differences in perception can be found depending on the environmental variation. One most famous example is fish vision in deep sea. Populations of the same species living at different depths exhibit visual sensibilities to different wavelengths (Lythgoe and Partridge 1989). Perception specialization allows adaptation to specific environments. But animals need to learn what they are able to perceive in order to survive. Animals would not be able to behave as they do without the ability to learn. Animal behavior presents a number of different kinds of learning in response to their own environment. This specialized perception systems is the basis of learning adaptive process in which animal develop specific learning ability in regards of what they have to face in nature. It shows the existence of biological constraints on learning that are drawn by the evolutionary history of the species concerned. For instance, the idea that there may be a number of different, adaptively specialized kinds of learning or cognition is related to the principle that learning and memory consist of more than one system. A broad definition of learning is a change of representation of the world as a result of experience and can be expressed by a modification of behavior. Associative conditioning is the most studied form of learning. The ability to acquire associations is largely represented in nature because it allows animals to adjust their foraging, predator avoidance, social behavior and other multiple behaviors. There are two categories of associative learning. First, classical Pavlovian conditioning refers to the modification of behavior in which a neutral stimulus, called conditioned stimulus (CS) is associated with a second stimulus that induce a particular response, known as the unconditioned stimulus (US) and the response to the US is called the conditioned response (CR). Pavlovian conditioning is based on the paired presentation of a sensory stimulus CS (light, tone, odor…) and the US. The US is can be appetitive (reward), aversive (punishment). Animal learns that the presence of the CS is predictive of the US and develops a specific

behavior to the CS in regard of the relevance of the US. Temporal contiguity between the CS and US is a key parameter to define the rule of the association (Rescorla and Holland 1972, Rescorla 1988). It is generally accepted that two events that are contiguous are more likely to become associated than two events that are separated. Most studies on associative learning use simultaneous CS and US exposure but it has been demonstrated that a slight temporal asynchrony in which the CS precedes the US can be better for enhancing learning (Batson *et al* 1992, Blaisdell *et al* 1998, Esmoriz-Arranz *et al* 2003). Moreover, a negative contingency (US precedes CS) would induce that the CS predicts the absence of US (Baeyens 1993, Yarali et al 2009). Another category of associative learning known as operant conditioning is described as the process by which animals learn about causal relationships between events and behave appropriately in response (Rescola 1988). Operant conditioning describes how animals associate stimuli with their own motor actions. In this kind of learning, the response does have a consequence. The response can be strengthened or weakened depending if the animal is confronted to positive or negative reinforcements respectively. Different basic phenomena influence the ability to form associative learning. These processes are related to the question about how multifeatured events are processed First, the phenomena of overshadowing is sometimes observed after two neutral elements are combined to form a compound neutral stimulus and then the compound stimulus is converted into a compound CS by repeatedly pairing it with a US. The compound CS is considered as a simple CS during conditioning but when the elements are tested separately, one element produces a stronger conditioned response than the other. In certain species, some stimuli naturally overshadow other stimuli depending on the sensory requirements specific to each species. For instance, in rats, tones tend to overshadow lights because rats use preferentially auditive than visual cues in their environment (Takigasaki 1995). Another important aspect of associative Pavlovian learning is that the simple contiguity between CS and US produces poor conditioning unless that CS also bears an informational relation to the US. An example is the phenomenon of blocking first reported by Kamin (1968). Blockin*g* occurs when one of the elements of a compound CS begins already fully conditioned. For instance, if an animal learns that a light predicts food and that, later, another stimulus (tone) accompanies the light so that both are associated with food, then the new element (tone) is blocked because it conveys no new

information about the US. In this case, the light is said to have blocked conditioning to the tone. The model of Rescorla-Wagner predicts that the amount of association strength of a CS would be proportional to the difference between the maximal strength induced by the US and the current associative strength of all CSs. Another model has emerged in the study of associative learning. Pearce configural model suggests that animal processes compound as new whole entity different from each element of the compound (Pearce 1994). These models have been tested in a particular task: the discrimination learning. The basic discrimination protocol consists in two CSs: one reinforced and another one not reinforced. In a discrimination task called negative patterning, a first stimulus A is reinforced, another stimulus B is reinforced but a compound AB is not reinforced. The model of Rescola-Wagner would not solve that kind of protocol because AB would be more excitatory than the stimuli A or B alone. Nevertheless, vertebrates as rats (Holland *et al* 2000), pigeons (Rescorla 1991), rabbits (Kehoe and Graham 1988) and humans (Young *et al* 2000) are able to learn that kind of configural representation. To explain these results, the compound needs to be perceived as the summation of its element, plus a unique stimulus specific to the compound (Kimmel and Lachnit 1991, Ludwig and Lachnit 2002). Then, even if the elements are excitatory when they appear in compound, the unique cue can gain enough inhibitory strength to cancel out their effect. The configural theory of Pearce can explain easily how animals are able to solve negative patterning (Pearce 1994). As the compound is treated as a unique stimulus different from its element, animals attribute associative strength to the compound but less to the elements alone. However, this model accepts partial similarities between the compound and its elements which allow a partial generalization. Within species, these models are not exclusive and each provides better performance in specific conditions. There is some evidence that the configural model is suited for complex discrimination whereas there are other situations for which the elemental perception is a better strategy in order to respond adequately to complex environment (Miller *et al* 1995). Complex learning was often assimilated to higher functions and elaborated nervous systems but it is now accepted that even insects with relatively simpler neuronal networks than vertebrates are able to perform complex cognitive tasks (Chittka 2009). For instance, Deisig *et al* have demonstrated that honeybees are able to solve negative patterning discrimination using odor compound in

the proboscis extension reflexe protocol (Deisig *et al* 2001). Other studies have shown that lobsters are able to use flexible perception processes to analyze and respond to compound stimuli (Derby *et al* 1996, Livermore *et al* 1997). In their experiment, the way the mixture was perceived differs according to the salience of each element. In nature, stimuli salience is variable as environment and experience change dynamically. Some theories emphasize that multiple CSs must compete for internal representations of learning about other stimuli (Mackintosh 1975). Then, learning must consider varying CS salience because discriminative stimuli compete for attention. A general learning theory must address the external and internal factors that influence how the brain allocates attention and apprehends the environment to select, store and retrieve information for generating adaptive behavior.

The brain utilizes its anatomical resources to organize peripheral inputs and facilitates the recognition and discrimination of behaviorally relevant odor blends. Multiple approaches as single-cell electrophysiology, multi-unit recording or population studies were performed to describe the neural basis of olfactory coding and particularly in insects. The anatomical organization of olfactory systems determines that information about odorant stimuli is first deconstructed by the receptor array (ORC: Olfactory Receptor Cells) located at the periphery, processed in the neuronal circuits (AL: Antennal Lobe) and finally reconstructed as a unitary odor percept at higher-order neuronal centers (Fig 1).

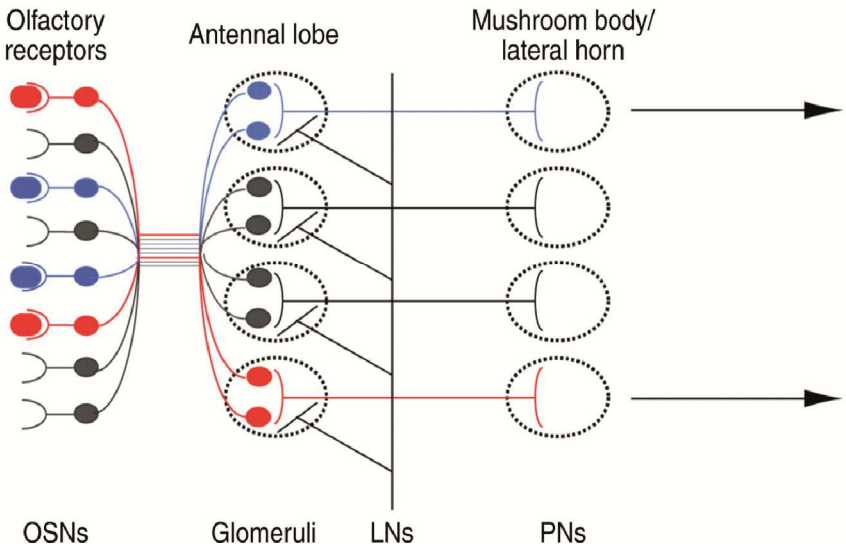


Figure 1 Organization of the Drosophila olfactory system. OSNs bind to odorants and send the information to glomeruli, innervated by local interneurons (LNs). The information then travels to higher centers in the brain, the mushroom body and lateral horn, through projection neurons (PNs) (modified from Pellegrino and Nakagawa 2009).

However, little is known about the mechanisms by which this system produces a perceived image of an odor mixture. The chemical composition of a compound odor is analyzed by transforming the information of a complex chemical mixture into the combinatorial activation patterns of different types of ORC. Once the information about an odor is distributed into different ORC channels, a process of regrouping may occur at early stage in AL. The AL consists of a species-specific number of glomeruli that collect the convergent axonal inputs of ORC expressing the same olfactory receptor protein (Mombaerts *et al* 1996, Vosshall *et al* 2000). The combinatorial receptor pattern for odors is also transmitted to the first processing stage and is represented by odor specific patterns of glomerular activity. When two odors are presented in a mixture, the neural representation of the mixture approximates but slightly differs from the sum of the responses to the components. This is caused by inhibitory interactions arising from the network of local inhibitory neurons connecting glomeruli. Thus, in the case of odor mixtures, specific odor-suppression phenomena that are proper to each odor combination appear. As more components are added to the mixture, such inhibitory interactions become more apparent (Joerges *et al* 1997). As elemental and configural processing are not mutually exclusive, central processing of an odor mixture may result in a unique representation, while still preserving information about single components. Elemental and configural processing have been revealed to be processed in the primary olfactory areas, the antennal lobe and more precisely in the second-order output neurons in different species (moth: Christensen *et* al 1989, 1991, 1995; Drosophila: Silbering and Galizia 2007, Silbering *et al* 2008). In honeybees, the AL network is built by three types of neurons: olfactory receptors neurons, projections neurons and local interneurons and is thought to reformat the input signal such that distinction of stimuli is increased. The projections neurons form the AL output to the higher processing centers and representation of mixtures seems to be more configural in the projections neurons (Deisig *et al* 2010). In 2012, Meyer and Galizia showed that there are elemental and configural neurons in AL, though the same neuron may fall into either category depending on the stimulus used such that the fastest neurons have elemental responses, and neurons with configural response properties respond later (Meyer and Galiza 2012). Other studies suggested that configural perception requires further integration at the

central level as mushroom bodies in honeybees (Lachnit *et al* 2004). In 2003, Komischke *et al* showed that bilateral olfactory input is necessary to solve negative patterning (Komischke *et al* 2003). As the olfactory circuits remain unconnected until the mushroom bodies, this result implies that integration of non-elemental cue also occurs after the AL in olfactory pathways. In Drosophila, few studies hold on elemental and configural perception of olfactory mixture. Are fruit flies able to perform non-elemental perception or just elementally perceive complex environment?

From learning to memory

In order to influence behavioral performance, learned information must be retained from one occasion to the next. The process of memory deals with the question about how information is stored, retained and retrieved. The example of food-storing bird has been intensively investigated for quite a long time (Shettleworth 1993, Pravosudov 2006). Birds are able to remember where they have previously hidden their food and this spatial memory has been found to correlate with a larger hippocampus, which is the center of spatial information processing than in non-storing species of birds. This is a good example of adaptive specialization at the memory level. Ethologists considered memory processes as they address some questions about what information is useful in an environment or what determines the condition of their expression in behavior. However, to understand how memory occurs in the wild, it is necessary to investigate the dynamics for the formation of long-lasting associations based on laboratory experiments.

Since the beginning of the 20th century, important characteristics of the memory process have been described. It first has been demonstrated that memories have different durations and proposed a model of memory formation in two phases: the first, labile and expressed for a short time, is called short-term memory (STM) and the second, robust and long-lasting is called long-term memory (LTM). The treatment of information from a STM to a LTM state needs a specific mechanism called consolidation that allows storage and stabilization of the memory trace in the brain (Squire 1987, Menzel 1990, for review see McGaugh 2000, Dudai 2004). A second characteristic of memory formation is that repetition of a learning assay influences

memory strength. For instance, in a classical aversive Pavlovian conditioning, Drosophila are trained to associate an odor to a shock. One cycle of training induces STM formation but not long-lasting memory whereas 5 cycles of training enhance LTM formation (Tully *et al* 1990). In the same way, honeybees require multiple trials of conditioning to form long-lasting form of memory in a proboscis extension response conditioning protocol (Menzel *et al* 2001). Not only the number of trials, but also the temporal dynamics of these assays influences memory formation. Spaced training (repeated sessions with a rest interval between each) lead to a better long-lasting memory in terms of strength and duration than massed assay (repeated sessions with no rest interval) (Frost *et al* 1985, Hintzman 2010). The last universal behavioral characteristic is that treatments such as electroconvulsive shock, administration of anesthetics or protein synthesis inhibitors slow or block memory consolidation (Davis and quire 1984). The effect of inhibitors has led to the notion that LTM stabilization is protein synthesis dependent (Flood *et al* 1977, Andrew 1980; Squire *et al* 1980, Akahane and Amakawa 1983, Rosenzweig and Bennett 1984). This requirement is consistent with the neurobiological view that memory consolidation is mediated by long-lasting structural changes in synaptic morphology. Genetic studies have also been made to determine the genetic bases of memory phases and in particular in *Drosophila*. The genetic bases of memory formation have been studied after the classical associative conditioning of an odor avoidance response in *Drosophila*. In 1976, the first mutant with deficit in learning ability *dunce* was found (Dudai *et al*, 1976). The mutants *dunce* and *rutabaga* (Livingstone *et al* 1984) have revealed that single gene mutations can disturb completely associative learning and memory and later, Tully *et al* have shown that these disruptions affect functionally distinct memory phases (Tully *et al* 1990). This mutant-based genetic dissection of memory processes in Drosophila has distinguished 4 memory phases using the Tully associative protocol in which flies are trained to associate an odor to a punishment (electric shock) (Fig.2). The first two phases are the short-term memory (STM) and the middle-term memory (MTM). These two forms can be disturbed by anesthetics or cold shock. Beside these two labile phases, there are two forms which are insensitive to anesthesia treatment: anesthesia-resistant memory (ARM) and long-term memory (LTM). ARM is induced after a massed protocol whereas LTM is induced after a spaced protocol. Unlike ARM, LTM requires protein synthesis to be stable.

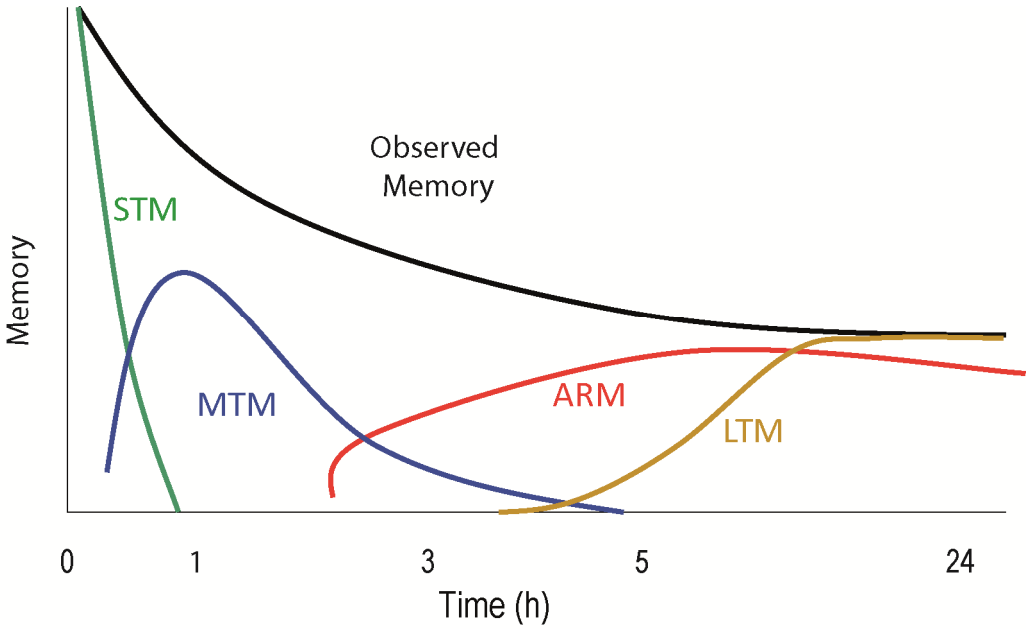


Figure 2 : Four functionally distinct memory phases have been detected underlying the observed retention scores. Short-term memory (STM), middle-term memory (MTM), anesthesia resistant memory (ARM), and long-term memory (LTM) appear sequentially, and each has a progressively slower rate of decay (from Dubnau and Tully 1998)

Specific mutants for ARM and LTM have also been described: *radish* disrupts ARM (Folkers *et* al 1993, 2006) and *crammer* disturbs LTM formation (Comas *et al* 2004). Other mutants are known to be involved in LTM activity. This protein synthesis dependent form is specifically disturbed in the transgenic flies expressing a dominant negative isoform of a cAMP-reponsive element-binding protein (CREB) (Yin *et al* 1994).However, Perazzona *et al* demonstrated that the transgene reported to enhance LTM in the previous study carries a deletion of one base and that this mutation produces the formation of a stop codon (Perazzona *et al* 2004). They overexpressed a corrected CREB protein but failed to enhance LTM formation. In this study, only the overexpression of a CREB repressor impairs LTM formation. Other studies in rodents have also demonstrated the implication of this transcription factor in LTM (Bourtchuladze *et al* 1994). Because genetic manipulation of CREB in both mammals and flies affects LTM formation, this protein may have a long evolutionary history of participating in memory formation.

At the neuronal level, memory trace formation happens in the mushroom bodies (for review see Heisenberg 2003, Davis 2004). In 2001, Pascual and Preat have demonstrated that a specific region of the mushroom bodies is required to form LTM using a specific structural mutant *ala* and that ARM formation is

not disturbed by the mutation. Since the 90s, neurobiologists debate about the properties of these two forms of memory and also their interaction. Two views have emerged with different conclusions. Some researchers think that ARM and LTM formation are completely independent with two molecular pathways acting in parallel (Tully *et al* 1994, Fig. 3a) whereas others suggest an inhibitory system in which ARM formation block LTM enhancement (Isabel *et al* 2004, Fig. 3b). In this last hypothesis, ARM would act as a gating mechanism which ensures that LTM is induced only after a spaced protocol. LTM and ARM are supported by parallel pathways: the former is cAMP-dependent, and the latter is rsh-dependent. After massed conditioning, ARM is hypothesized to prevent the formation of LTM. After spaced conditioning, LTM replaces ARM. These hypotheses concern the neurobiological processing of memory formation but more recently, evolutionary aspects of memory formation are studied in order to determine how evolution has led to such a complex multiphasic system.

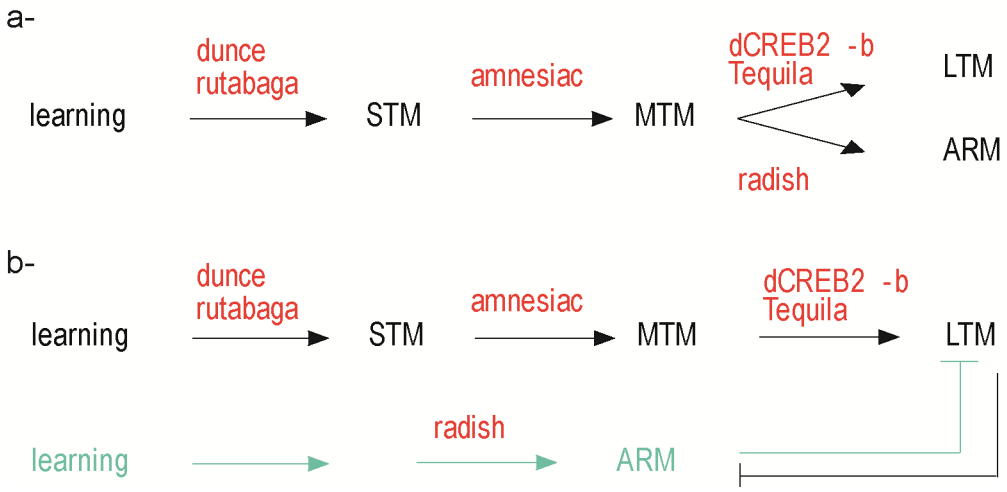


Figure 3 Model of competitive memory phases. (**a**) A model that holds that ARM and LTM coexist after spaced conditioning. (**B**) A model that postulates mutually exclusive consolidated phases.

The existence of multiple forms of consolidated memory in Drosophila is an interesting tool to investigate how information is stored depending on the environment and what are the interactions between the different memory phases.

Forgetting and extinction: two processes of memory update

Environment rarely stays in a constant state but can changes rapidly. Animals do not have to adapt their behavior to their environment only once in their lifetime but to make multiple adaptations in response to environment modifications. It seems there are no ecological advantages to keep information which is not useful anymore and there are different mechanisms by which memory can be modified. Long time after an associative conditioning, even if association with appetitive or aversive US can be retained surprisingly well over time (Gale *et al* 2004), it is possible that animals forget details about either the CS or the US (Riccio *et al* 1984). There are different reasons that explain the forgetting phenomenon. One possibility is that the memory trace might fade away which is called trace decay. This reason is not very surprising because it seems difficult to believe that the brain stores memory traces forever if they are never used. Another reason that does not involve decay over time is interference. Memory trace of information can be disturbed when conflicting information is learned at some other point in time. There are two kinds of interferences depending on when the conflicting information is learned. Proactive or retroactive interference occurs if the conflicting information is learned respectively before or after the target information (Mensink 1988, Bouton 1993). For instance, an experimenter gives to people two lists of words to memorize and then can ask to remember either the first or the second list (Postman *et al* 1968). In proactive interference, the first list interferes with acquisition and storage of the second and in retroactive interference, the second list interferes with the first one. One process in which interference may be important is memory extinction. In classical associative conditioning, the response to the CS remains high as long as it still predicts the US. However, if the US is removed, the conditioned response to the CS disappears (Bouton 2004). For instance, when an auditory cue is associated with the presence of food, rats exhibit a conditioned response which declines if the food is no longer presented (Bouton and Peck 1989). Extinction is a crucial process in behavioral modification in response to changing environment. An important aspect of extinction is that it does not involve destruction of the original memory. After extinction of a memory and then presentation again of the signal, it is sometimes possible to recover the conditioned response. This effect is known as the spontaneous recovery (Quirk 2002, Rescorla 2004). Then, extinction is assimilated as an active process that

consists in the consolidation of a new memory between the CS and the absence of the US. Extinction is different from forgetting. In extinction, behavior is lost because of direct experience of a disconnection between the CS and the US whereas forgetting consists in a drop of behavior because passage of time. However, it seems that, after consolidation of an associative memory, the presentation of the CS without the US does not extinct automatically the original memory. Reactivation destabilizes the original memory trace. Once labile, this trace can also be stabilized again through a process called reconsolidation, so that the conditioned response is maintained (Misanin *et al*, 1968; Mactatus *et al*, 1979; Nader *et al,* 2000; Sara, 2000; Dudai, 2006). Reconsolidation and extinction thus lead to two opposite outcomes. Whether one or the other is induced is likely to depend on different factors (Alberini *et al*., 2006). In 2004, Suzuki have demonstrated that the strength and duration of the reactivation as well as the strength and age of the original memory are important parameters such that younger and weaker memories tend to be more easily reconsolidated (Suzuki et al 2004). The two processes seem to have distinct molecular requirements but how environmental context influences one or the other process is still not elucidated. Moreover, the reconsolidation process has not been detected in some systems and the similarity between consolidation and reconsolidation is strongly debated (Dudai 2006). Even if some studies suggests that these processes are different and require different pathways and brain region activations (Nader *et al* 2000*,* Alberini 2005), others think that reconsolidation is just a repetition of the consolidation (Cammarota 2004).

Evolution of learning and memory processes

The concept of learning and memory adaptation assumes that the capacity to acquire and store information over time is adaptive, meaning that the systems that enable learning and memory are functionally designed. Another important aspect of adaptation is that learning and memory are products of the evolution because the ability to learn and memorize is likely to be influenced by the fitness relevance of the information and task involved.

In nature, we can imagine that better learning and memory abilities represent a benefit for survival (Papaj and prokopy 1989; Dukas 1999) and so, that natural selection should be favored, but response to

selection has also genetic constraints. A lack of genetic variation can limit the appearance of relevant alleles and decrease the probability of selection. Ecologically, we can also assume that, even with genetic variability high enough to allow evolution of better learning ability, this potential can be more costly than beneficial for the animal, and so, deleterious for survival. These constraints can be addressed by imposing experimental selection on behavioral abilities in laboratory. In artificial selection, a behavior of interest is quantified for multiple individuals and the ones that produce the target behavior are selected as breeders to produce the next generation. Experimental selection has been performed for different levels of behavior from simple behaviors to complex cognitive processes. For instance, rats were selected for their learning performance in mazes. Tryon separately mated those that learned quickly ("bright") and those that did not ("Dull"). Offspring of "bright" parents were bright and offspring of "dull" parents were dull and this difference increased over succeeding generation. Even if these results are strongly dependent on the kind of environment in which rats were raised, it suggests these phenotypes can be inherited (Tryon 1940). The genetic potential to evolve is tested by giving conditions favorable for improvement. For instance, Mery and Kawecki imposed a selection regime on *Drosophila* that favored the ability to associate a flavor to an oviposition substrate with an aversive bitter taste. After 12 generations, selected flies learned the task better than unselected flies (Mery and Kawecki, 2002). This evolutionary potential is not always used in a natural environment because it happens that the benefit of higher cognitive functions could not be high enough to compensate for the costs. Learning and memory are complex processes which are known to be associated with costs or trade-off (Dukas 1999, 2004). Usually, most discussions hold on the cost induced by the acquisition of information during learning. This cost consists in the energy and time used by the neuronal machinery to create the learning trace in the brain and also the risk to make some mistakes. But learning performance is also related to costs which reflect evolutionary trade-off between the learning process and other ecological aspects of the animal. For instance, selected flies for oviposition site choice also exhibit a decline of larval competitive ability (Mery and Kawecki 2003) and also shorter longevity (Burger *et al* 2008). Moreover, the trade-off observed with longevity is symmetrical. Flies selected for longer lifespan were found to have low learning ability. This kind of trade-off is related to the brain which is very costly in terms of

energy for its development, maintenance and function. This organ is very plastic and presumably requires more energy in case of behavior improvement. The increase of energy expenditure would be compensated by a decrease in others functions such as growth, survival or immune system. So, individuals with genetically high learning ability could pay this cost even if they do not exercise their ability. These costs could be seen as pleiotropic effect of genes influencing learning ability and other phenotypic traits. Little is known about the evolutionary costs and trade-offs associated to memory ability, although it is the balance of these benefits and costs that determines whether natural selection favors improved cognition.

Description of the thesis:

Within the last four years, I focused my work on the organization in "modules" of animal cognition through different mechanisms from perception to memory update. The concept of modular organization is used to interpret complex behaviors as the interaction between different modules controlling more basic behavior. I based my study on olfactory associative learning and memory of *Drosophila melanogaster* using behavioral and evolutionary approaches to understand how, in fruitflies behavior, complexity can arise from a relatively simple nervous system. This manuscript is organized in four chapters.

The first chapter addresses the question about the modularity of compound perception in complex discrimination learning protocols. Using different discrimination tasks, I investigated the different ways to perceive a compound from the elemental theory in which the mixture is the sum of its element to the configural theory which assumes that the compound is represented as a new whole entity.

The second and third chapters hold on the phenotypic plasticity of the olfactory memory system. First, I evaluated the genetic variability of learning and memory ability in a panel of 40 inbred lines in order to reveal eventual pleiotropic effects between the different memory phases and secondly I developed an artificial selection on specific form of consolidated memory ARM or LTM. Artificial selection would help to reveal the existence of different interacting memory modules and also eventual trade-offs with other phenotypic traits reflecting the cost of an improved ability to consolidate.

In the last chapter, I tested flies' ability to update already consolidated memory. The system of extinction/reconsolidation is very well described in vertebrates but few studies investigated these mechanisms in invertebrates. I used the particular memory system existing in *Drosophila* to investigate the interaction between consolidation, reconsolidation and extinction processes.

Materials and Methods

Conditioning assay:

We used a classical aversive olfactory conditioning assay (described in Mery and Kawecki 2005). Flies were conditioned to associate one of two odors (CS = 3-octanol or 4-methylcyclohexanol, SIGMA) with a mechanical shock (US). Conditioning was performed on groups of 50 adult flies (sexes mixed) aged 3-5 days from eclosion. Training assay consist in cycles and are each composed by 4 phases :

1° flies were exposed for 1min to one of two odor (CS+) accompanied by mechanical shock (2000 rpm vibration pulses of 1s duration, delivered every 5s by a test tube shaker)

2° 1min rest during which flies received humid air flow (no odor)

3° flies received the second odor (CS-) without shock

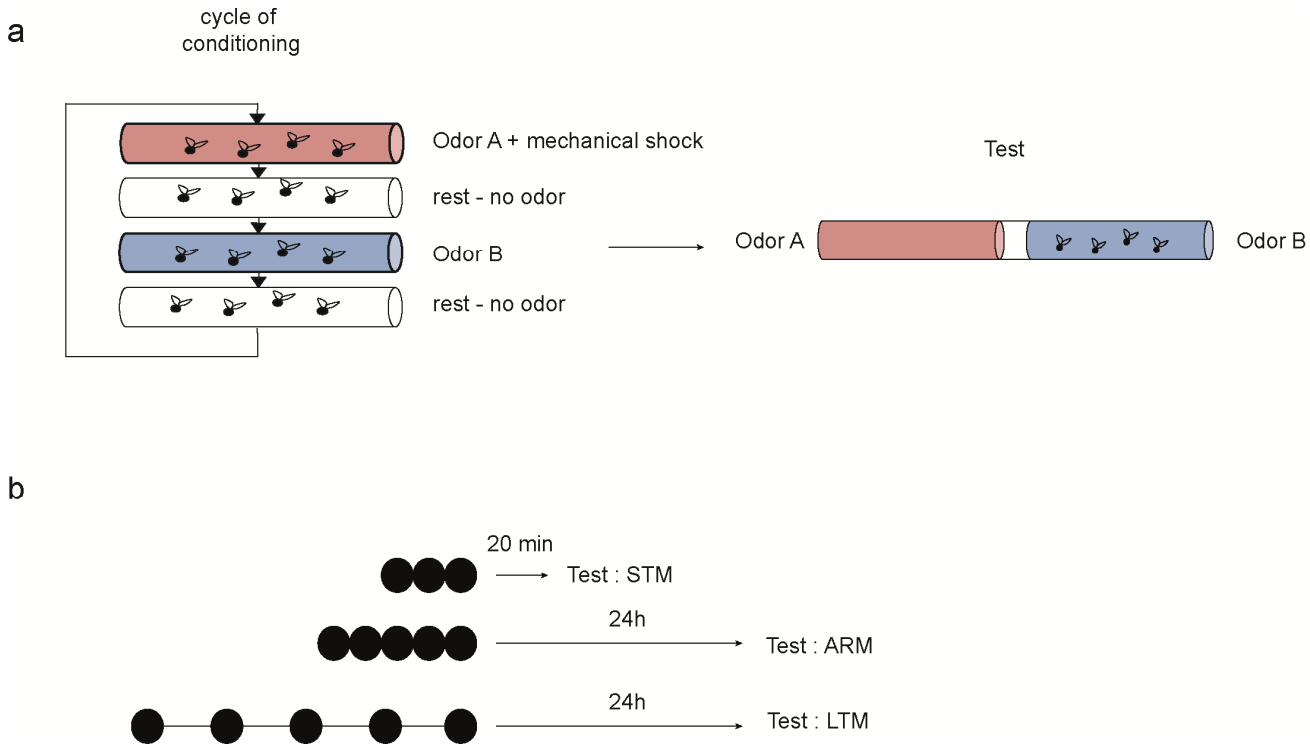
4° another 1min rest period

Depending on the type of memory tested, 3 or 5 cycles of conditioning were applied. STM were performed with 3 cycles immediately one after another. Consolidated forms of memory (ARM and LTM) required 5 cycles either separated by a 20min interval (LTM: spaced protocol) or not separated by time interval (ARM: massed protocol). Half of the groups were conditioned with OCT as CS+ and the other half with MCH as CS+.

Retention measurement:

Learning and memory test consisted in a choice between the CS+ and the CS- in a T-maze. After conditioning (20 min for STM and 24h for LTM and ARM), groups of flies were transferred in the central part of the T-maze in which they were exposed to two divergent currents of air (one carrying the CS+ and one

carrying the CS-) from opposite arms of the T-maze. During 1 min, flies chose between the two odors, then they were trapped, killed and counted. For each measurement, a memory score was calculated as the difference in the proportion of flies choosing octanol when conditioned to avoid methylcyclohexanol versus when conditioned to avoid octanol.



Chapter 1 – Compound processing in *Drosophila*

Sensory perception of complex environment seems to be a crucial process in order to survive. Animal need to be equipped with sophisticated sensory systems and also high ability of learning to be able to process complex pattern of stimuli. Some invertebrate models as drosophila, even with simpler neuronal system than vertebrates, are known to support large range of behavior but their ability to solve more complex task remains unknown. In this study, we address the question about odor compound process in *Drosophila melanogaster*. Compound perception can be explained by different approaches from the pure elemental theory in which a mixture is perceived as the summation of its element to the configural theory which assume that a compound is perceived as a new entity different from its element. We tested these theories investigating flies' ability to learn discrimination involving associative conditioning of a binary odor compound with mechanical shocks in three protocols with different complexity. We first demonstrated that flies are able to learn mixture and also to generalize each element to the compound. Another protocol that involves overlap of elements in the discrimination task suggests that flies do not only use elemental perception of odor compound. However, we tested in a specific protocol called negative patterning discrimination that require configural perception to be solved and flies were not able to perform this discrimination.

Introduction

Natural environment is composed of complex blends of stimuli. For instance, pollinators are attracted by plants with floral displays composed of visual, tactile, olfactory and gustatory stimuli and use the different sensory modality to detect flowers (Kulahci *et al* 2008, Leonard *et al* 2011). Animals are rarely confronted to environment with only one stimulus but usually complex compound. Animals are always confronted to environment in a sensory background and have to distinct the presence or absence of the stimulus of interest in order to behave adequately. They have to learn how these stimuli are associated and the relevance related to each of them. These circumstances address the question about how animals process compound stimuli in natural environment.

Since 1970s, different ways of interpreting perception of such complex environment have emerged. All the point of views exists between two extremes hypothesis. The first model is the elemental perception of compound in which mixture is perceived just as the sole summation of each element of the compound (Rescola and Wagner 1972) and the more complex view consist in a configural perception suggesting that each compound is interpreted as a whole with its own properties different than the elements' properties (Pearce 1987, Pearce 1994).

These different models have been intensively tested in a particular approach: the discrimination learning. The basic discrimination protocol consists in two CSs: one reinforced and another one not reinforced. A compound can be used as the conditional stimulus reinforced or not and tested in protocols with different complexity. The elemental and configural models differ in the prediction they make for these protocols. For instance, in feature positive (AB^+ / A) or negative (A^+ / AB) discrimination (Pearce and Wilson 1991), the response to one stimulus A is controlled by the presence or absence of another stimulus B. Elemental perception predicts that this discrimination would result in B gaining either negative or positive associative strength because it is the only reliable predictor of the outcome even if we should consider the importance of the different of salience between A and B. Configural representation should predict that the compound AB is associated or not to the US. Another protocol called patterning discrimination produces different predictions with elemental and configural perception. Patterning protocols consist in associative

conditioning involving a mixture and each element composing the mixture. In the positive patterning (PP),a mixture AB is reinforced and the element alone A and B are not reinforced and in the negative patterning discrimination (NP), the element A and B alone are reinforced but once mixed are not reinforced. Interestingly, elemental and configural perception can both explain PP, but NP can only be possible if we consider a configural representation of the compound AB. The Rescorla-Wagner model cannot explain NP because there is no way for both A and B to be excitatory while their compound is neutral or inhibitory. In fact, AB should be even more excitatory than A or B alone. However, vertebrates as rats (Holland 2000), pigeons (Rescorla 1991), rabbit (Graham *et al*, 1988) and even human (Harris *et al* 2009) are able to perform NP, so mixture perception assume that the compound AB may also creates a unique, configural cues that function in the same way as normal stimuli: this alternative approach is known as the unique cue theory. Recently, invertebrate models were used to investigate these elemental and configural representation of stimuli compound in a specific task: the patterning discrimination. Honeybees have found to be able to solve positive and negative patterning (Chandra and smith 1998, Deisig *et al* 2001) and other studies have shown that the lobster is also able to use elemental and configural representation of compound (Derby *et al* 1988, Livermore *et al* 1997). In this last model, the way to perceive depends on the saliency of each representation in a particular environment which means that lobsters are able to specifically use elemental or configural perception in regard of their efficiency.

The question about how animals perceive their environment is still debated both within and across sensory modalities. Within modality, vertebrate and invertebrate models have already been investigated to understand the choice between these alternatives and particularly in the case of odor mixture perception. The model Drosophila is generally used to investigate olfactory learning and memory ability using classical Pavlovian olfactory conditioning. In this paradigm, flies learn to associate an odor (CS) to an electric (Tully *et al* 1994) or mechanical (Mery and Kawecki, 2005) shock (US). We note that this learning is a discriminative task implying two CS; one reinforced (CS+) and one not reinforced (CS-). We decided to describe mixture perception of flies using binary compound in this paradigm.

In this study, we performed three different protocols to investigate how flies perceive binary compound depending on the complexity of the task.

- The first experiment describes the ability to associate a mixture to a punishment and discriminate element composing a mixture AB by training flies to avoid a mixture AB in preference to a third odor C
- The second experiment concerns the ability to make a distinction between an odor alone or in presence of a second odor.
- The third experiment consists in testing patterning discrimination.

Material and Methods:

Fly stock and maintenance:

Our fly stock was derived from a wild-type *Drosophila melanogaster* population collected in the center of France (Chavroches) in 2006 and maintained in lab on standard food under a 12h/12h light/dark cycle. Flies were 3-5 days old in all experiments.

Odor quality and quantity:

In this study, 3 odors were used: Isoamylacetate, Ethylacetate and Benzaldehyde. We chose these odors in regards of previous experiment showing significant associative learning (Reaume *et al* 2011, Yarali *et al* 2009). To avoid odor bias, we tested different concentration on naïve flies and decided on the concentration which produces an equal distribution in both arms of the T-maze. These tests were performed on all the combinations using the same amount of odor. Finally, the concentration inducing the smaller bias was 300 µL of odor diluted in 150 mL of paraffin oil. In each protocol, the odors are represented by 3 letters A, B and C. All the combinations using the 3 odors were performed and then, we pooled the data in order to express learning score not specific to one odorant. For all conditioning and tests, flies received the same amount of odors between CS+ and CS- in conditioning and tests.

Conditioning assay:

The 3 experiments were performed in the classical olfactory conditioning. In each protocol of conditioning, the CS+ was always represented by the sign + after the odor or mixture punished. Reciprocal training was never performed but we assessed all the tests with naive flies in order to focus on the avoidance of the CS+ and not the eventual effects of the CS- attractive learning on flies. Memory scores were calculated as the difference in proportion of CS+ avoidance between the conditioned and naïve flies. Each letter A, B or C represents a same quantity of odor as, for instance, the mixture AB contains as much odor as the element CC.

Statistic analysis:

Statistical analysis were performed using the software SPSS. For each learning score, we calculated the 95% confidence intervals using t-scores for a Student-t distribution with df = N-1. Differences of learning scores between groups from different conditioning or test were analyzed by Tuckey *post hoc* test. Univariate ANOVA were also used to test the effect of conditioning on specific tests.

Results

Experiment 1: Mixture perception

In this experiment, we address the question whether a binary mixture conditioning is perceived by flies and whether the presence of a second odorant cue during training or conditioning could interfere on the learning task. Two different conditioning were performed.

- A first group was trained to avoid a binary compound in preference of a third odor CC: AB^+ vs CC. Twenty minutes after three conditioning cycles, flies were tested either for their ability to avoid the mixture (AB vs CC) or to avoid one element of the compound (AA or BB vs CC). Every odor represented once A, B or C so, we pooled the data of the tests AA vs CC and BB vs CC. In order to express in our learning score the specific ability to avoid the compound, the reciprocal training CC^+

vs AB was not performed and the learning score was calculated as the difference in the proportion of conditioned flies avoiding the CS+ minus the proportion of naïve flies avoiding the CS+.

- A second group of flies were conditioned to avoid an element alone AA in preference of a second odor CC: AA vs CC. Flies were tested for three different choices twenty minutes after three cycles of conditioning: AA vs CC, AB vs CC and BB vs CC in which B represents an odor never previously exposed to the flies. In the same way than the first conditioning, learning score were calculated as the difference of avoidance between conditioned and naïve flies.

The results obtained are presented in Figure 1 and Table 1.

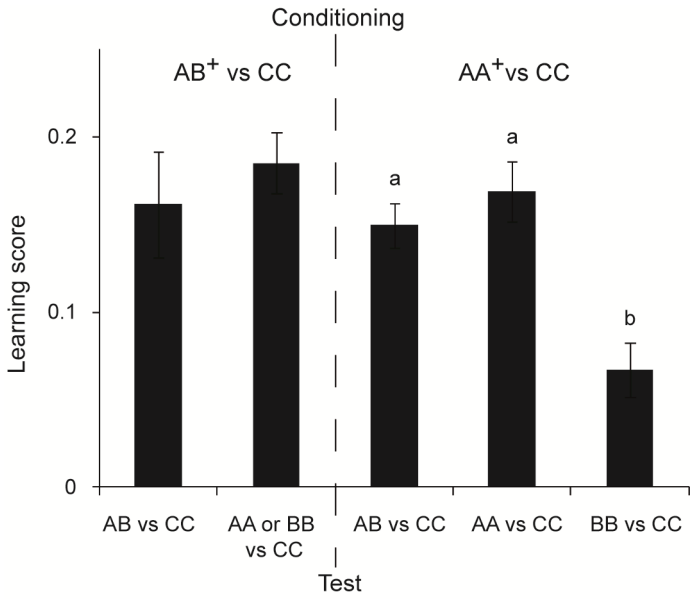


Figure 4: Learning scores after two different training: AB^+ vs CC and AA^+ vs CC. In all tests, the learning score is expressed as the ability to avoid the first odor or compound (e.g. in the test AB vs CC, the score represents the ability to avoid AB). Tuckey *post hoc* analyses were performed for the conditioning AA^+ vs CC.

Training	Test	N	PI	95% CI
AB^+ vs CC	AA vs CC	48	0.17 (± 0.05)	[0.10-0.22]
	AB vs CC	96	0.18 (± 0.05)	[0.15-0.22]
AA^+ vs CC	AA vs CC	96	0.16 (± 0.03)	[0.13-0.21]
	BB vs CC	96	0.05 (± 0.03)	[0.03-0.10]
	AB vs CC	96	0.17 (± 0.04)	[0.12-0.17]

Table 1: Shock avoidance score**s** after two different training**s** AA^+ vs CC and AB^+ vs CC (+ denotes the odor or mixture reinforced during conditioning). For all tests, we calculated the 95% confidence intervals on the means of learning scores indices using t-scores for a Student–t distribution with df = N - 1. PIs (Performance Index) are expressed as mean ± error standard mean. N: number of replicate per test. CI: Confidence interval.

After a conditioning AB^+ vs CC, flies showed a significant avoidance of the mixture and also of the odor composing the mixture. Moreover, there was no significant difference in the score of avoidance of the mixture or the odor alone ($F_{1,\ 143}$ = 0.54, P = 0.46). Flies that are trained to avoid the odor AA in the conditioning AA^+ vs CC were able to avoid not only the conditional stimulus AA but also the compound AB and there was no significant differences between both scores ($F_{1\ ,191}$ = 0.45, P = 0.5). However, after this training, even if flies showed significant score in the test BB vs CC, flies' response is lower than in the other tests.

In this experiment, flies conditioned to avoid a mixture were also able to avoid its elements. The Rescola-Wagner elemental model which assumes the compound is perceived as the sum of its element (AB = A + B) implies that each element is associated to the US, and then is able to produce the adequate behavior. However, the Pearce model implies that partial generalization is also possible between the compound and its elements (Pearce 1987, 1994). This result reflects the ability to generalize from a compound to its elements.

Experiment 2: Feature discrimination

The second experiment consisted in testing flies to solve problems known as feature positive and negative discrimination. This protocol is an extension of the first one in which the third odor CC was replaced by an odor of the compound.

- Feature positive discrimination: the odor AA alone is not reinforced but if this odor is simultaneously presented with another stimulus B, then reinforcement is delivered. Experimentally, it consists in a training AB^+ vs AA. Twenty minutes after 3 cycles of conditioning, flies were transferred in the T-maze for three different choices. We tested flies' choice AB vs AA, AB vs BB and BB vs AA.

- Feature negative discrimination is the opposite of the positive version: the odor AA alone is reinforced whereas the compound AB is not reinforced: AA^+ vs AB. Twenty minutes of three cycles of this conditioning, we also tested three different choices: AA vs AB, AB vs BB and AA vs BB.

- In control to these experiments, we also conditioned flies in unique odor training. In the feature positive discrimination, we called this protocol BB^+ vs AA and in the feature negative discrimination, this protocol was called AA^+ vs BB. But each odor was once A and once B, so the two protocols BB^+ vs AA and AA^+ vs BB are obviously the same thing and data were pooled.

  The score obtained are reported in the Table 2.

Training	Test	N	PI	95% CI
AB^+ vs AA	AB vs AA	96	0.21 (± 0.02)	[0.17, 0.26]
	AB vs BB	96	0.002 (± 0.02)	[-0.04, 0.04]
	BB vs AA	96	0.03 (± 0.03)	[-0.02, 0.08]
BB^+ vs AA (or AA^+ vs BB)	AB vs AA	191	0.18 (± 0.01)	[0.13, 0.18]
	BB vs AB	191	0.19 (± 0.02)	[0.19, 0.25]
	BB vs AA	191	0.24 (± 0.03)	[0.22, 0.28]
AA^+ vs AB	AA vs AB	96	0.18 (± 0.02)	[0.15, 0.22]
	AB vs BB	96	0.06 (± 0.02)	[0.02, 0.09]
	AA vs BB	96	0.25 (± 0.02)	[0.21, 0.29]

Table 2: Learning score after overlap conditioning AB^+ vs AA and AA^+ vs AB (+ denotes the odor or mixture reinforced). For all tests, we calculated the 95% confidence intervals on the means of learning scores indices using t-scores for a Student –t distribution with df = N - 1. PIs (Performance Index) are expressed as mean ± error standard mean. N: number of replicate per test. CI: Confidence interval.

After the feature positive discrimination AB+ vs AA (Fig. 2), flies showed significant score of avoidance of the compound. However, flies were not able to respond after the tests AB vs BB and BB vs AA. After the unique odor conditioning BB+ vs AA, flies had significant score for the three tests but the score was significantly higher in the test BB vs AA than in the two other tests AB vs AA and BB vs AB. The learning score to the test AB vs AA is not different for both training using mixture and odor alone ($F_{1, 287}$ = 1.82, P = 0.18).

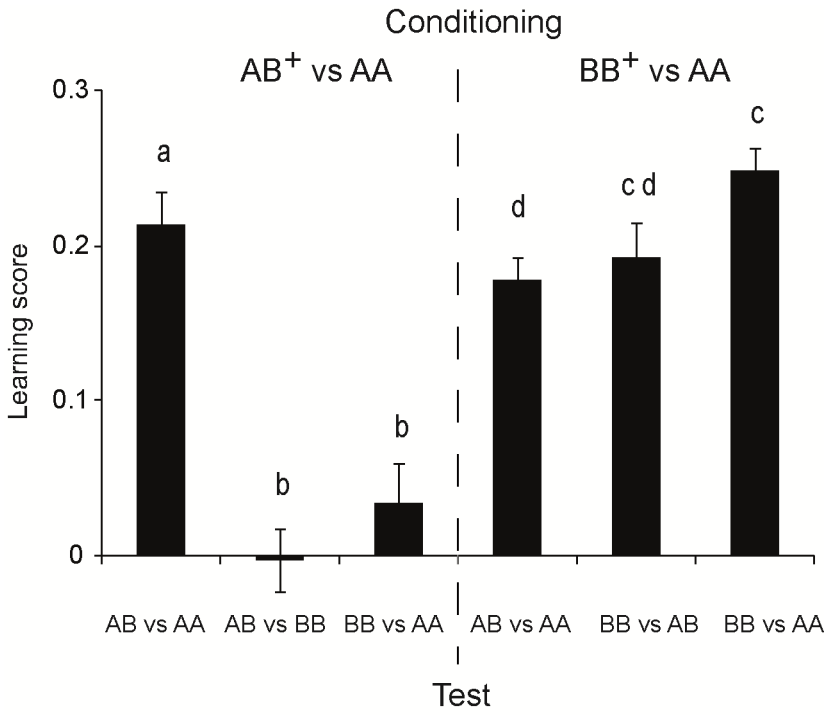


Figure 2: Learning scores after two different training: AB^+ vs AA and BB^+ vs AA. In all test, the learning score is expressed as the ability to avoid the first odor or compound (e.g. in the test AB vs AA, the score represents the ability to avoid AB). Tuckey post hoc analyses were performed for each conditioning session.

After a feature negative discrimination, flies were able to avoid the element alone AA in preference of the compound AB (Table 2, Fig. 3) and they also avoid efficiently AA in the test AA vs BB. However, even if the score is significant, the response to AB in the test AB vs BB is lower than the other test. Moreover, there was no effect of the conditioning protocol on the score after a test AA vs AB ($F_{1, 287}$ = 1.86, P = 0.17). Flies respond similarly to the test AA vs AB if they receive the protocol AA^+ vs AB or the protocol AA^+ vs BB.

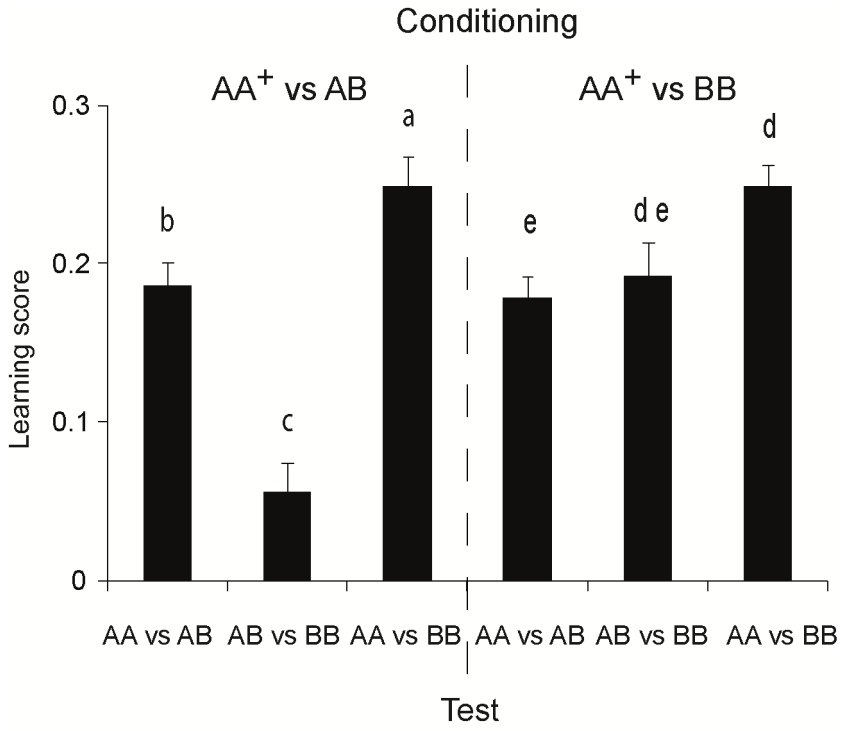


Figure 5: Learning scores after two different training: AA^+ vs AB and AA^+ vs BB. In all test, the learning score is expressed as the ability to avoid the first odor or compound (e.g. in the test AB vs AA, the score represents the ability to avoid AB). Separate Tuckey post hoc analyses were performed for each conditioning session.

Predictions of feature discrimination learning differ according to the way flies perceive the compound. In feature positive discrimination (AB^+ vs AA), the Rescola-Wagner model assumes that the element B is the more relevant stimulus in the discrimination task and would produce a stronger associative strength than A. The response in the test AB vs AA is consistent with this idea but the fact that flies were not able to respond to B alone in preference to the odor A in the test BB vs AA is in contradiction with the prediction. However, it seems logical that flies did not avoid AB in the test AB vs BB in regard of the presence of B in both sides. Results in the feature negative discrimination seem to be in agreement with an elemental model of perception. The attribution of a positive value to the odor B would explain the high level of avoidance of AA in the tests AA vs AB and AA vs BB. The low level of avoidance in the test AB vs BB can be explained by the presence of B on both sides. This low score may be a response to the higher concentration of B in the element BB than in the compound AB.

A configural representation means that the compound AB is perceived as new whole entity different of its element (AB = X). Such a mode of representation predicts that only the compound should be avoided and not the elements if presented alone. However, in feature positive discrimination (AB^+ vs AA), flies were not able to avoid AB if the other choice during the test was B. Nonetheless, results in feature negative discrimination can be explained by a configural perception. Flies were trained to avoid an odor alone AA in preference of the compound AB, so a positive value is attributed to the compound. However, in the test AB vs BB, even if the score is low, flies should not avoid the compound.

In the unique cue approach, the compound is perceived as the sum of its element plus a specific configural stimulus U (AB = A + B + U). The salience of each component in this equation is important to explain our panel of result. For instance, if we consider in the feature positive discrimination (AB^+ vs AA), that the component B is overrepresented than A or U because it was the only relevant stimulus, we can explain why flies were not able to respond to the test AB vs BB because B is represented in both side. But it does not explain why flies did not avoid B in the test BB vs AA. Another hypothesis is that the component A and B had similar value that could explain the absence of response in the test BB vs AA but with these parameters, flies should have been able to avoid AB in the test AB vs BB, because of the summation effect of the associative strength attributed to A and B.

Experiment 3: Patterning discrimination

In this third experiment, we tested flies' ability to solve positive and negative patterning discrimination.

- We first investigated whether flies were able to solve positive patterning (PP). In PP, flies were exposed to a reinforced compound AB^+ and to non reinforced element A and B. Flies received three cycle of conditioning and each cycle consisted in successive exposition of compound and element such as AB^+ / AA / AB^+ / BB. Twenty minutes after conditioning, flies were tested for their ability to avoid the mixture in preference of AA or BB.

- We also trained flies in the negative patterning discrimination task (NP) in which the compound AB was not reinforced but the elements A and B alone were shocked. Flies were exposed to three cycles of AB / AA^+ / AB / BB^+. Then, we tested flies ability to avoid each element alone AA or BB in preference of the mixture AB.

The scores obtained are reported in the table 3 and figure 4.

Training	Test	N	PI	95% CI
PP AB^+/AA/AB^+/BB	AB vs AA	48	0.2 (± 0.02)	[0.20, 0.30]
	AB vs BB	48	0.25 (± 0.03)	[0.15, 0.25]
NN AB/AA^+/AB/BB^+	AA vs AB	48	-0.03 (± 0.03)	[-0.09, 0.02]
	BB vs AB	48	-0.06 (± 0.03)	[-0.12, 0.01]

Table 3: Learning score after Positive (PP) and Negative (NP) Patterning (+ denotes the odor or mixture reinforced). For all tests, we calculated the 95% confidence intervals on the means of learning scores indices using t-scores for a Student –t distribution with df = N - 1. PIs (Performance Index) are expressed as mean ± error standard mean. N: number of replicate per test. CI: Confidence interval.

In PP discrimination, flies avoided the mixture efficiently in preference to the elements alone AA or BB. After a NP discrimination task, it appears flies were not able to solve this problem by avoiding the elements. Nonetheless, the scores were not different from 0 (Table 3).

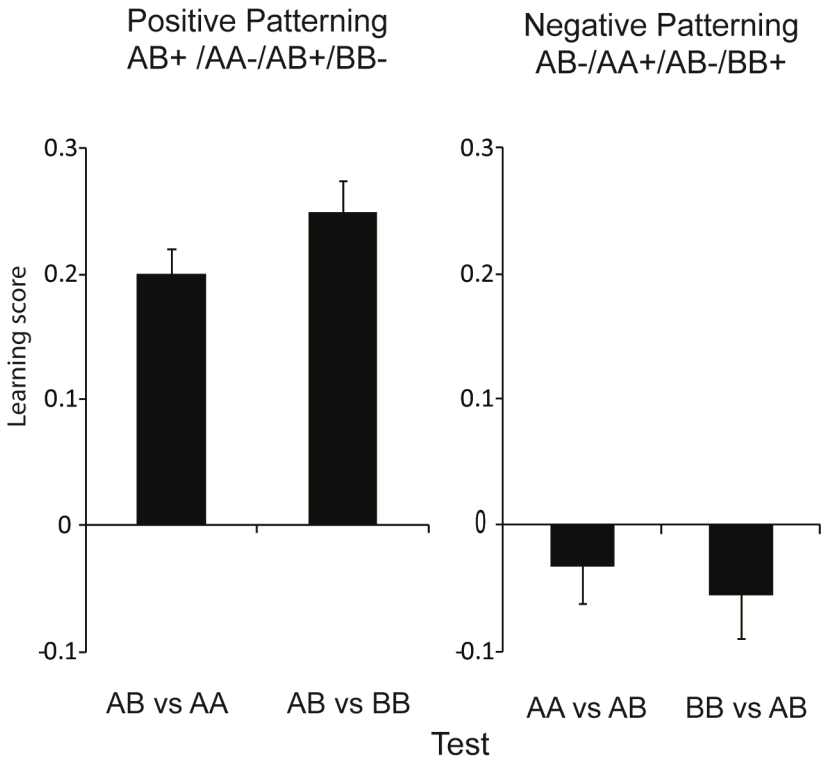


Figure 4 Learning scores after positive and negative patterning discrimination. In all test, the learning score is expressed as the ability to avoid the first odor or compound (e.g. in the test AB vs AA, the score represents the ability to avoid AB). Differences between test for each protocol were tested with univariate ANOVA analysis and no effect of test were observed (Positive patterning: $F_{1, 94} = 2.587$, P = 0.11; Negative patterning: $F_{1, 94} = 0.264$, P = 0.61).

The elemental theory can explain our result in the positive patterning. The associative strength of each element could be below the threshold needed to induce conditioned response but once added during the presentation of the mixture, they might result in an associative strength above the threshold required to induce the conditioned response. However, the null score after a negative patterning protocol is not consistent with the elemental perception. In negative patterning the elemental model predicts that the associative properties of A and B will summate so that the response to AB would be consistently stronger than the response to A or B alone and induce an avoidance of the compound AB. However, flies did not avoid significantly the mixture. In the unique cue approach, the cue created by the compound AB would acquire negative associative strength and eventually result in little or no responding during the compound which is consistent with our result. Obviously, a configural representation of the mixture in negative

patterning discrimination would result in a significant avoidance of the elements alone because the elements were perceived differently than the compound.

Conclusion

In this study, we assessed a range of protocols involving mixture perception in order to assess the question about different models of perception used to solve discrimination learning in an associative conditioning. We first discovered that flies are able to associate a binary compound to a mechanical shock and that they are able to generalize from a compound to each element (experiment 1). In experiment 2 (feature discrimination) and 3 (patterning discrimination), even if some results cannot be directly explained, we demonstrated that flies are not able to perform negative patterning.

In nature, environmental variability implies that animals are rarely confronted twice to the identical stimulus (Shepard 1987, 1994). Thus learning would be useful only if animals are able to respond to stimuli that are perceptually similar but not identical to the one that was previously associated with the reinforcement. The first experiment demonstrated flies' ability to generalize from a compound to its elements in a quite simple discrimination task. However, in feature discrimination learning, flies were not all the time able to generalize from the compound to the elements when the mixture was shocked. The fact that there was an overlap of odors in the discrimination task seems to have blocked the generalization process. Generalization ability is correlated with the level of similarity between the stimulus learned and the one that animals have to respond but in discrimination learning, we assume that the CS- is also involved. After a conditioning AA+ vs CC, flies partially respond to the test BB vs CC which suggest that flies associated the CS- to a safe condition. Furthermore, in this experiment, the CS- is completely different from the compound, so we assume there is no difficulty to respond to a stimulus partially similar to the CS+. However in feature positive discrimination, the CS- and the CS+ already have one common element that can block the generalization when a stimulus similar to the CS+ is presented during the test. This result indicates that generalization ability is dependent on the number of common elements between the CS+ and the CS-. In

2003, Deisig *et al* developed a modified version of the unique cue theory to explain how the number of common elements between the reinforced and the non-reinforced stimulus influences perception in honeybees. Other studies demonstrated the flexibility of the generalization process. For instance, Livermore *et al* (1997) demonstrated that lobsters that are only trained to avoid a binary odor compound AX do not generalize from the mixture to its elements, but after being trained to avoid a compound AX in preference of a compound AY, animals are able to generalize from AX to X and from AY to Y. This result indicates that lobsters use elemental perception in order to behave adequately to the task but when lobster are just trained to avoid a compound AX without CS-, lobsters produce a configural representation of the compound. So, the generalization seems to be a plastic system depending on the task.

In order to demonstrate configural perception in animals, negative patterning is the perfect discrimination learning because it can be solved only using configural representation of the compound. *Drosophila* were not able to perform negative patterning; however, the results in negative patterning indicate that flies were able to create a configural stimulus of the compound but the associative strength attributed to this cue is not inhibitory enough to compensate the excitatory associative strength attributed to the elements. We conclude that even if flies did not produce a pure configural representation of a compound, the elemental model is not the only way they use to process complex environment. The unique cue theory seems to be a model that can explain our results in feature positive and negative patterning discrimination. Previous studies suggest that the associative strength between the elemental or configural cue and the unconditional stimulus is dependent on its salience in the particular task they have to perform. In flies' perception, our results indicate that a compound is generally elementally perceived, but if this kind of perception is not sufficient to solve the discrimination, flies can attribute an associative strength to a configural cue specific to the compound even if this was not behaviorally efficient in our protocols. We also note that negative patterning discrimination is the only protocol in which flies must learn two different stimuli A and B. A possible explanation is that flies are not able to lassociate two different stimuli independently with the mechanical shock. A control conditioning should be performed using different odors for the CSs+ and the CS- (AA+/CC-/BB+/CC-) to test this hypothesis.

Recently, Young *et al* made the same experiment on flies and obtained the same pattern of results. The authors also used algorithm models to produce predictions about behavior response considering each model of perception. Simulations could be a useful tool in order to test how saliency of elemental or configural cue influences the way to perceive compounds in different contexts.

To conclude, elemental and configural processing do not seem to be exclusive. Various species from vertebrates to invertebrates are able to use both ways of perception to process complex environments. Animals adapt their perception to the context in order to produce the most adequate behavior in regard of the task. Elemental perception seems to be the basal model of perception but in environmental conditions that require partial or total configural perception, animals can shift their perception from elemental to configural. In honeybees, extended phases of conditioning induce this shift and we wonder if more cycles of conditioning in Drosophila would enhance configural representation. This study suggests that flies do not only elementally perceive complex environment but also use a form of non-elemental perception. The genetic bases of specialized perception need to be investigated to understand how evolution acts on this process. Unlike honeybee or others invertebrates generally used in that kind of study, Drosophila genome is intensively described and genetic construction can be easily obtained which suggests that perception studies in Drosophila could bring new information about adaptation to complex environments.

Chapter 2- Genetic variability of olfactory learning and memory ability

Cognition is generally assimilated to the processes by which the animals perceive, learn, remember and act in consequence to environmental information. A lot of studies investigated the neuronal network and the molecular pathways producing and regulating these mechanisms. However, the genetic bases that maintain phenotypic variation among these processes and the genetic interaction existing between memory phases remains unknown. Here, we quantified learning and memory abilities in 40 wild-derived lines of *Drosophila melanogaster* and report a high variation of general cognition resource allocation probably caused by the variance of inbreeding depression among the lines. Moreover, these lines were also evaluated to other phenotypic traits as longevity or starvation resistance in other laboratories in order to identify eventual pleiotropic effect among traits. Comparisons between cognitive and life history traits variation did not indicate genetic correlations which could be explained by a strong pattern of gene by environment interaction.

Introduction

Natural populations are able to express a large range of phenotypic variation for different aspect of animals' biology from behavior to physiology. Phenotypic variation is mostly due to segregating alleles at many interacting genes with environment effect (Falconer and Mackay 1996). Investigating the genetic basis of such variability seems crucial to understand the relation between DNA and phenotype and further adaptive evolution of quantitative traits. The ultimate aim of this field is to elucidate the genetic underpinnings that control the interdependence of multiple phenotypes, and to address the question of the genetic basis of genotype by environment interactions.

An international project initiated by Trudy Mackay (North Carolina University) has proposed to study the genotypic and phenotypic variation of a natural population of *Drosophila melanogaster*. The Mackay lab has derived a set of 345 inbred lines from Raleigh (NC) natural popation by inbreeding isofemale lines to homozygosity by 20 generations of full sib mating; such that each line represented a homozygous genotype which differs from the other lines. The homozygosity of a subset of 40 of these lines has been verified by analysis of microsatellite markers and this core set of lines comprises the genetic reference panel. This project proposed the sequencing of this panel and, in parallel, the collection of extensive information about complex trait phenotype. These lines create a new innovating genetic tool for the Drosophila community. These lines can be evaluated for different complex traits which give the opportunity to test pleiotropic effects among traits. Physiological, morphological and behavioral traits were measured in Trudy Mackay lab and other research groups. Comparisons between the genetic structure and the phenotypic variation would help to identify genetic modules involved in different complex traits and finally to find some candidate genes. It also addresses the important question about the genetic basis of gene by environment interactions.

In our lab, we quantified learning and memory capacities in this reference panel. Heritability of learning and memory ability has already been demonstrated through experimental selection in oviposition site choice (Mery and Kawecki 2002, Dunlap and Stephens 2009) which is based on an olfactory Pavlovian training. These studies demonstrated that learning ability is variable enough in natural populations to respond to selection. Another protocol using olfactory stimuli is the classical aversive olfactory conditioning

in which flies are conditioned to associate an odor (CS) to a mechanical shock (US) (Mery and Kawecki 2005). Considering the olfactory memory formation in this protocol and the debate about the interaction between the phases, a genetic study of these processes would help scientists to dissect memory phases and understand these interactions. We addressed the question of the phenotypic variability of this cognitive trait among the lines. These results could provide 1) a better understanding about the genetic dynamics and the variability of memory phases and 2) a knowledge of the relationship between memory ability and other phenotypic traits.

Materials and methods

Fly stock and maintenance:

40 inbred lines from a natural population (Raleigh, North Carolina) were derived by 20 generations of full-sib mating. These lines do not contain residual heterozigosity according the whole genome marker analysis. This reference panel is viable and maintained in lab under a cycle light/dark 12h/12h.

Statistical analysis:

For each measurement, a memory score was calculated as the difference of the proportion of flies conditioned to avoid MCH and choosing OCT during the test versus the proportion of flies conditioned to avoid OCT and choosing OCT. To compare memory scores, all proportions were angularly transformed before statistical analyses.

Results and discussion

Variation in learning and memory phases ability in 40 wild-derived inbred lines:

Learning and memory abilities were evaluated on this panel of 40 wild-derived inbred lines. Short-term memory (STM), anaesthesia resistant memory (ARM) and long-term memory (LTM) were tested in a classical olfactory conditioning (see Materials and methods). STM was tested 20 min after 3 cycles of conditioning whereas LTM and ARM were tested 24h after either 5 spaced cycles or 5 massed cycles respectively. First, we quantified variation among these lines (Fig. 1) and found substantial genetic variation in STM, ARM and LTM (Table 1 : STM: $F_{1,39} = 2.346$, $P < 0.001$; ARM: $F_{1,39} = 1.691$, $P < 0.01$; LTM: $F_{1,39} = 1.348$, $P < 0.05$).

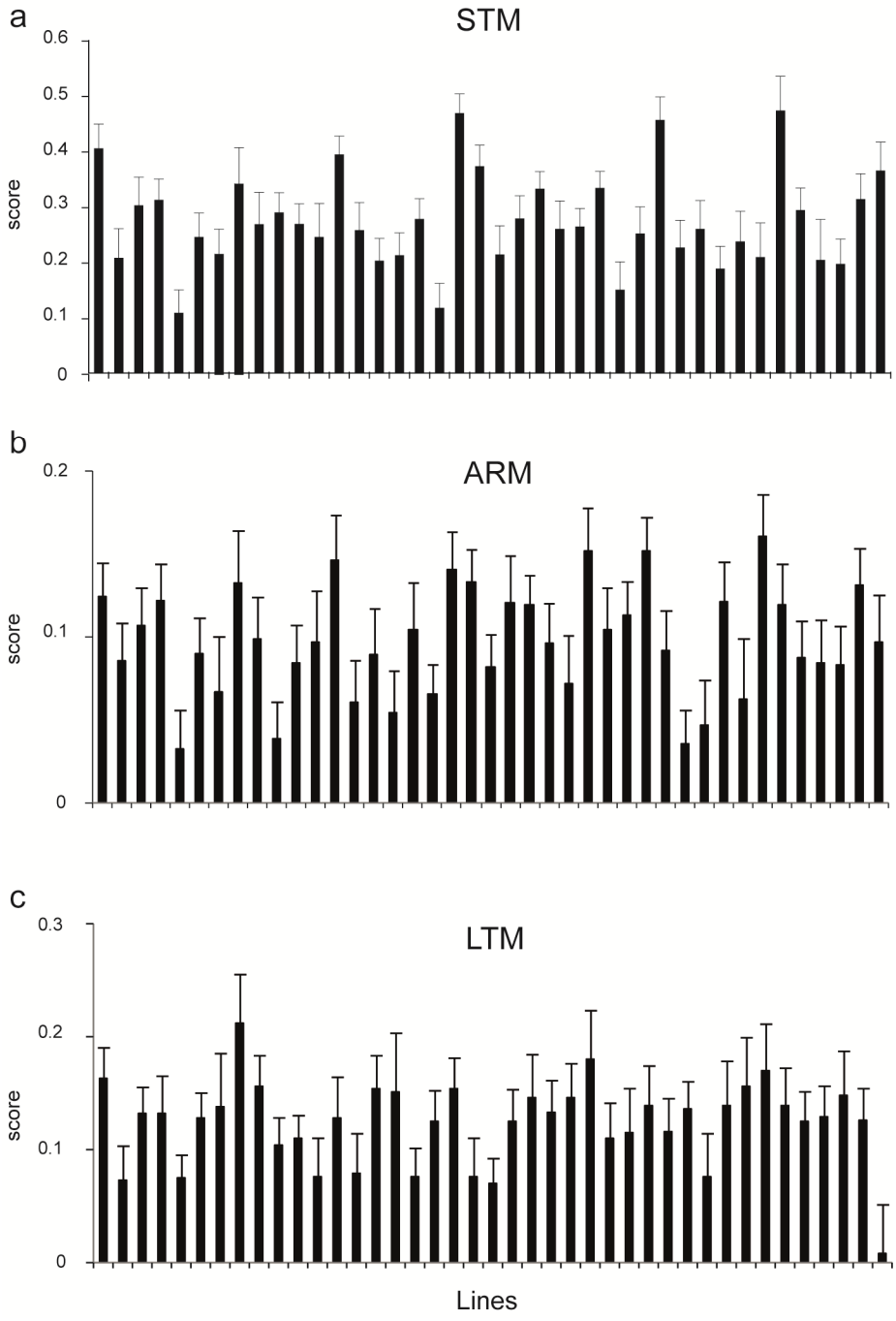


Figure 1: Variation for learning and memory phases among 40 wild-derived inbred lines. **(a)** Short-term memory (STM) (N > 12) **(b)** Anaesthesia resistant memory (ARM) (N > 25) **(c)** Long-term memory (LTM) (N > 25) **(a-c)** Scores are expressed as mean ± s.e.m.

Trait	Mean	σ^2_L	σ^2_E	σ^2_P
STM	0.25	0.0065****	0.039	0.045
ARM	0.113	0.0016**	0.019	0.02
LTM	0.154	0.0022*	0.019	0.021

Table 1 Estimates of genetic variance. σ^2_L: among lines variance component. σ^2_E: environmental variance component (within replicates of lines). σ^2_P: total phenotypic variance ($\sigma^2_L + \sigma^2_E$). (*: P < 0.05, **: P < 0.01, ****: P < 0.001)

This panel of inbred lines allows strict comparison of phenotypic complex traits. We asked whether there were significant genetic correlations among these memory phases, as would occur if segregating alleles have pleiotropic effects on these traits in the same or opposite direction. Genetic correlations between STM and both consolidated forms ARM and LTM is high (Fig. 2 and Table 2) which indicates that lines with a better STM also have a better ability to consolidate memory.

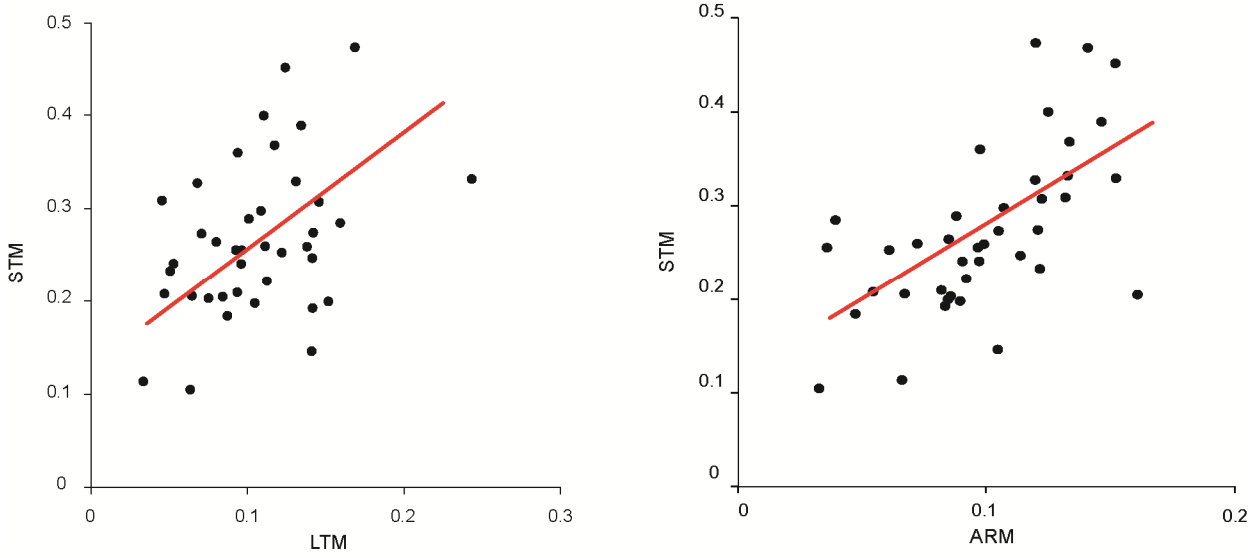


Figure 2 Genetic correlation between STM and either ARM **(a)** or LTM **(b)**. Each dot represents one of the 40 wild-derived inbred lines.

We also address in this study the question about the interaction between ARM and LTM which is still unclear. The comparison showed a significant positive correlation between ARM and LTM (Fig. 3 and Tab. 2).

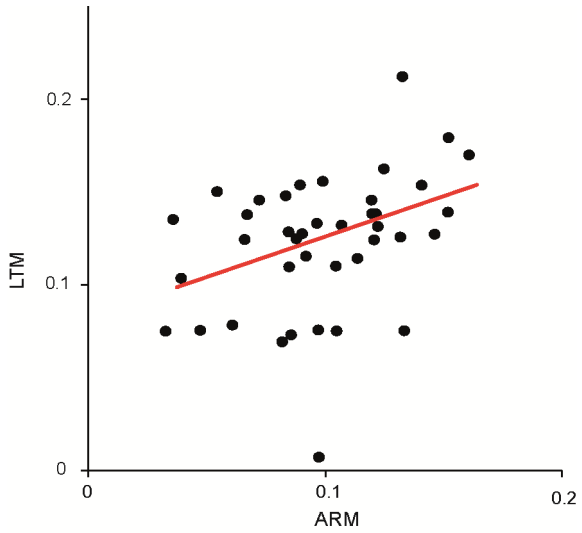


Figure 3: Genetic correlation between ARM and LTM among the 40 inbred lines

	STM	ARM	LTM
STM	1	0.643**	0.367*
	ARM	1	0.346*
		LTM	1

Table 2: Genetic correlations between STM, ARM and LTM (*: P < 0.5, **: P < 0.01)

We observed in this panel of wild-derived inbred lines a significant genetic variability of learning and memory abilities. Moreover, comparisons between phases demonstrated positive correlations.

The first point to discuss in this study concerns the origin of this panel. Inbreeding is known to affect life history traits (Latter and Sved 1994, Latter *et al* 1995, Crnokrak and Roff 1999, Keller and Waller 2002, Lyons *et al* 2009) causing inbreeding depression. Consequences of inbreeding depression on cognitive capacities are documented by few studies for different processes : deficits in parental behavior in mouse (Margulis and Altmann 1997, Margulis 1998), courtship behavior in the housefly (Meffert and Bryant 1991). The effect of inbreeding on learning has been examined in rats, where it causes a deficit of spatial learning ability (Harker and Whishaw 2002) and fruit flies in which inbreeding causes a mild effect on olfactory learning (Nepoux et al 2010). Among the inbred lines, we observed some lines with morphological and

physical deficits (small flies, bad locomotion, few eggs …) and noted that these lines were the ones that obtained low learning and memory scores. Inbreeding may have created a high level of variance in general cognitive abilities among the lines and the positive correlation among the phases reflects the variability of general cognitive functions more than the variability specific to each phase. In this panel, we should consider that memory abilities are certainly under the dependence of unrelated traits as locomotion or olfaction. Considering that the variability for these other traits is high, the correlations observed between the memory phases therefore reflect the correlations between cognition and these unrelated parameters. Previous examples on sex functions showed that phenotypic trade-off between the allocation to the sex function depends on two main factors: the amount of variation in the reproductive resource budget and the amount of variation in sex allocation (Van Noordwijk and De Jong, 1986, Scharer *et al* 2005). In our cognitive system, we can make the hypothesis that the phenotypic correlation between the memory phases depends on both the variation in cognitive resource budget related to other fitness-related parameters and the variation in resource allocated specifically to each phase (Fig. 4). Whether the variation in cognitive resource budget is higher or lower than the variation in resource allocated to the memory phase would result in a positive or negative correlation respectively. We supposed that the variation in cognitive resource budget is strong in regard of the inbreeding which could explain the positive correlations between the different memory phases and that these correlations do not reflect the interaction between the memory modules.

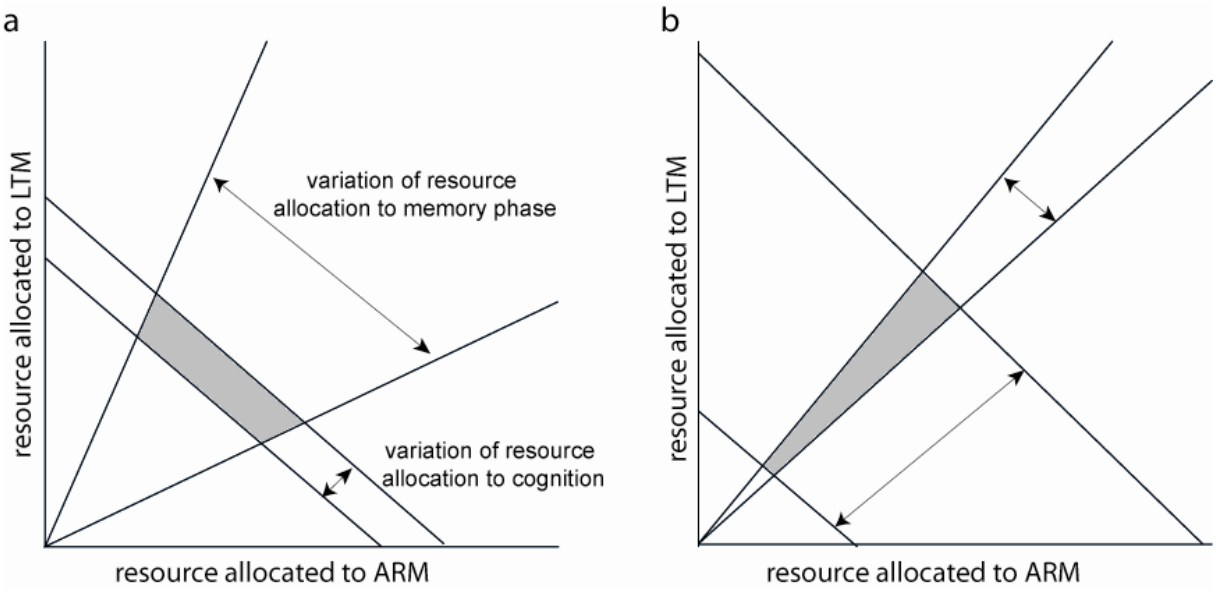


Figure 4 Graphical representation of the effect of the variation of cognition resource budget on the correlation between ARM and LTM ability. The shaded areas depict the allocation values we expect to observe given different combinations of variation in budget and memory allocation. Note that the trade-off between ARM and LTM allocation can only be observed if the variation in the memory phase allocation is large and the variation in the budget is low (left panel). Failure to control for resource budget therefore leads to a positive correlation (right panel) (adapted from van Noordwijk & De Jong, 1986).

Another way to explain these correlations is related to the protocol. We tested STM abilities applying three cycles of conditioning and we tested flies at least 20 minutes after the beginning of the training. Then, in the tests, flies may express not only STM but also ARM ability which creates a bias in the correlations observed.

Genetic correlation among phenotypic traits:

In Trudy Mackay lab, they also quantified multiple complex traits as longevity, starvation resistance, chill coma recovery (resistance to cold).

	ARM	LTM	LS	CCR	SR
STM	0.643**	0.367*	- 0.071	0.221	0.202
	ARM	0.346*	- 0.027	0.197	- 0.045
		LTM	- 0.109	0.048	- 0.056
			LS	0.12	0.29*
				CCR	0.4*
					SR

Table 3 Genetic correlation between phenotypic traits including learning and memory phase and other phenotypes quantified in Mackay lab (LS: Lifespan, CCR: Chill coma recovery, SR Starvation resistance)

They found substantial variations for all traits they quantified, with estimates of H^2 between 0.25 and 0.54 (Ayroles *et al* 2009). We asked whether there were significant genetic correlations between these traits and our data on learning and memory ability, as would occur if segregating alleles have pleiotropic effect on two traits in the same or opposite directions. The comparison between these phenotypes and our data did not indicate any significant positive or negative correlations (Table 3). This lack of correlations does not necessarily mean that there is no interaction between the traits. A strong effect of a gene by environment interaction can explain the absence of genetic correlations by the presence of a high environmental variability. The other phenotypic traits had been evaluated in different environments and particularly were fed with different foods. Interaction between behavior performance and laboratory environment has been found to produce strong effects. Crabbe *et al* (1999) studied the influence of laboratory environment on six behaviors in several inbred lines of mice and demonstrated systematic differences in behavior across laboratories. Comparisons between the cognitive performance and phenotypic traits may only reflect a strong effect of gene by environment interactions that cannot allow determining the genetic bases of pleiotropic effect between traits. Quantifying others traits, in particular olfaction and locomotion, in my lab would have provided different results that may correlate with the memory scores. Then, the positive correlations between the memory phases could result more on other traits variability than on the direct interaction between the phases.

In parallel of the phenotype description, they assessed whole-genome variation transcript abundance of each of these lines and found that the genome is highly correlated at the transcriptional level, which imposes constraints on the evolution of transcriptional genetic networks. They obtained 10 096 variable transcripts which were genetically correlated among the lines and grouped them into 241 modules using an identification method of separable clusters of highly interconnected genes (Ayroles *et al* 2009). Finally, they compared these genetic modules with the variation of complex traits. Unfortunately, when our behavioral data were compared to the transcriptional networks, no modules seemed to be associated with learning or memory phenotypes whereas other traits have shown significant correlations with transcriptional modules as alcohol sensitivity (Morozova *et al* 2009), sleep (Harbison *et al* 2009) or aggressive behavior (Edwards *et al* 2009).

In conclusion, this panel of 40 wild-derived inbred lines demonstrated significant correlated phenotypic variability of memory abilities that may be explained by general cognitive variation among the lines certainly related to others fitness-related traits. Even if this was not demonstrated in these lines, we suppose that natural variation of these mechanisms could also be explained by specialized adaptive modules which respond to particular environments. Moreover, the lack of correlated response between olfactory memory abilities and other phenotypic traits among these lines suggests a strong pattern of gene by environment interaction.

Chapter 3-Evolutionary trade-off between two forms of consolidated memory

Memory is a complex and dynamic process composed of different phases. Its evolution under natural selection likely depends on a balance between fitness benefits and costs. Learning and memory capacities have been found to trade-off with other fitness related traits but very little is known about the pattern of genetic correlations among different memory phases which may strongly affect cognitive evolution. In Pavlovian aversive olfactory conditioning in Drosophila, two separate forms of consolidated memory phases can be formed: Anesthesia Resistant Memory (ARM) and Long-Term Memory (LTM). ARM is formed after repeated conditioning sessions followed one another immediately, whereas LTM formation requires time intervals between conditioning events. Both memory types last more than 24h but, unlike ARM, LTM requires *de novo* protein synthesis to be induced. In recent years, several studies have focused on the differences between these long-lasting memory types, but how they evolve and interact remains unknown. Furthermore, at the functional level, whether ARM and LTM are processed in parallel or are mutually exclusive has been intensively debated.

In this study we report that fly populations selected specifically for improvement of one consolidated memory phase show reduced performance for the other memory phase. This is the first evidence of an evolutionary symmetrical trade-off between two memory phases for the same learning task. Such trade-offs may have an important impact on the evolution of cognitive capacities and help explain why evolution has maintained variations of two consolidated memory phases. On a neural level, these results support the hypothesis that mechanisms underlying these forms of consolidated memory are, to some degree, antagonistic. We also addressed the question about the cost induced by the experimental selection and evaluated some phenotypic traits. Even in no effect of selection were observed on fecundity, larval development or oxidative stress resistance, longevity measurements have shown a specific constitutive cost of LTM improvement on male longevity.

Introduction

Memory is a product of evolution and the degree to which information is maintained in the brain varies among species and among different types of behavior. Its evolution under natural selection likely depends on a balance between fitness benefits and costs (Dukas 1999). Learning and memory capacities have been found to tradeoff with other fitness-related traits as longevity or larval competition (Roth *et al.* 2010; Burger *et al.* 2008; Mery and Kawecki 2003). Burger *et al.* have shown that selection for longer life in *Drosophila* induces a decrease in learning capacities at young age. Interestingly, they also demonstrated that this trade-off seems to be symmetrical because flies selected for high-learning abilities have a shorter lifespan than unselected flies.

In *Drosophila*, memory formation is not a direct flow from short-term to long-term storage. Evidence points instead to a complex, multiphase pathway of memory consolidation. These findings raise the question of the functional and evolutionary relationships among, as well as the genetic basis of, these different components of memory. The evolution of learning and memory is thus likely to depend on the genetic diversity of each memory phase and on the pattern of genetic correlations among these different memory phases. Moreover, the genetic network existing between the different memory phases and lifespan need to be described to understand how adaptive behavior lead to life history trait evolution.

In Pavlovian aversive olfactory conditioning in *Drosophila*, two separate forms of consolidated memory phases can be formed: Anesthesia Resistant Memory (ARM) and Long-Term Memory (LTM). ARM is formed after repeated conditioning sessions following one another immediately, whereas LTM formation requires time intervals between conditioning events. Both memory types last more than 24h but, unlike ARM, LTM requires de novo protein synthesis to be formed (Tully *et al.* 1994). Screening for mutants with learning deficiencies has led to identification of a number of loci whose products are involved in the processes of learning and memory in Drosophila (Dubnau and Tully 1998). Such mutants are an invaluable tool in uncovering neurobiological and molecular bases of these phenomena. However, these mutant alleles typically have other deleterious effects (Waddell and Quinn 2001, Sokolowski 2001) and would presumably be strongly selected against in natural populations. It is an open question whether the genetic variation for

learning and memory segregating in natural populations involves milder alleles of those loci, or some other loci (Tully and Quinn 1985, Tully *et al.* 1994). As this genetic variation acts on natural selection, uncovering its genetic bases is important for understanding microevolutionary changes of learning ability. In the present study, we directly addressed the question of the genetic diversity and correlation between ARM and LTM by imposing an artificial selection regime on populations of *Drosophila* for either specific ARM or LTM improvement and determining the extent to which selection on one memory phase affects formation of the other memory phase. Unlike ARM, LTM formation is known to be costly and to induce a decrease of conditioned flies' longevity (Mery and Kawecki 2005). Knowing that learning ability trades with other traits, we quantified longevity, fecundity, stress resistance and development time to demonstrate eventual constitutive costs of LTM improvement.

We report that fly populations selected specifically for improvement of one consolidated memory phase show reduced performance for the other memory phases suggesting an evolutionary symmetrical trade-off between two memory phases for the same learning task. The existence of such trade-offs may have an important impact on the evolution of cognitive capacities. Moreover, LTM improvement has specific constitutive costs that decrease longevity in males and so, males selected for ARM improvement showed extended lifespan.

Materials and Methods

Fly stock and maintenance:

Our base stock population was derived from a wild-type *Drosophila melanogaster* population collected in the center of France (Chavroches) in 2006 and maintained in the lab under 12h/12h day/light cycle. All flies used were 3-5 days old after emergence.

Selection regimes:

The experiment consisted of two selection regimes ARM and LTM (Fig. 1). For each selection regime, eight replicate lines were selected for specific memory improvement whereas eight other lines were kept as

control lines. We selected memory abilities under the protocol of olfactory associative conditioning. Every generation, each line was divided into two groups. One group was conditioned to avoid OCT and the other to avoid MCH. Each group was conditioned on one day and was tested on the next day (Fig. 1). For the ARM selection regime, a massed conditioning protocol was used whereas a spaced conditioning protocol was used for the LTM selection regime. For the selected lines, only flies going towards the CS- odor were kept for breeding the next generation. For the control lines, flies choosing either the CS- or the CS+ were kept. To avoid differential inbreeding between control and selected lines, the same number of flies was kept for control line X ($1 \leq X \leq 8$) as the number of flies making the "correct choice" for selected line X. For each line, after testing and counting, selected flies were grouped and kept for three days on standard food medium. On day 4, they were allowed to oviposit on standard food and the eggs were kept to form the next generation.

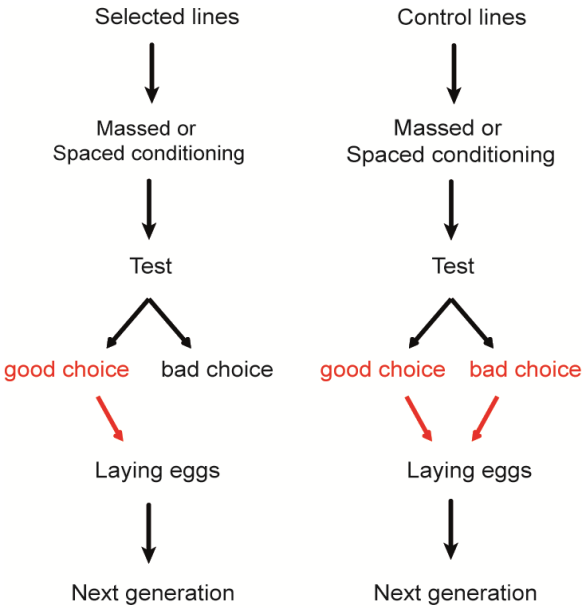


Figure 1: Experimental selection design: From a natural population, 16 lines were conditioned in the Pavlovian olfactory paradigm; then, 24h later, memory retention was tested in a T-maze. Only flies making the correct choice were kept to breed the next generation. Control lines were bred using a random selection of flies after the test.

For each measurement, a memory score was calculated as the difference of the proportion of flies conditioned to avoid MCH and choosing OCT during the test versus the proportion of flies conditioned to

avoid OCT and choosing OCT. To compare memory scores, all proportions were angularly transformed before statistical analyses (Sokal and Rohlf, 1995). Differences between scores were tested using the SPSS software. ANOVA were performed on memory scores for each selection regime by including selection type (control *vs.* selection) as the fixed factor and the line as a random factor nested within selection type.

Behavioral assay with electric shock:

Selected and control lines for both regimes were tested in a protocol of associative learning in which flies associate an odor to an electric shock. This protocol is similar to the one used to perform the selection except for the US. In the conditioning phase, groups of 50 flies were placed in training chamber covered with electrifiable copper grid. Flies then were exposed sequentially to two odors (3-octanol (52µL in 160 mL of paraffin oil) or 4-methylcyclohexanol (58µL in 160 mL of paraffin oil)), which were carried through the training chamber in a current of air, and received electrical shocks during exposure to the first (CS+) but not the second (CS-) odor. Flies first were exposed for 60 s to the CS+ (either OCT or MCH), during which time they received the US (twelve 1.5 s pulses of 60 V DC electric shock at 5 s interpulse intervals). After the CS+ presentation, the chamber was flushed with fresh air for 45 s. Then, flies were exposed for 60 s to the CS- (either MCH or OCT), which was not paired with the US. After the CS- presentation, the chamber again was flushed with fresh air for 45 s. This procedure constituted one training cycle. ARM was induced after 5 massed cycles and LTM was induced after 5 cycles spaced by 15 min interval. The protocol of testing is identical to the one used for the selection experiment.

Longevity:

After emergence, groups of 50 virgin males or females were transferred into a box containing one vial of food. We changed food once per week and counted the number of dead flies twice per week. We measured longevity on the two selection regimes (8 control and 8 selected lines for each regime) with two boxes per sex and per line (32 lines, 2 sexes, 2 replicates = 128 boxes).

The effect of selection regimes on longevity was analyzed by comparing mortality rate of each group (selected versus control) using a package specific to mortality rate analysis on the software R derived from Winmodest (Pletcher 1999). For each sex, we first used Akaike's Information Criterion to select the most parsimonious model and found that the logistic model is the best suited to our data. Then, for each sex, we fitted this model:

$$\mu = \frac{ae^{bx}}{1 + a\frac{s}{b}(e^{bx} - 1)}$$

where μ is the instantaneous mortality rate at age x; a, b and s are the parameters of the model: a is the initial mortality rate, b is the rate at which mortality increases with age and s is the deceleration of the mortality rate. The observed mortality rate were estimated by

μ_x = - ln (p_x) / Δx where p_x is the proportion of flies surviving from age x to age $x + \Delta x$.

Fecundity:

To assay fecundity, virgin females were isolated for each selected or control line and kept in vials with fresh food until they are 3 days old. Then, groups of 5 females were separated and mixed with 3 wild-type males for 2 days. After mating, flies were transferred in boxes where females were allowed to lay eggs on a Petri dish filled with agar for 24h. We added an attractive odor to the agar to stimulate oviposition (banana odor). We measured fecundity of the 4 replicates for each line in each treatment (8 selected versus 8 controls) in the two selection regimes (ARM versus LTM). This assay was performed after 42 generations of selection.

We analyzed the fixed effect of treatment and the random effect of replicate lines nested within the treatment (selected or control) for both selection regimes on the number of laid eggs, using a generalized linear model.

Stress resistance:

To assay paraquat resistance, we isolated groups of 20 flies and kept each group in vials with fresh food. One day after, we transferred groups of flies in new vials without food but containing filter paper imbibed with paraquat solution (33mM) diluted in 5% sucrose solution. After 30h of treatment, flies were removed from the vial and dead flies were counted. We measured mortality rate for 5 replicates of each selected or control lines for both regimes of selection (5 replicates, 2 treatments of 8 lines each, 2 regimes of selection = 160 vials). We analyzed the fixed effect of treatment and the random effect of lines nested within each treatment using a generalized linear model.

Larval development:

To assess larval development, groups of about 100 flies from each selected or control line were transferred in boxes where females were allowed to lay eggs in one Petri dish filled with agar for 12h. From each Petri dish, 5 groups of 30 larvae were isolated and transferred in vials containing standard food. We evaluated the time of development by counting the number of lies emerging each 12h until no flies emerged anymore. Statistical analysis was perfomed on the time for 50% rate of emergence and also the final percentage of emergence using univariate ANOVA using SPSS software.

Results

Trade-off between ARM and LTM

We performed two independent artificial selections on ARM and LTM. Every generation, groups of adult flies were conditioned to associate one of two odors with an aversive mechanical shock. Five training sessions were performed, either separated by 20 min intervals (spaced protocol) or not separated by time intervals (massed protocol) depending on the selection regime (LTM *vs*. ARM selection regime, respectively). Twenty-four hours after conditioning, the flies' choice between the two odors was tested in a T-maze in order to assess their memory of the odor-shock association (Tully *et al.* 1994). For each selection regime, 16 lines derived originally from a single stock population were used. Eight of these lines were selected for

improved memory and eight were kept as control lines. Selection for improved memory was performed by breeding only flies which avoided the odor previously associated with the shock. Control lines were also subjected to conditioning and testing, but the flies used to breed the next generation were chosen at random after testing (Fig. 1).

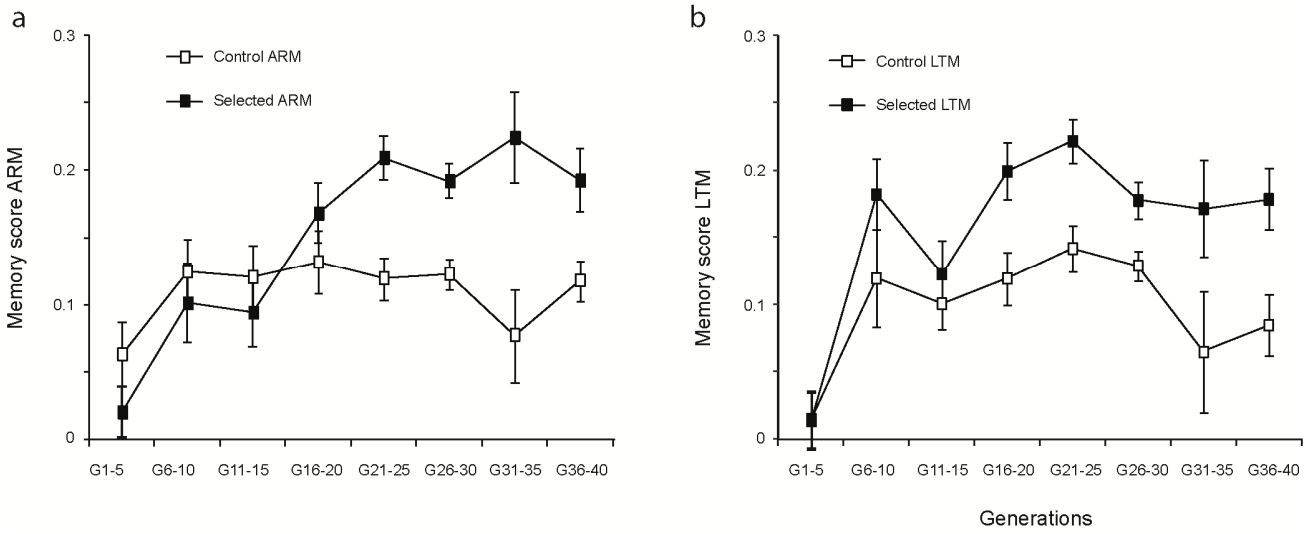


Figure 2: Memory score in the course of the experimental selection (means ± s.e.m). Each dot represents the mean score for 5 generations of selection regime. (a) ARM scores of selected for improved ARM (black squares) and control (white squares) lines over generation. (b) LTM scores of selected for improved LTM (black squares) and control (white squares) lines over generation.

After 23 and 28 generations of selection we measured ARM and LTM for all lines with 5-9 replicates per line. As expected, consolidated memory phases increased over generations accordingly to the selection regime (Fig. 2).When tested after 23 or 28 generations (Fig. 3), lines selected for improved ARM had higher memory scores than respective control lines 24h after massed conditioning (Fig.3A, Generation 23: $F_{1,14}$ = 16.6, P = 0.001; Generation 28: $F_{1,14}$ = 5.3, P = 0.040), and lines selected for improved LTM had higher memory scores than control lines 24h after spaced conditioning (Generation 23: $F_{1,14}$ = 7.6, P = 0.01; Generation 28: $F_{1,14}$ = 15.6, P = 0.001). At these generations, we decided to evaluate ARM and LTM in the selected lines for LTM or ARM improvement respectively and surprisingly, compared to control lines, lines selected for improved ARM had lower 24h memory scores when subjected to spaced conditioning (Fig. 3B,

Generation 23: $F_{1,14}$ = 5.6, P = 0.023; Generation 28: $F_{1,14}$ = 10.05, P = 0.007) and lines selected for improved LTM had lower 24h memory scores when subjected to massed conditioning (Generation 23: $F_{1,14}$ = 6.4, P = 0.016; Generation 28: $F_{1,14}$ = 11.4, P = 0.004).

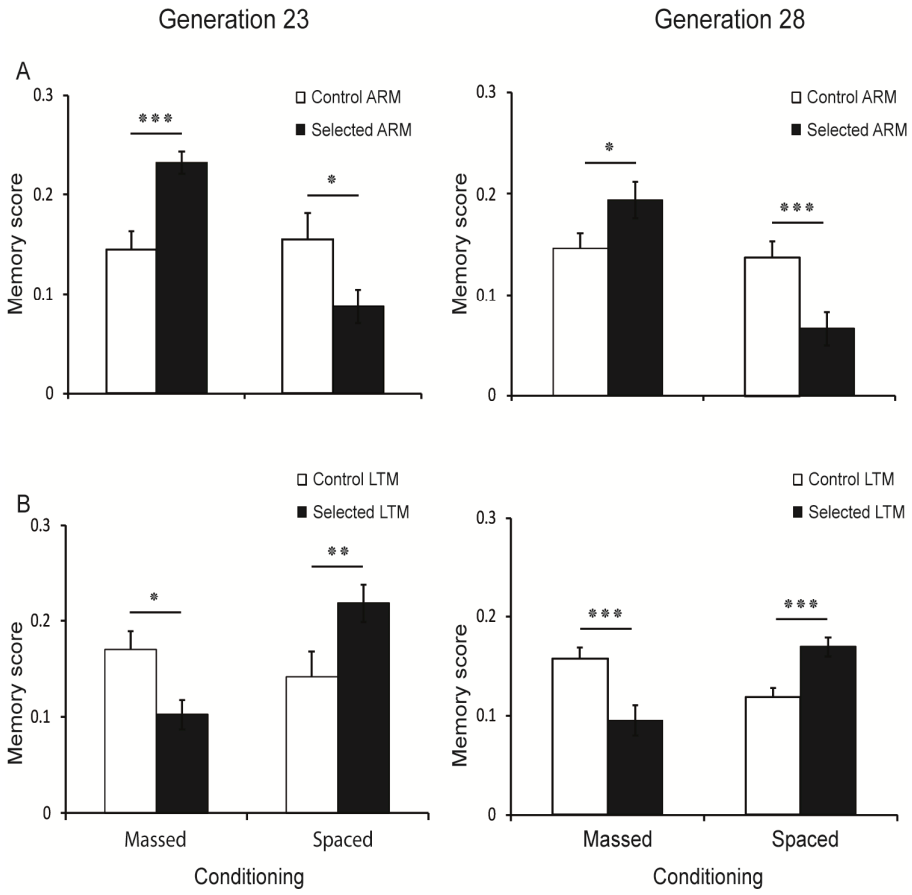


Figure 3 : Memory scores 24h after massed or spaced training for control and selected ARM (A)or LTM (B) lines after 23 or 28 generations of selection. (Mean ± s.e.m based on variation among lines, n=5-9 per replicate line) * indicates a significant difference between control and selected lines. (*: P < 0.05; **: P < 0.01; ***: P < 0.001).

We also looked at the distribution of each selected line for both regimes (Fig. 4). At generation 23, comparison of LTM and ARM ability for all the selected lines showed a significant negative correlation (r = -0.66, P = 0.005).

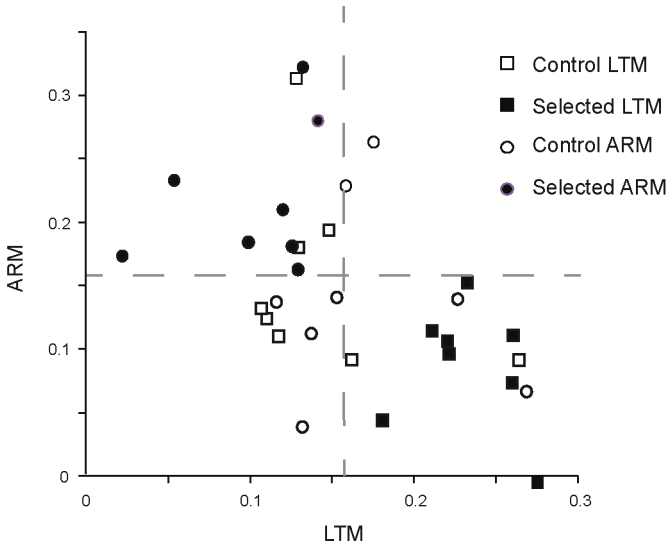


Figure 4: Generation 23: distribution of selected and control lines for both regimes of LTM and ARM scores.

Interestingly, in both selection regimes, selected lines also showed short-term memory (STM) improvement compared to control lines when measured 20 min after three massed conditioning cycles (Fig. 5: ARM selected lines: $F_{1,14} = 11.58$, $P = 0.01$; LTM selected lines: $F_{1,14} = 12.75$, $P = 0.01$). We observed no differences in olfaction between control and selected lines.

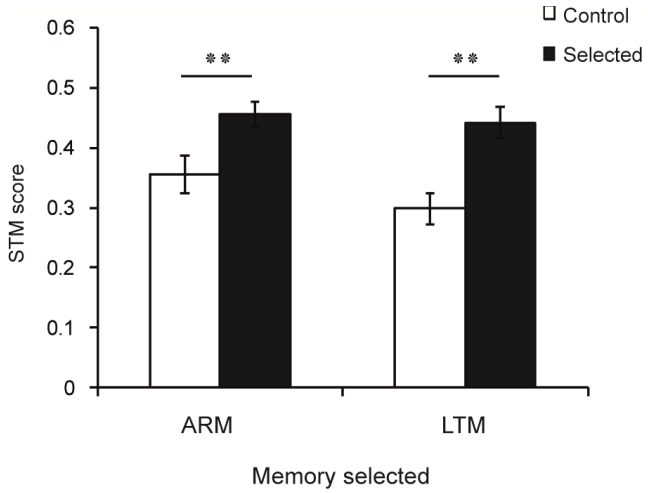


Figure 5 : Short Term Memory score after 3 massed cycles on selected and control lines after 23 generations of selection. (Mean ± s.e.m based on variation among lines, n=4 per replicate line). Selected lines shows higher scores than control lines in both selection regimes (*: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$).

We also tested our selected and control lines in the original aversive conditioning using electric shock as the US (Fig. 6). The same pattern of results was obtained. After 30 generations of selection, flies selected for improved ARM had higher score when tested after a massed conditioning ($F_{1,14} = 7.9$, $P = 0.014$)

but lower score after a spaced conditioning ($F_{1,14}$ = 4.1, P = 0.039). Flies selected for improved LTM had higher scores after a spaced conditioning ($F_{1,14}$ = 6.8, P = 0.021) but lower scores after a massed conditioning ($F_{1,14}$ = 17.4, P = 0.01).

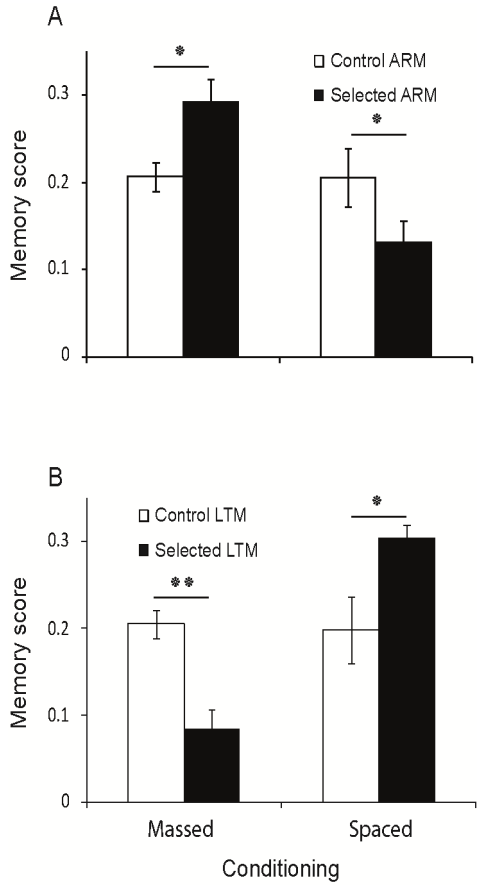


Figure6: Memory scores 24h after massed or spaced training for control and selected ARM or LTM lines after 30 generations of selection using electric shock as US. (Mean ± s.e.m based on variation among lines, n=5-9 per replicate line) * indicates a significant difference between control and selected lines. (*: $P < 0.05$; **: $P < 0.01$).

Correlated response in longevity

Longevity: To investigate the effect of selection regime for memory ability on lifespan, we measured the longevity of group sof males or females from all selected and control lines. With analysis based on half-life, male selected lines for improved LTM died sooner than control lines (Fig. 7: $F_{1,14}$ = 5.45, P = 0.034) whereas males selected for improved ARM expended significantly their lifespan (Fig. 8: $F_{1,14}$ = 4.84, P = 0.042). Selected females for both regimes did not show significant differences in longevity compared to their respective controls (ARM: $F_{1,14}$ = 0.92, P = 0.76; LTM: $F_{1,14}$ = 2.53, P = 0.13). In males, the effect of both

selection regimes was primarily the result of a difference for the initial mortality rate (parameter a) and we found that the effect of selection for ARM improvement also consisted in an increase of the rate at which mortality increases with age (parameter b). In females, mortality rate analyses did not reveal effect of the selection for both regime.

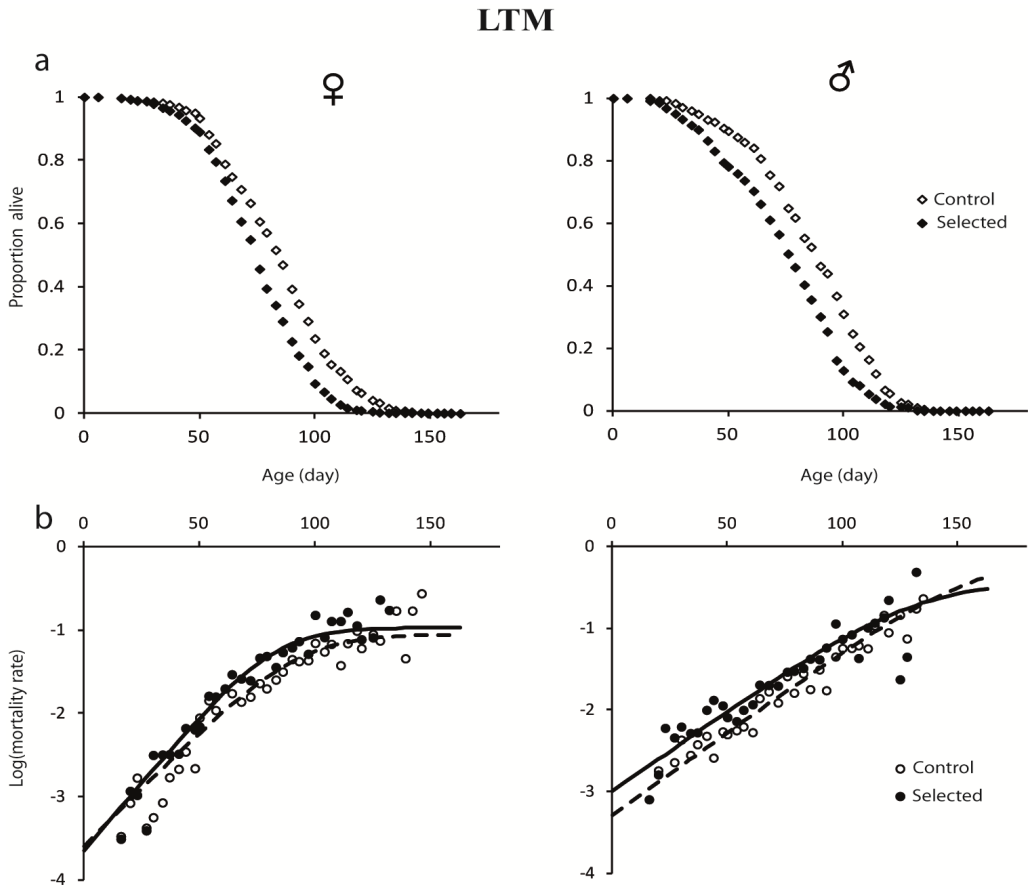


Figure 7: Correlated response in longevity to selection for improved LTM ability. (a) Age-specific survival for virgin females (left panel) and males (right panel) of control and selected lines is expressed by the mean proportion of flies alive ± s.e.m.. (b) Age-specific mortality rate for both selected and control lines for males and females. Regression lines represent for each sex the most parsimonious logistic model (Control: continuous lines and Selected: dashed lines).

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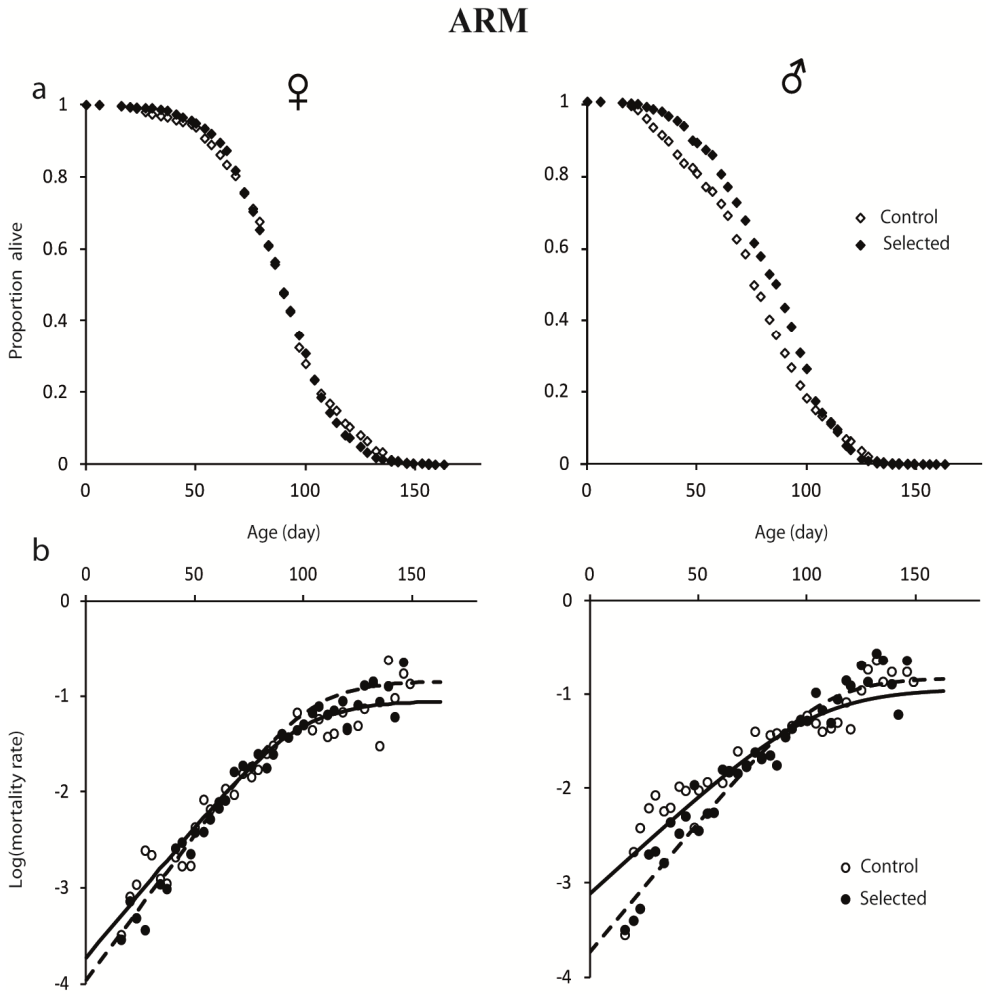

Figure 8: Correlated response in longevity to selection for improved ARM ability. (a) Age-specific survival for virgin females (left panel) and males (right panel) of control and selected lines is expressed by the mean proportion of flies alive ± s.e.m.. (b) Age-specific mortality rate for both selected and control lines for males and females. Regression lines represent for each sex the most parsimonious logistic model (Control: continuous lines and Selected: dashed lines).

Correlated response with other phenotypic traits

Larval development: Whatever the regime of selection, the duration of larval development to adult flies was as long in selected as in control lines (Fig. 9A and 9B).The rate of emergence at the end in comparison with the number of eggs did not show any significant differences between the control and selected lines (ARM: $F_{1,79} = 3.12$, $P = 0.08$; LTM: $F_{1,79} = 2.81$, $P = 0.1$)

Stress resistance: We quantified stress resistance by testing the mortality rate when exposed to a powerful oxidative agent: paraquat. Ingestion of paraquat by flies is known to cause oxidative damages that mimic aging (Phillips *et al.* 1989). After 30h of paraquat treatment, there was no effect of the regime of selection on mortality (Fig.9C: LTM selection: $F_{1,14} = 0.03$, P = 0.8; ARM selection: $F_{1,14} = 0.1$, P = 0.76).

Fecundity: There was no effect of selection for improved ARM or LTM on the number of eggs laid at young age (Fig.9D: LTM selection: $F_{1,14} = 0.51$, P = 0.47; ARM selection: $F_{1,14} = 2.14$, P = 0.16).

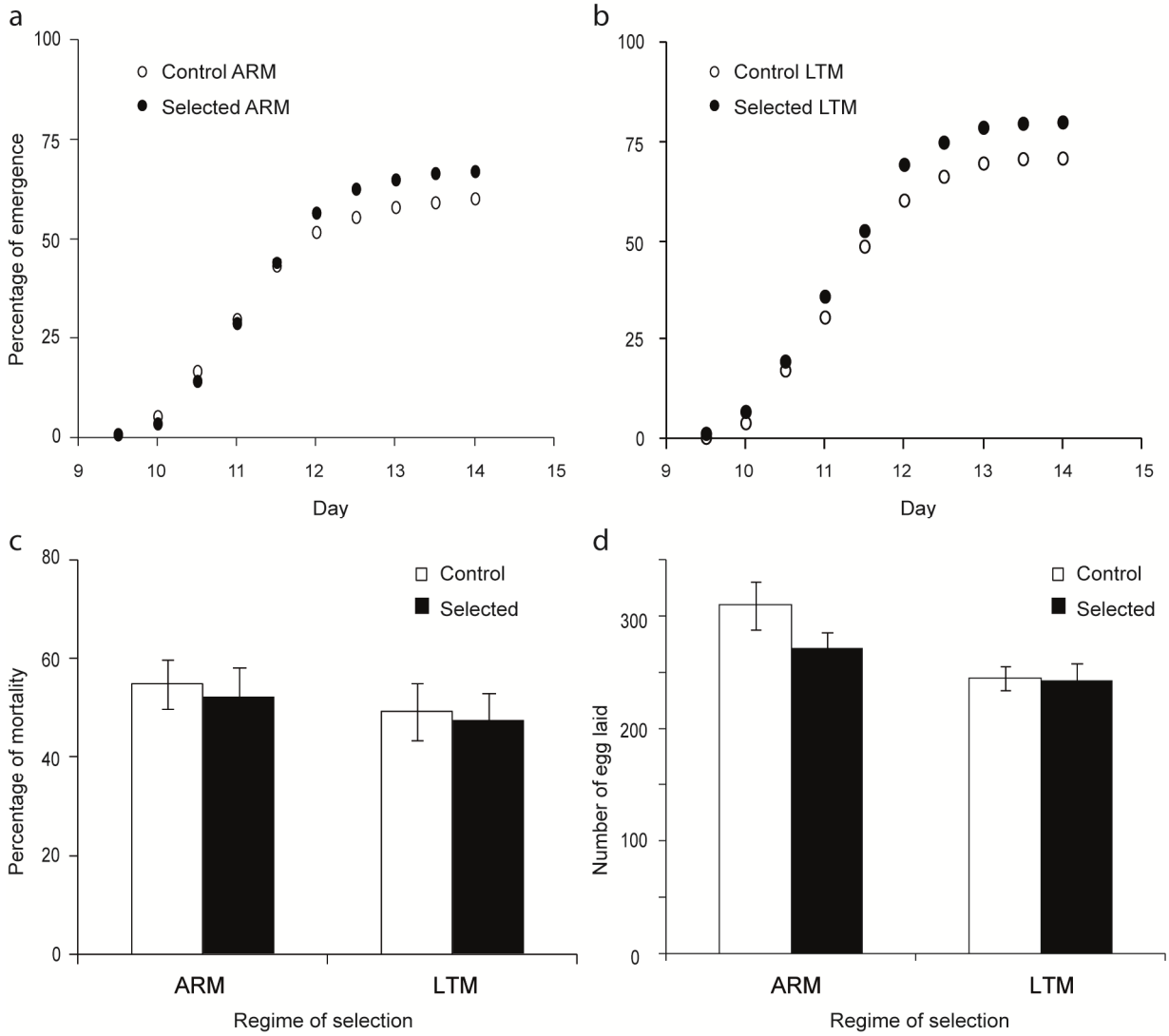

Figure 9: correlated response in other life-history traits to selection for improved ARM or LTM (mean ± s.e.m.) (a-b) development (N = 5 per line), (c) percentage of death after oxidative stress (N = 7 per line) and (d) fecundity (N = 4 per line).

Discussion

In this study, we report that, for a single cognitive task, selection for improvement of one specific consolidated memory phase induces an increase in performance for the selected memory phase but a decrease in performance for the other memory phase. Moreover, selection for improved LTM decreases male longevity whereas ARM selection extends male lifespan.

Evolutionary trade-off between ARM and LTM

Studies on variation of memory capacities between closely related species or populations of the same species have mostly compared global performances for cognitive tasks without focusing on the memory dynamics. Smid and coworkers (Smid *et al.* 2007) recently found evidence for species-specific memory dynamics by comparing two close parasitoid species. In a classical conditioning set-up, *Cotesia glomerata* formed exclusively LTM whereas *C. rubecula* formed both ARM and LTM. The present study suggests strong genetic variation for the two memory phases and the possibility for natural selection to act on memory dynamics.

The main result of this study is the observation of an evolutionary symmetrical trade-off between two memory phases for the same cognitive task. Correlated responses to selection may be due to pleiotropic effects of genes targeted by selection or to genetic hitchhiking of alleles at loci closely linked to the target genes (Falconer and Mackay 1996). We believe the second hypothesis to be unlikely. Because of the large base population it is not likely for strong linkage disequilibrium to have arisen by drift, except when one of the alleles involved had been very rare, present only in several copies in the gene pool. But such an allele would be unlikely to be sampled in all replicate lines, and we would not see a consistent response. On a functional level, the mechanistic relationship and functional significance of the different forms of consolidated memory is still under debate. Neurogenetic studies in vertebrates and invertebrates usually suggest that memory formation proceeds through several functionally distinct memory phases. In *Drosophila,* the most common protocol to study learning and memory processes is based on the association between an odor and an electric shock. In this protocol, mutants at the *radish* locus have disrupted ARM

(Folkers *et al.* 1993) but largely normal LTM (*Tully et al.* 1994) whereas mutations at some other loci (e.g., *crammer* (Comas *et al.* 2004)) selectively impair LTM. Disruptions of the transcription factors dCREB2, Adf1, or Notch block LTM without affecting ARM (Yin *et al.* 1994, Ge *et al.* 2004). These findings support the view that consolidated memory can be genetically dissected into functionally distinct ARM and LTM phases, which co-exist in flies. However, more recently, Isabel et al. (2004) found that LTM formation induces active erasing of ARM. These authors proposed that ARM and LTM are mutually antagonistic, and that ARM interferes with LTM formation. In mammalian systems, various memory types, including hippocampus-dependent memory, are enhanced by cAMP/PKA activity, whereas a different type of memory, working memory, is inhibited by PKA (Ramos *et al.* 2003, Dash *et al.* 2007). To compare our data to the previous genetic results in *Drosophila*, we tested our selected and control lines in this paradigm using electric shock and obtained the same trade-off which means there is not specificity of the selection for one aversive stimulus. In this way, we can say that our results support the hypothesis of a dynamic interaction between ARM and LTM.

Interestingly, we observed an increase of STM in both selection regimes. Recently, interaction between early and late memory traces has been investigated and Trannoy *et al* (2011) have shown that after an appetitive learning, LTM can be formed independently of STM. They proposed a parallel processing of the two traces. The improvement of STM in our selected lines then means that selection did not only act on specialized modules ARM and LTM but also on the general cognitive ability.

LTM and lifespan: antagonistic pleiotropic interaction

In order to identify eventual constitutive costs reflecting trade-off evolution, we evaluated various fitness-related phenotypes. Even if no specific effect of memory improvement wasobserved on fecundity, stress resistance or larval development, results on longevity reveal some differences depending on the form of memory selected. We evaluated the interaction between memory phases and longevity measuring lifespan of selected lines for each form of consolidated memory. Males selected for improved LTM which have low scores in ARM die sooner than control lines and the males selected for improved ARM live longer. First, the fact that some flies died sooner than control flies could be explained by an inbreeding effect due to

multiple mating with siblings. However, an inbreeding depression would not have allowed an improvement of cognitive performance, so, I will exclude this hypothesis of my discussion. These results rather suggest that LTM specifically trades with longevity and that the selection response seems to be based on genes with pleiotropic effects on both LTM and lifespan. Moreover, the consequence of the selection regime is opposite depending on the memory selected.

The kind of memory induced strictly depends on the environment in which the animal lives. Whether the events are spaced over time or not would favor LTM or ARM formation respectively. We can assume that if an animal live in a stable environment, it would be beneficial to develop adaptive specific modules in order to behave adequately to this particular environment. For instance, if aversive event are repeated and spaced over time, a better LTM ability would improve animal fitness. However, LTM requires *de novo* protein synthesis to be stabilized and its formation is known to be costly compare to ARM consolidation (Mery and Kawecki 2005). In this study, aging of untrained selected flies for LTM is affected which demonstrates a constitutive cost of LTM improvement by artificial selection. These costs are not related to the process of memorization of information but rather reflect the interaction between memory ability and other life history traits. Such evolutionary trade-off could be explained by the energetic cost of the central nervous system. Thus, one hypothesis would be that selection of a costly form of memory as LTM modifies the resource allocation from survivorship to cognitive ability and so, affects the animal fitness. Another hypothesis would be that selection has induced inbreeding within selected lines but it is not consistent with the fact that these lines also express higher LTM ability. This constitutive cost is only observed in males. Others studies have obtained similar sex-specific costs associated to improvement of learning and memory performance. Burger *et al* evaluated longevity of flies selected for improved ability to associate a flavor of an oviposition substrate with an aversive bitter taste and female longevity was found to be decreased in selected lines. However this protocol involves a female-specific behavior that can explain this sex-specific cost. In our experimental lines, we cannot exclude that males were just more sensitive to environmental stress than females. The experimental conditions during the longevity measurements were very permissive (the last fly died after 5 months), then females may just have lived easily and, without stressful environmental conditions, did not

express the trade off. However, two questions can be addressed about this sex-specific trade off. First, constitutive costs could have been attributed to another behavior more related to female biology. For instance, longevity and fecundity are known to be strongly related in female Drosophila (Dick *et al* 2011) so we expected to observe a trade-off with fecundity but no differences were observed in the number of eggs laid in the selected population. Secondly, we can wonder how selection has affected memory ability in each sex. In control lines, we already know that there are no differences between male and female memory scores, but separate conditioning on both sex of selected lines needs to be performed to eventually detect sex-biased specialization. Nonetheless, this work reveals the first trade-off specifically related to one kind of memory: LTM. This specificity is consistent with the idea about the existence of different memory modules that govern behavior in response to different environments.

Even if no specific alleles are officially known to have antagonistic effect on both memory processes and longevity in Drosophila, some pleiotropic effects have been observed for alleles that affect learning abilities (Mery 2007) and lifespan (Nuzhdin et al, 1997). Although some genes were found to be specifically responsible of each consolidated memory, few studies investigated alleles affecting both memory and longevity. For instance, the factor of transcription crh-1, the *C.elegans* homologue of CREB, expression level changes allow maintenance of memory ability with age in longevity mutants and are specifically involved in LTM formation. These findings suggest that CREB could be a good candidate to regulate the interaction between memory and longevity. Selected lines for specific memory phases would be a perfect tool to investigate this question.

The understanding of the level of interdependence among the different memory phases is fundamental for a better comprehension of the evolution of cognition. The symmetrical evolutionary trade-off between two consolidated memory phases observed in our selected lines may open new perspectives on the study of the evolution of memory and its constraints. It may also explain the natural variation of the different memory phases observed between closely related species. Moreover, these experiments give us more knowledge about genetic bases of life history traits evolution and their pleiotropic effects. Linking

neurobiology, ecology and evolutionary biology will open new perspectives on how natural selection can model animal cognition.

Chapter 4- A switch from ARM consolidated memory to LTM-like reconsolidation and extinction in Drosophila

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It is generally accepted that, after learning, memories stabilize over time and integrate into long-term memory (LTM) through the process of consolidation, which depends on *de novo* protein synthesis. Besides, studies on several species have shown that reactivation of already stabilized LTM can either make this memory labile and then re-stabilize it (a process called reconsolidation) or inhibit it (extinction). However, the identity of both processes and their interactions with consolidation are still under debate. Regarding memory stabilization, *Drosophila* offers a striking exception since, in these species, LTM is not the sole stable form of memory. Under specific learning conditions, anaesthesia-resistant memory (ARM) can be formed through processes yet unknown but that are resistant to cycloheximide, a classical protein synthesis inhibitor that impairs LTM. Here, we took advantage of this dichotomy to ask whether both ARM and LTM could be extinguished and/or reconsolidated. We also studied whether two forms of memory extinction and reconsolidation exist in flies, as for memory stabilization. We show that either reconsolidation or extinction can be induced after olfactory conditioning in *Drosophila*, depending on the number of reactivations as in other species. Furthermore, regarding the effect of cycloheximide, the ARM/LTM dichotomy for stabilization does not apply to extinction and reconsolidation. Blocking protein synthesis interfered with both processes regardless of whether initial stabilization was sensitive (LTM) or not (ARM) to cycloheximide. These results thus show that *Drosophila* is a useful model to tackle these questions, and that reconsolidation is not necessarily a mere repetition of consolidation.

Introduction

Many studies have shown that the maintenance of memories over long periods requires consolidation, a crucial phase which allows the learned information to become resistant to interfering treatments (Duncan, 1949; Dudai and Morris, 2000; Dudai and Eisenberg, 2004). Until recently, it was considered that, after a single consolidation phase, memories remained resistant to interference (McGaugh, 2000). However, several studies, mostly based on Pavlovian conditioning, have challenged this view. After consolidation of a memory of an association between a conditioned and an unconditioned stimulus (respectively, CS and US), further presentations of the CS alone (reactivation) can destabilize it. Once made labile, the initial memory trace (CS-US) can be stabilized again through reconsolidation (Misanin *et al*, 1968; Mactatus *et al*, 1979; Nader *et al,* 2000; Sara, 2000; Dudai, 2006), so that the behavioral response to the CS is maintained. Alternatively, reactivation can lead to extinction, a decrease of the response resulting from the consolidation of a new memory of the association CS-no US (Suzuki *et al*, 2004).

Reconsolidation and extinction thus lead to opposite behavioural outcomes. Whether one or the other process is triggered depends on several factors (Alberini *et al*., 2006). In particular, as shown in rats by Suzuki and colleagues (2004), the strength and age of the original memory is important, as is the strength of the reactivation phase (number/ duration of reactivation trials), such that younger and weaker memories are more easily reconsolidated. In species usually studied, like consolidation, extinction and reconsolidation are processes of memory stabilization, and require *de novo* protein synthesis. However, what their underlying mechanisms are is still under debate. In particular, the question of the similarity between consolidation and reconsolidation remains disputed (Alberini, 2005; Nader *et al.*, 2005; Alberini *et al*., 2006; Eisenhardt and Menzel, 2007; Moore and Roche, 2007; Stollhoff *et al*, 2008).

To clarify the relationships between these three processes, we studied some of their properties in *Drosophila melanogaster*, which provides genetic tools that should ultimately help to define important aspects of their cellular and molecular bases. We used the common learning paradigm of classical (Pavlovian) olfactory conditioning, in which the CS is an odorant and the US a shock, whether electric (Tully *et al*., 1994) or mechanical (Mery and Kawecki, 2005). A mutant-based genetic dissection of memory has

distinguished four kinds of memory using electric shock as US (Tully *et al*., 1994). Beside the labile short-term memory (STM) and middle-term memory (MTM) are two forms of stabilized memory: a decremental, cycloheximide (CXM)-insensitive memory (anesthesia-resistant memory, ARM) and a non-decremental, CXM-sensitive (protein synthesis dependent) long-term memory (LTM). They can be induced experimentally through multiple CS-US presentations during conditioning: ARM is produced when no delay separates successive presentations of the CS paired with the US (massed protocol) while a sufficient delay (spaced protocol) produces both ARM and LTM (Tully *et al.*, 1994). This study aimed to analyze the effect of reactivation on these two forms of memory, by evaluating whether extinction and/or reconsolidation could be induced by different numbers of reactivation trials, and whether the nature of the initial memory had an impact on these processes.

Materials and Methods

Fly stock and maintenance*:*

Adult wild-type *Drosophila melanogaster* flies collected in the center of France (Chavroches) in 2006 and maintained in the lab on standard food were used for all experiments.

Reactivation:

Reactivation was performed 24h after conditioning (as described in Material and method). This phase consisted in successive cycles of presentation of the CS+ alone without mechanicals shocks. Each cycle consisted of a 1-min presentation of the CS+ followed by a 1-min rest (without odor). The resting period prevented habituation of the flies to the odorant. Flies received 0, 1, 3 or 5 reactivation cycles and were then transferred back into vials containing wet filter paper until the memory assay.

CXM treatment:

In order to assess how consolidated memory depends on the synthesis of *de novo* proteins, flies were fed for one hour with the protein synthesis inhibitor cycloheximide (CXM, Sigma-Aldrich, Lyon, France) dissolved into sweet water (20% sucrose) just before the conditioning or reactivation phase. Control flies were fed with sweet water only during the same amount of time. We used a dose (35 mM) previously shown to efficiently block LTM (Tully *et al*., 1994, Mery and Kawecki, 2002).

Statistical analysis*:*

For each measurement, a memory score was calculated as the difference in the proportion of flies choosing octanol when conditioned to avoid methylcyclohexanol versus when conditioned to avoid octanol. For statistical comparison of the memory scores, all proportions were angularly transformed before the analysis (Sokal and Rohlf, 1995). Differences between memory scores were tested with a univariate ANOVA. For comparisons among reactivation cycle number, we used planned orthogonal contrasts and t-tests were employed for mean comparisons. All statistical analyses were performed using SPSS.

Results

Massed and spaced conditioning induce stable ARM and LTM

As a first step, we verified whether, using our protocol with a mechanical shock, we could obtain a CXM-resistant memory (ARM) after massed training and if spaced training also induced a CXM-sensitive memory (LTM). In our conditions, both massed and spaced training protocols of associative conditioning induced stable memory, as observed 24h and 48 h later (Fig. 1). Flies treated with CXM before conditioning showed significantly reduced response levels compared with controls when the spaced protocol was used for conditioning (univariate ANOVA: $F_{1,14} = 10.3$; $P < 0.01$) but not with the massed protocol ($F_{1,14} = 0.2$; $P >$

0.05), thus indicating that LTM was formed only in the former case. However, once LTM was established, it became resistant to the blockade of protein synthesis: when applied 24h after spaced conditioning, CXM treatment did not affect response levels when tested 48h post-conditioning ($F_{1,70}$ = 0.9 ; P > 0.05). We conclude that, in our experimental conditions, (1) the two protocols induce different stable memories (both ARM and LTM after spaced training and only ARM after massed training) and (2) as expected, protein synthesis was required only for LTM formation, but not for its maintenance nor for ARM.

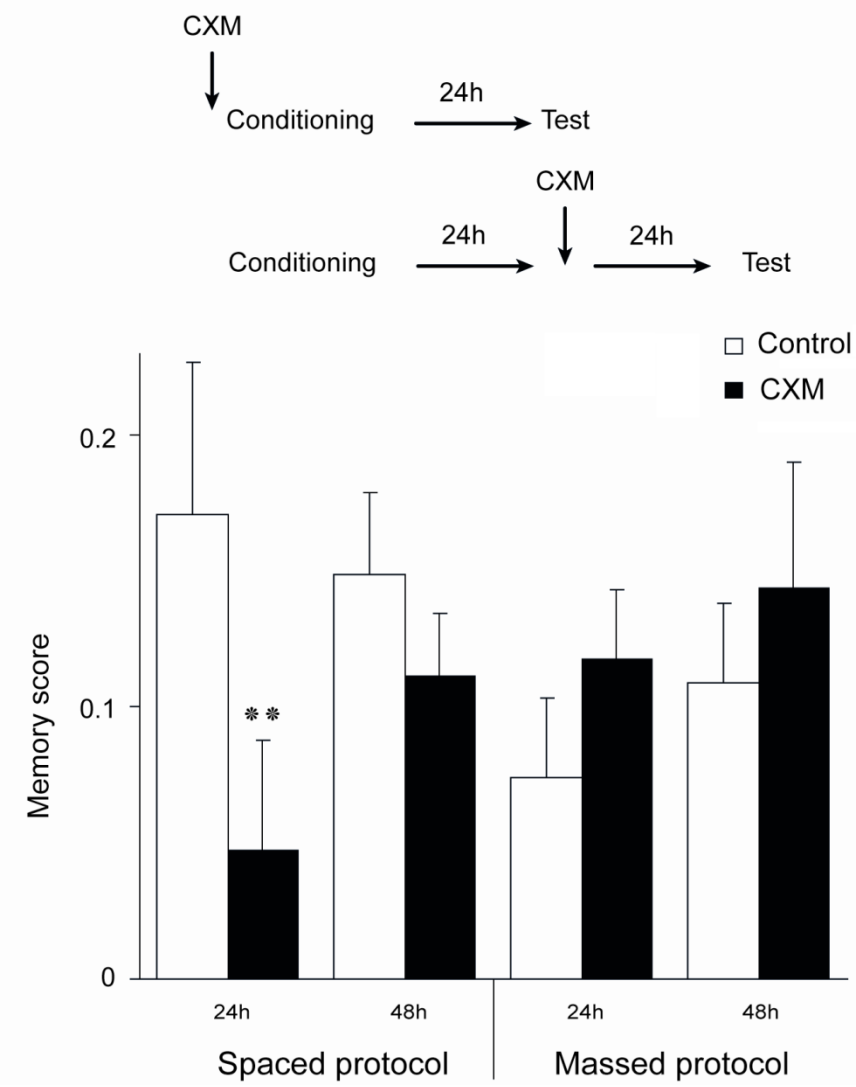

Figure 1: Effect of CXM treatment after massed or spaced training. *Spaced protocol*: memory was tested 24h after conditioning (CXM just before conditioning : n=44 per group) or 48h after conditioning (CXM 24h after conditioning : n=36 per group). *Massed protocol*: memory was tested 24h after conditioning (n=16 per group) or 48h after conditioning (n=16 per group) (*** : p < 0.01).

Moderate reactivation of ARM triggers a protein synthesis-dependent reconsolidation

We then investigated the effect of the number of reactivation cycles on both stabilized memories. 24h after a massed or spaced conditioning protocol, a variable number of reactivation cycles (see Materials and Methods) were performed, and the response levels of the flies were measured in a memory test 24h later (i.e. 48h after conditioning) (Fig. 2). One reactivation cycle did not affect memory, as shown by response levels similar to those of flies not submitted to any reactivation (*0 cycle* groups), whatever the conditioning protocol (planned contrast: LTM: $P > 0.1$; ARM: $P > 0.1$). Besides, whether they had been treated with CXM prior to the presentation of CS+ had no effect (Fig. 2: *1 reactivation cycle*, $P > 0.1$ in both cases), thus confirming previous studies showing that a short reactivation does not affect initial consolidated memory.

When reactivation consisted of three cycles, the response levels observed 24h later differed significantly according to the protocol used for conditioning. After a massed protocol, untreated flies submitted to a three-cycle reactivation responded at levels similar to those of non-reactivated flies (Fig. 2A). However, memory was abolished if flies had been treated with CXM prior to reactivation. This suggests that the initial memory trace was de-stabilized by the 3-cycle reactivation, and then consolidated through new protein synthesis. This is the first evidence, to our knowledge, of reconsolidation in *Drosophila*. Interestingly, the memory formed by the massed protocol conditioning was CXM-resistant (Fig. 1) but it became CXM-sensitive after a three-cycle reactivation. Thus, it appears here that different mechanisms of stabilization were triggered after acquisition and after reactivation.

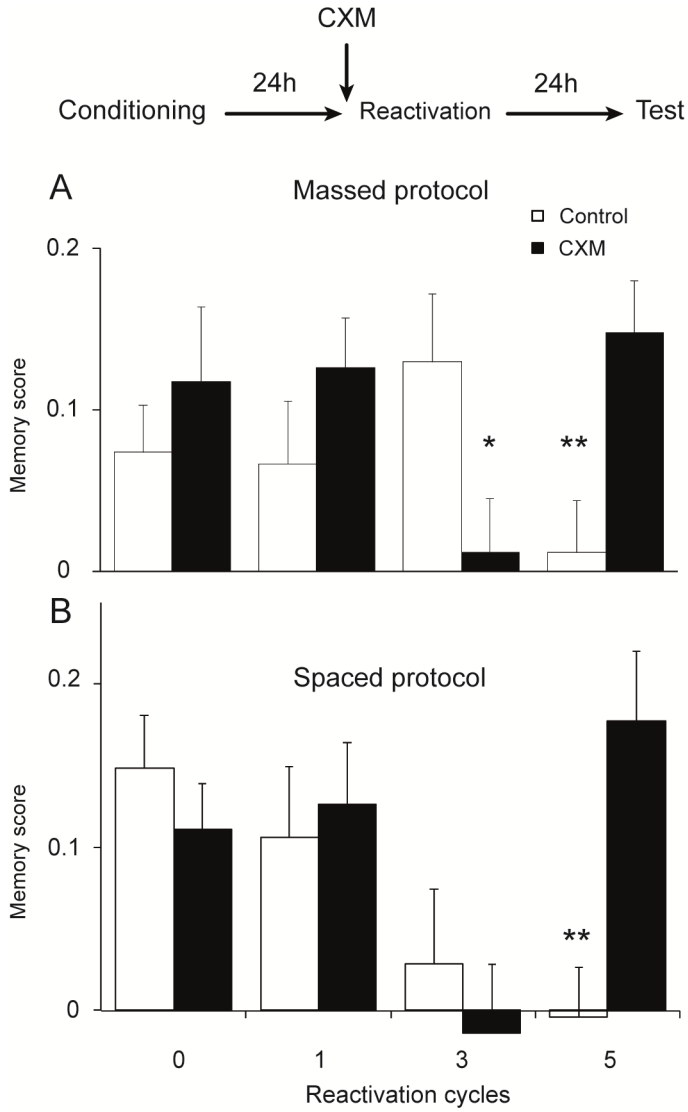

Figure 2: Effect of CS+ reexposure on stability of consolidated memory after a massed (A) or a spaced (B) protocol.A- 0, 1, 3 or 5 cycles of CS+ reexposure after massed conditioning (control group: n=16 per group; CXM treated group: n= 15-16 per group)B- 0, 1, 3 or 5 cycles of CS+ reexposure 24h after spaced conditioning (control group: 0 cycle, n=36; 1 cycle, n=12; 3 cycles, n=12; 5 cycles, n=20; CXM treated group: 0 cycle, n=36; 1 cycle, n=12; 3 cycles, n=12; 5 cycles, n=19) (** : $p < 0.05$; *** : $p<0.01$).

When tested 24h after a spaced protocol conditioning and three reactivation cycles, neither CXM-treated nor control flies showed any significant memory (Fig. 2B). As this suggested that in such conditions the memory was less stable, we measured responses to the CS+ at shorter delays (0, 5 or 8 h after reactivation) to test whether a memory trace could be detected at earlier times. However, surprisingly we observed no significant memory at any of these time points. From this, we conclude that, under our experimental conditions, when the initial memory is induced by spaced protocol (CXM-sensitive), three cycles of reactivation must disrupt it completely. This result is contradictory to previous studies which found

a strong reconsolidation effect after reactivation on protein synthesis dependent acquisition memory (Nader et al 2000).

Intense reactivation of both ARM and LTM produces protein synthesis-dependent extinction

When the number of reactivation cycles was increased to five memory of the CS-US association was abolished 24h later in all untreated flies, regardless of the protocol used to form the acquisition memory (*t*-test: $P > 0.1$ in both cases) (Fig. 2). This drop in memory was dependent on protein synthesis in both cases, since CXM-treated flies showed response levels similar to those of unreactivated flies (planned contrast: $P > 0.1$ in both cases). Thus, extinction induced by a 5-cycle reactivation resulted in the active consolidation of a new memory, CS-noUS, expressed by a drastic drop of the response levels to the CS+. It is noteworthy that the requirement for protein synthesis in the stabilization of this new memory occurred independently of the involvement of protein synthesis in the consolidation of the acquisition memory, since similar results were obtained when the acquisition memory was CXM-resistant (Fig. 2A) and CXM sensitive(Fig. 2B).

Reconsolidation and extinction are specific to presentation of the CS+

Finally, we tested whether the observed reconsolidation and extinction processes were specific to the presentation of the CS+, by presenting the CS- instead during the reactivation cycles. As opposed to 3 cycles of CS+ presentation, which induced significant CXM-sensitive reconsolidation of ARM (Fig. 3A, ANOVA: $F_{1, 15} = 9.35$, $P < 0.01$), three cycles of CS- presentation 24h after massed conditioning did not affect the response of the flies whether they had been treated with CXM or not (Fig. 3A: $F_{1,14} = 0.16$, $P > 0.05$). Similarly, while extinction occured after 5 CS+ reactivation cycle, i.e. flies showed no significant memory (*t*-test: ARM: $t_{1, 7} = -1.9$, $P > 0.05$; LTM: $t_{1, 7} = -0.035$, $P > 0.05$), flies exposed to 5 CS- reactivation cycles still responded significantly to the CS+ (ARM: $t_{1, 7} = 4.55$, $p < 0.01$; LTM: $t_{1, 7} = 4.22$, $P < 0.01$) (Fig. 3B). Thus, extinction of both

ARM and LTM could be induced by a sufficient number of presentations of the CS+ only. We therefore conclude that, when occurring, reconsolidation and extinction were specifically triggered by repeated presentations of the CS+.

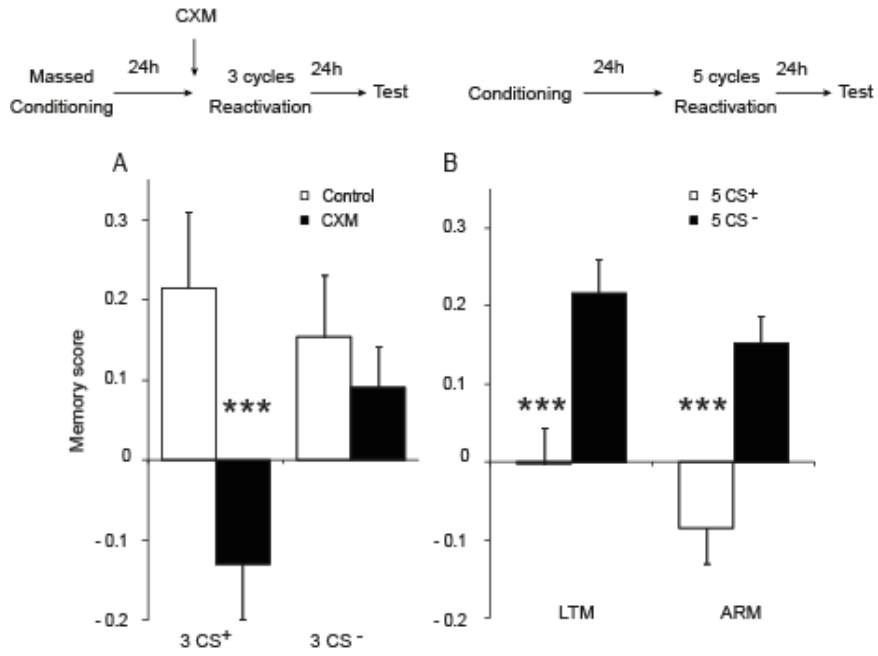

Figure 3: Specificity of CS+ exposure (A)- Effect of 3 CS+ or CS- exposure 24h after a massed conditioning (ARM). CXM treatment is administrated just before reactivation (3CS+: control group, n=8; treated group, n=8; 3CS-: control group, n=8; treated group, n=8) (B)- Effect of 5 CS+ or CS- exposure 24h after spaced (5CS+, n=8; 5CS-, n=8)or massed conditioning (5CS+, n=8; 5CS-, n=8).

Discussion

In this study we addressed for the first time the question of the relationship between consolidation, reconsolidation and extinction using *Drosophila melanogaster*. Our results clearly show that both reconsolidation and extinction exist in this model. This opens new perspectives for the understanding of the molecular and cellular aspects of such processes. The wide array of genetic tools now available in this model organism can be used to tackle these questions, as it allowed dissecting several aspects of memory stabilization (e.g. Keene and Waddell, 2007). Within this perspective, we have started investigating the relationships between extinction, reconsolidation and stabilization of acquisition memory. For this, we took advantage of a potentially unique aspect of *Drosophila* cognition: the existence of two forms of stabilized memory, ARM and LTM, respectively resistant and sensitive to the CXM protein synthesis inhibitor

(Margulies *et al*., 2005). We asked whether this dichotomy extended to reconsolidation and extinction. Our findings show that (1) whether reconsolidation or extinction takes place upon reactivation of the stable memory depends on the intensity of such reactivation, (2) both processes can be built whether the memory initially stabilized was CXM-sensitive or resistant except reconsolidation after spaced training which was not observed in this study, (3) both are CXM-sensitive even if the stabilization of the acquisition memory was not, as in the case of ARM, and (4) extinction or reconsolidation occurs specifically when reactivation involves the presentation of the CS+.

The onset of reconsolidation or extinction depends on the intensity of reactivation by CS+ presentations:

Taken together, our findings show that after formation of an acquisition memory through repeated CS-US pairings, further reactivation of this memory can modify the flies' response to the CS. The observed change is variable, depending on the intensity of reactivation (here, the number of reactivation cycles). This is in accordance with previous studies on other organisms (crab: Pedreira and Maldonado, 2003; fish: Eisenberg and Dudai, 2004; rat: Suzuki *et al.*, 2004; Power *et al.,* 2006; bee: Stollhoff *et al.,* 2005). Here, while a weak reactivation (1 cycle) did not modify the expression of the acquisition memory (CS-US), a moderate (3 cycles) or intense (5 cycles) reactivation could induce, respectively, reconsolidation of the acquisition memory or extinction. The latter is generally considered to result from the formation of a new memory (extinction memory) corresponding to the new association of the CS with the absence of US. This view is comforted by our demonstration that the CS+ is required to induce the observed drop of conditioned responses (CS- presentations had no effect). Besides, extinction is an active process involving protein synthesis, since a CXM treatment maintains stable levels of response after 5 reactivations. We interpret this as the expression of the original acquisition memory when the formation of the new extinction memory is repressed, in accordance with previous work (e.g. Suzuki et al., 2004; Stollhoff *et al.*, 2005). Thus, in *Drosophila* as well, extinction corresponds to the consolidation of a new memory trace that is sensitive to protein synthesis inhibitors rather than to the erasure of the initial trace.

If extinction is not induced, the original memory can be maintained over days once formed and stabilized, and is resistant to CXM treatment, independently of the conditioning protocol. However, we observed reduced response levels in CXM-treated flies after 3 cycles of reactivation (following a massed conditioning). This result thus indicates that moderate reactivation can de-stabilize the originally CXM-resistant memory and turn it into a labile, CXM-sensitive, memory. This effect of moderate reactivation has been observed in a wide range of studies and has been interpreted as a reconsolidation process (for review Tronson *et al*, 2007). However, in this study it is surprising that we could not reveal such a reconsolidation after spaced training acquisition, since both CXM-treated and untreated flies maintained low levels of response when identical reactivation conditions followed spaced conditioning. Although reconsolidation should lead to a stable memory, we tested the unlikely possibility that memory had been reconsolidated indeed but then had been quickly erased during 24h-delay prior to testing. Our results clearly show that no expression of memory could be observed, even immediately after reactivation.. This is at odds with those of previous studies which found a strong reconsolidation effect after reactivation on protein synthesis-dependent acquisition memory (Nader *et al.,* 2000). It is neither likely that extinction occurred, since this drop in expression of the CS-US memory was observed also in CXM-treated flies. Indeed, CXM should have repressed extinction memory and allowed normal response levels, as after 5 cycles of reactivation (see above), though we cannot discard that some CXM-resistant extinction might have occurred. Neither can we exclude the fact that under these conditions reconsolidation and extinction might have co-occurred. This remains an open question that will require further studies. Still, our main result remains that both extinction and reconsolidation could be demonstrated experimentally in *Drosophila*. As in other species, these opposite processes share a common requirement for protein synthesis, and are triggered by different intensities of reactivation (Eisenhardt and Menzel, 2007).

Reconsolidation does not recapitulate consolidation in Drosophila:

Interestingly, *bona fide* reconsolidation – depending on protein synthesis - could be observed after reactivation of acquisition memory of the ARM type, which had been stabilized independently of protein

synthesis. If reconsolidation was underlied by mechanisms identical to those of consolidation, we would expect two possible outcomes. If one considers that ARM stabilization does not require protein synthesis and is thus not proper consolidation, reconsolidation should not occur after ARM formation. Alternatively, considering that ARM was consolidated in a CXM-resistant manner (if consolidation is taken *sensu lato* as stabilization), reconsolidation should occur likewise. However, our results show that, in flies submitted to a massed conditioning followed by 3 cycles of reactivation, two different events occurred: first, an ARM-type acquisition memory was formed and stabilized (through CXM-resistant mechanisms yet unknown); second, this memory was reactivated and destabilized, then reconsolidated into a CXM-sensitive form of memory. Even if considering that CXM does not fully block protein synthesis (Tully, 1994), it remains clear that we are in presence of two different forms of memory, and that the stabilization of the CXM-sensitive reconsolidated memory must involve different processes from those contributing to the formation of the original CXM-resistant acquisition memory. This conclusion confirms the view that reconsolidation is not repetition of consolidation (Nader *et al., 2000;* Alberini*, 2005*), and extends it to *Drosophila*, a new model thus available now for the study of these questions.

A molecular switch triggered by reactivation?

In our experimental conditions, acquisition could lead to two different forms of memory depending whether massed (ARM) or spaced trials (LTM and ARM) were used during conditioning, in accordance with previous studies (Tully, 1994). On the contrary, it is remarkable that, as far as we have observed, post-reactivation processes appear more unified as they required protein synthesis in all our experiments. In particular, both massed and spaced conditioning could be followed by the formation of an extinction memory which was disrupted by CXM, regardless of the nature of the initial memory (LTM and/or ARM). More specifically, it is striking that an ARM-type memory is not sensitive to the protein synthesis inhibitor for its stabilization, but that its extinction and reconsolidation are. Thus, as proposed above for reconsolidation, we conclude that molecular pathways involving protein synthesis could be recruited for extinction even if they had not been required initially for the formation of the acquisition memory. Hence, extinction and

acquisition memories can be stabilized through distinct underlying mechanisms. Interestingly, we used a reactivation protocol in which the CS+ presentations followed each other without delay. This can thus be viewed as a massed conditioning protocol for the acquisition of the CS-noUS memory (extinction memory). The rule that massed training leads to CXM-resistant memory would then not apply to extinction, for which a CXM-sensitive memory trace does not necessarily require spaced trials to be established. We can therefore conclude that post-reactivation processes such as extinction and reconsolidation involve protein synthesis in such a way that they can be disrupted by CXM, even if they follow the stabilization of ARM (CXM-resistant), at least under our experimental conditions. This implies that a switch between different signaling pathways may take place upon reactivation, as those involved initially in consolidation may not be used. Perazzona *et al* (2004) have proposed that antagonistic molecular cascades (cAMP-dependent for LTM and cAMP-independent for ARM) could underlie the formation of ARM and LTM following acquisition. According to their model, ARM would serve as a gating mechanism for LTM formation. These authors showed that if LTM was formed, it implied that ARM should be erased. Our results suggest that this may also apply to LTM-type extinction and reconsolidation memories. This competition is still under debate, and whether it is symmetrical remains an open question, as inhibition of LTM by ARM has only been hypothesized (Isabel *et al*, 2004). Interestingly, we find that de-stabilization of LTM can be followed by consolidation of extinction memory through protein synthesis. Future work with mutants should help to test the implication of cAMP-dependent and independent pathways in post-reactivation processes.

Conclusion and perspectives

During my thesis, I focused my work on the different processes of integration of environmental information in animal cognition. I demonstrated learning and phenotypic plasticity of the olfactory systems at different levels from perception to memory update.

Compound processing in Drosophila

In the first chapter, I addressed the question about flies' ability to process odor compounds and what kind of perception flies use to solve complex discrimination task. First, I demonstrated that flies are able to associate a compound to an aversive stimulus and also to generalize each element to the compound. Overlap and patterning discrimination have revealed that, even if flies are not able to form configural representation of an odor compound, flies do not use only elemental perception. The way to process compound is not likely to be a problem of choice between elemental or configural representation. It rather depends on the task the animal is performing. Previous researches suggested that the model of perception used depend on how much it is suited to use it in order to perform the discrimination. In the overlap discrimination, I assume that the different tests for a same learning task probably require different types of perception in order to behave adequately. Flies have to acquire and process the stimuli that will produce the good behavior but if flies are later confronted to a partially different environment, they must recall the relevant information of what they have learned. Previous studies (Rescorla, 1972, 1985, Kehoe *et al* 1988) proposed that the associative strength formed between the elemental or configural cue and the unconditioned stimulus depends on the salience of the configural and elemental representations. Others suggest that a compound is generally elementally perceived under normal conditions and can be configurally perceived if it is needed by the task (Saavedra 1975). All this theories suggest that a shift would exist from elemental to configural perception in conditions requiring configural representation. For instance, negative patterning experiments in honeybees show that the compound is first elementally perceived and that an extensive training can induce this shift and lead to a configural representation of the compound that allow to

perform the task (Deisig *et al* 2003). In Drosophila, Young *et al* (2010) performed extended training but did not observed apparition of configural representation. In natural environment, animals must be able to discriminate different complex blends, which suggests an ability to respond to specific combinations of stimuli. Intermediary conditioning using two different compounds AB and CD, one shocked and the other one not shocked, and testing the different combinations of mixtures could help us to determine whether flies are able to attribute an associative strength to specific combination of odorants. A variant of this experiment, the biconditional discrimination task (AB+/AC-/CD+/BD-) is generally used to test non-elemental perception of complex environment and could also be performed on fruit flies.

The inter-trial interval in the conditioning also seems to be another important parameter in positive and negative patterning discrimination on honeybee (Deisig *et al* 2007) but it has never been tested in Drosophila and so, needs to be investigated. The learning task intensity seems to have important impact on perception but it is not clear whether perception ability is directly related to the ability to form strong memory traces. Configural processing seems to require higher center integration in the brain (Komischke *et al* 2003) and our conditioning intensity could have been too weak to allow the expression of the configural cue. The lines we selected for improved ability to form long-lasting memory also exhibit better STM than their control. Then, testing these selected lines would help us to determine how the modulation of perception is related to learning and memory abilities. Another important question can be addressed about how the stimulus modality influences perception. In this discrimination experiments, I tested compound processing with one odorant modality but in natural environment, animals have to learn complex compounds with different modalities. Since the 90s, multimodal perception is investigated in various invertebrate models (fruit fly: Guo and Guo 2005, Yarali 2008; honeybee: Gerber and Smith 1998; cricket: Matsumoto and Mizunami 2004). For instance, Yarali *et al* used olfactory and visual stimuli in an associative learning to detect whether there is an integration of the visual cue in the olfactory memory in *Drosophila* but no interaction between both sensory systems was detected in larvae and adult flies (Yarali *et al* 2006, 2008). However, Guo and Guo found that visual and olfactory cue interact and that a crossmodal transfer occur during the memory process (Guo and Guo 2005). Knowing that learning can be divided in specialized

sensory modules, the question about the interaction between different sensory modalities in a compound would help to understand the modulation of sensory perception and at what level of information processing different modality interact.

Modulation of memory dynamic :

In the third chapter, I developed a regime of selection targeting specifically each form of consolidated memory and discovered that improvement of one form creates a specialization that is balanced by a decrease of the ability to produce the other form. Neurobiologists proposed two different models to explain ARM and LTM interaction. One model suggests a parallel processing of memory consolidation whereas the other model proposed that ARM and LTM are mutually antagonistic, and that ARM interferes with LTM formation (Isabel *et al* 2004). The interaction between ARM and LTM is clearly demonstrated in our results and the trade-off reflects the antagonism between both consolidated memories. The second important result from this artificial selection was to discover the constitutive cost related to LTM improvement. LTM formation was already known to be costly (Mery and Kawecki 2005) but, to my knowledge, it is the first time that a constitutive cost has been attributed to a specific long-lasting memory phase. Even if this cost on longevity was only observed in males, we suppose females may express this cost on another phenotype. In order to have a complete documentation about life history trait interaction with cognitive ability, changes in other fitness-related traits need to be investigated.

The genetic potential of improved learning and memory ability depends first on the genetic variability within population. The evaluation of learning and memory abilities on 40 inbred lines showed that phenotypic variability can be explained either by the variance of resource allocation to general cognitive abilities related to other fitness-related trait (olfaction, locomotion …) or by the variance of resource allocation to specific learning or memory modules. These two levels of adaptations should be affected by artificial selection. Results in the second and third chapters look controversial but can be explained by different levels of adaptation. Our regime of selection specifically affected ARM or LTM abilities but the observed improvement in STM ability whatever the regime could be explained by a general cognitive ability

improvement. It may be important to understand how much our evolutionary experiment acted on general learning processes and on specific memory modules. Our experimental lines must be tested for their ability in another learning task. For instance, the oviposition learning task tests flies' ability to associate a flavor of an oviposition substrate with an aversive bitter taste (Mery and Kawecki 2002). This protocol is more ecologically relevant than our aversive conditioning because it involve a choice of the best place to oviposit. In contrast to our classical conditioning, the oviposition site choice protocol consists in an operant learning. In this protocol, the temporal frequency of the events is not controlled, so it is not possible to determine if flies store information in LTM, ARM or both. Testing our selected and control lines in this protocol would help us to first determine the kind of memory formed in the oviposition site choice and secondly understand the degree of specialized or generalized improvement of cognition in our experimental population.

At the functional level, response to selection is mediated by molecular, physiological and neural changes (Whener *et al* 2001). Identification of genes or molecules responsible for the response to selection can be a starting point to understand how these changes occur. Even if gene mapping has been found to be difficult, it is now possible to determine every gene expression using microarray technology. Comparison between gene expression patterns and behavioral trait ability in same animals would help to find evidence that particular genes are involved in a behavior. However, several factors limit the advantage of such a technique to study responses to selection because it ignores the possibility that changes in allele frequencies are likely to occur in selected lines at loci unrelated to the target phenotype. Another approach to reveal the molecular changes consist in making quantitative proteomic. Microarray techniques assume that changes in gene expression directly underlie the change of behavior; however, the advantage of proteomics is that the real functional molecules of the cell are being studied. Strong gene expression, resulting in an abundant mRNA, does not necessarily mean that the corresponding protein is also abundant or indeed active in the cells. (Cox and Mann 2007). In collaboration with Alberto Pascual (Neuroscience Institute - Seville) and Thomas Préat (ESPCI – Paris), we intend to perform two-dimensional fluorescence difference gel electrophoresis (Unlu *et al* 1997). This technique consists in the extraction and electrophoresis of head protein from our selected and control lines in order to detect quantitative changes in protein expression.

Microarray and quantitative proteomic experiments would give some evidence that particular genes are related to behavioral variation. Once the genes are identified, mutants for these genes can be generated and tested to confirm their role in the behavior.

Consolidation, extinction and reconsolidation:

In the fourth chapter, I study the ability to update a memory in response of a modification of the environment. Extinction and reconsolidation are two opposite processes that occur after reactivation of a consolidated memory. I developed an experimental protocol to test how these processes are induced in *Drosophila* and what parameters favored one or the other.

After a classical conditioning, we destabilized consolidated memory by exposing conditioned stimulus without the unconditional stimulus. I showed that the number of reactivation cycle enhances opposite mechanisms such as few reactivation cycles lead to the reconsolidation of the original memory whereas extended reactivation lead to the consolidation of an extinction memory. Moreover, I demonstrated that, whatever the consolidated memory induced, its reconsolidation or extinction is protein synthesis-dependent suggesting that the reconsolidation is not just a repetition of the previous consolidation because a weak reactivation of ARM induced its reconsolidation into a LTM-like memory. The reconsolidation or extinction memory is LTM-like but its similarity with LTM needs to be investigated. Mutants disrupting specifically LTM: *tequila* or *crammer* could be tested in this protocol. After a massed conditioning, these mutants express normal ARM; then it is possible to destabilize ARM and test whether the reconsolidation and extinction are impaired or not. In the case these processes are impaired, it would mean that reconsolidation leads to the re-stabilization of the information in LTM and that extinction is the consolidation in LTM of the new association.

Even if these results give important indications about the similarities and differences among these three processes of stabilization, their interaction still remains unknown. For instance, the PTSD syndrome (Post Traumatic Stress Disorder) concerns people who, after strong traumatic events, cannot dissociate the conditioned stimulus with the traumatic outcome anymore (see review Yehuda and Ledoux 2007). The

underlying mechanism of this disorder still remains unclear: is it a problem of extinction that cannot occur anymore or because of a systematic reconsolidation of the traumatic association? Extinction is assimilated as a consolidation process of a new association between the CS and the absence of the US and I wonder whether selection for improved ability in consolidation process favored either reconsolidation or extinction. Testing our selected lines for improved ARM or LTM would give new information on the dynamic of memory stabilization through different processes.

All the results exposed in this thesis indicate the power of the model Drosophila to address questions about animal cognition. Using this model, I have investigated different levels of integration of environmental information and demonstrated that animal behavior is governed by a variety of adaptively specialized cognitive modules. But these modules are not exclusive and interact all the time in order to produce the adequate behavior. Two kinds of plasticity are investigated in this study. Learning allows a modification of the behavior in response to past experience and this plasticity occurs within an individual lifetime. In contrast, when a phenotype is modified by the environment and the individual genotype, this plasticity can become adaptive if natural selection favors responses that increase animal fitness over generations. Even if the drosophila neuronal system seems less complex than in vertebrates, the neuronal mechanisms underlying learning process appears to be very similar. Thus, determining the molecular and evolutionary aspects of learning and memory in an invertebrate model could give new insight about how evolution processes have shaped animal cognition in vertebrates.

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Scientific posters

A memory switch in Drosophila

Presented at Gordon Research conference Genes and Behavior 2008 (Lucca - Italy)

Intraspecific variability of learning and memory in Drosophila

Presented at Insect learning and memory workshop 2009 (Roscoff – France)

Evolutionary trade-off between two consolidated memory phases in Drosophila

Presented at 13th ESEB congress (Tuebingen – Germany)

A memory switch in *Drosophila*

Consolidation, reconsolidation and extinction of memory processes in *Drosophila melanogaster*

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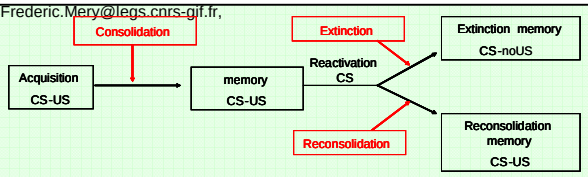
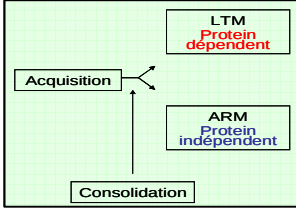
CONTEXT

Memory consolidation = process of stabilization over time

Memory retrieval may induce two process :

- reinforcement of the original memory = Reconsolidation
- consolidation of a new memory = Extinction





AIM In *Drosophila melanogaster*, two forms of consolidated memory have been demonstrated and can be independantly initiated :

- **LTM** protein synthesis dependent
- **ARM** protein synthesis independent

We took advantage of this dichotomy and, using classical olfactory conditioning, demonstrate:

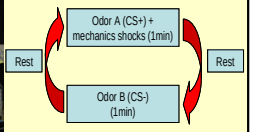
- **reconsolidation and extinction can occur after either ARM or LTM consolidation**
- **relationships between the three memory processes**
- **specificity of CS+ on ARM and LTM reactivation**

Materiels and Methods

Conditioning

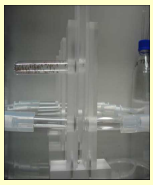


5 cycles massed (0 min interval between each cycles) or spaced (20 min interval between each cycles)



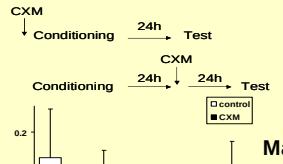
Conditioning cycle

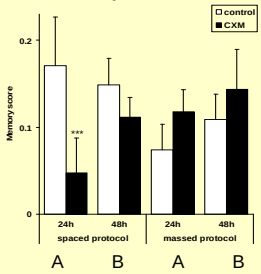
Test



Odor A **Odor B**

Treatment : Cycloheximide (CXM) = protein synthesis inhibitor





Massed protocol ➡ **ARM**

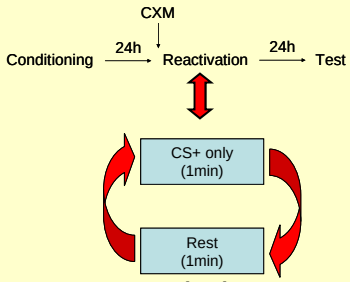
Spaced protocol ➡ **LTM**

A- Spaced protocol induces protein synthesis dependent memory formation : LTM

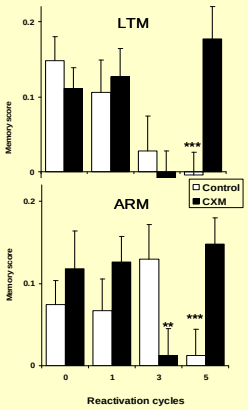
B- Once consolidated, ARM and LTM cannot be disrupted by protein synthesis inhibitor.

Results

1. Reactivation of ARM and LTM memory



Reactivation cycle



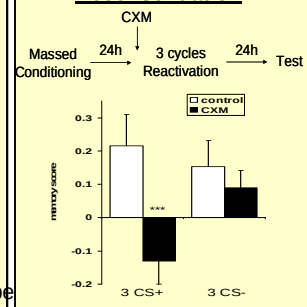
➡ **Reactivation can induce reconsolidation and extinction processes**

reactivation	1 cycle	3 cycles	5 cycles
LTM protein dependent	no effect	no memory	**Extinction** protein dependent
ARM protein independent	no effect	**Reconsolidation** protein dependent	**Extinction** protein dependent

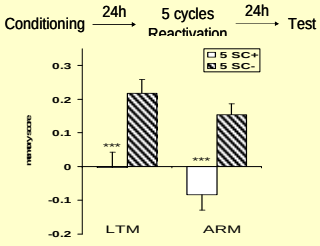
SWITCH

2. Specificity of CS+

Reconsolidation



Extinction



➡ **Reexposure effects are specific to the shock paired odor CS+**

DISCUSSION

➡ **In *Drosophila*, Reactivating an already consolidated memory destabilizes the association and induces either the reconsolidation or the extinction depending on reactivation condition. These induction are specific to the presentation of CS+.**

➡ **The existence of an ARM memory in *Drosophila* model allows us to show a switch from a consolidated protein synthesis independent memory to a protein synthesis dependent memory.**

Intra specific variability of learning and memory in *Drosophila*

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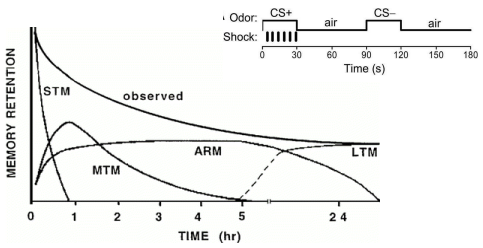
Natural populations are expected to show a wide range of phenotypic variation in cognitive abilities. Knowledge of this variation is important for understanding the adaptive evolution of neural systems . To answer this question, 40 inbred lines were derived from a natural population collected from Raleigh, North Carolina, USA (Mackay T.).

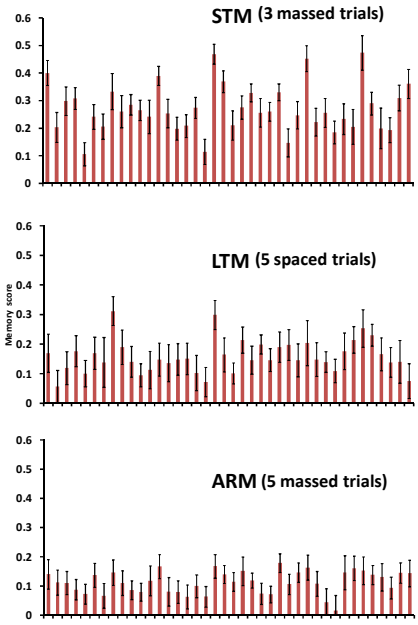
These lines are a huge collection of common polymorphisms affecting complex traits.

This study quantified variation among the 40 inbred lines for in learning and memory phase of a classical olfactory learning task.

Learning and memory in Drosophila

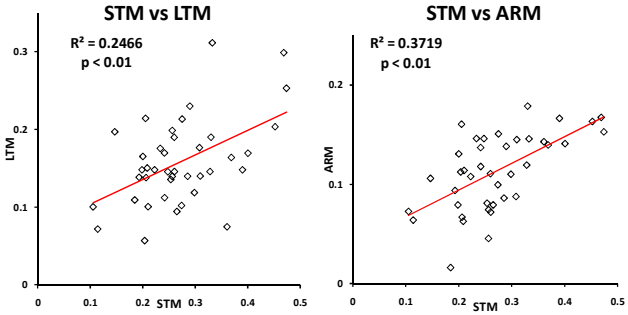


Variation in memory phenotype among wild-derived inbred lines

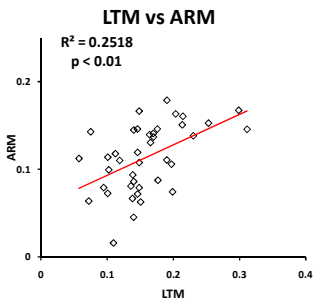


➡ Significant genetic variation for all memory phases

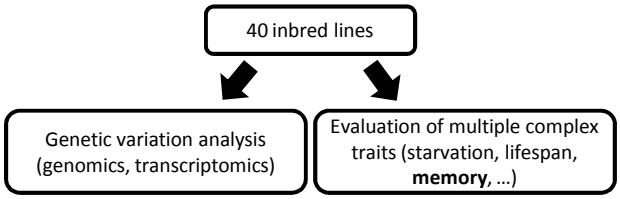
Genetic correlation between memory phases



Learning (STM) variability is positively correlated with both consolidated forms of memory (LTM and ARM), suggesting a pleiotropic effect of these traits in the same direction.

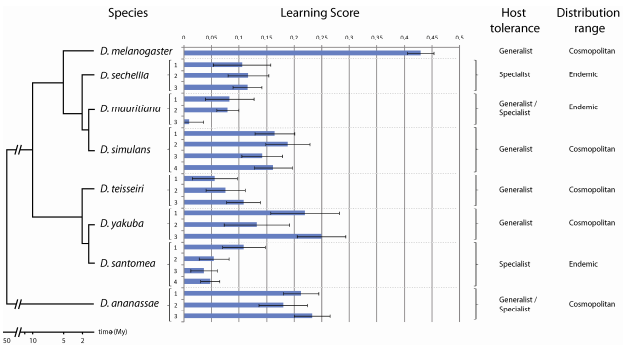


Future directions



At this low taxonomical level, the collection of information on complex trait phenotype may reveal pleiotropic effects between traits. Sequencing this genetic reference panel of 40 wild-derived lines can provide the opportunity to unravel the genetic basis of complex phenotypes.

Candidate genes for any complex trait can be identified by quantifying the trait phenotype in the reference panel of sequenced strains.



At a higher taxonomical level, the learning and memory polymophism could provide clues about ecological and life history factors promoting enhanced cognitive abilities over evolutionary time.

A5-Sy01-i016-R

Evolutionary trade-off between two consolidated memory phases in Drosophila

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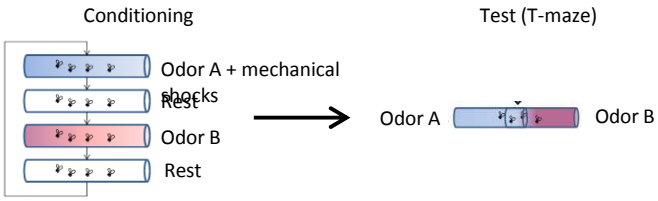
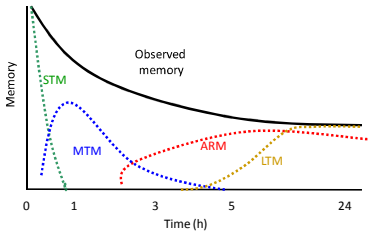
Memory is a complex and dynamic process composed of different phases. Its evolution under natural selection is likely to depend on a balance between its fitness benefits and costs. Learning and memory capacities have been found to trade-off with other fitness related traits but very little is known about the pattern of genetic correlations among different memory phases which may strongly affect cognitive evolution. Although a symmetrical trade-off has previously been observed between learning ability and ageing in *Drosophila* (Burger et al., 2008), how memory ability interact with longevity remains uninvestigated.

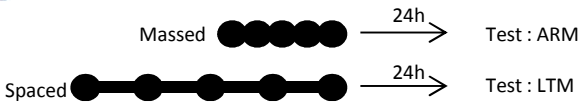
Olfactory aversive conditioning in Drosophila

Associative learning between an odor (CS) and a mechanical shock (US)

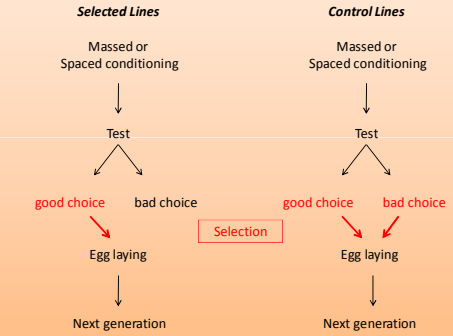




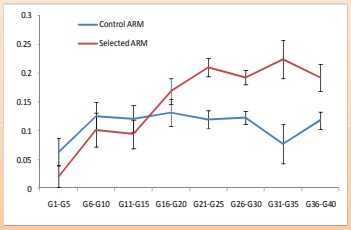
Dubnau et al. (2003) J. Neurobiol. 54:238



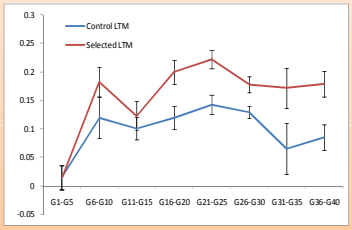
Experimental selection regime



Massed conditioning

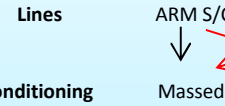


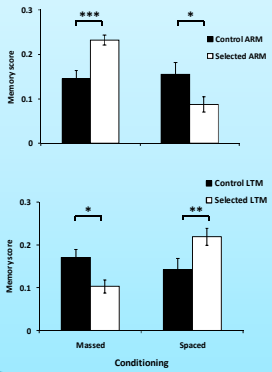
Spaced conditioning



After 20 generations, lines selected for improved memory showed better response than respective control lines 24h after massed or spaced conditioning.

Interaction ARM/LTM

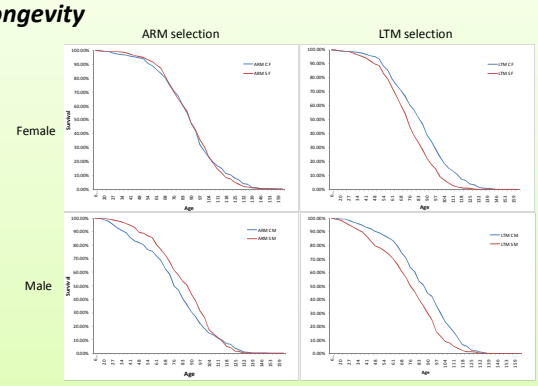




Surprisingly, compared to control lines, lines selected for improved ARM showed lower 24h memory when subjected to spaced conditioning and lines selected for improved LTM showed lower 24h memory when subjected to massed conditioning.

= Evolutionary Trade-off

Longevity



The longevity measurements in males have shown that selection for LTM improvement shortens lifespan whereas selection for ARM improvement increase population demography.

• Such trade-off may have important impact on the evolution of cognitive capacities. It would be consistent with the hypothesis that mechanisms underlying these forms of consolidated memory are, to some degree, antagonistic (Isabel G. *et al*, 2004). It may also help to understand why evolution has maintained two consolidated memory phases and open perspectives on the study of the adaptive value of these different memory phases.

• The effect of selection regime on longevity is antagonistic depending on the form of memory selected. In males, lifespan is shortens by improving LTM whereas selection for ARM improvement extends demography. This results demonstrate the constitutive cost of LTM on fitness.

Abstract

Cognition refers to the mechanisms by which animals acquire, store, process and act on information from the environment and this includes perception, learning, memory and decision making. Animals have their own perceptual world and adaptation seems to be crucial in order to survive by developing specialized ability in regard of the relevance of each sensory information. The process of storage is another mechanism important for adaptation because learned information can be retained from one occasion to the next. The underlying mechanisms of behavioral adaptation are based on learning and phenotypic plasticity. How this plasticity induces the formation of these adaptive specialized modules still remains unsolved.

The general aim of this PhD hold on the modularity and plasticity of olfactory learning and memory ability in *Drosophila melanogaster*. Drosophila is always confronted to complex environments with generally more than one stimulus that need to be associated with positive or negative reinforcements. In laboratory, it is possible to reproduce that kind of behavior in various protocols of associative learning. I tested adaptation processes at different levels of information processing. I demonstrate in this manuscript that adaptation occurs at each level: perception of complex stimuli, storage of relevant information and also update of memory trace not relevant anymore. Complex cognitive processes revealed the existence of adaptive modules more or less specialized that allows the animal to adapt to its specific environment. Moreover, artificial selection on specific memory ability demonstrates the implication of evolution in the modularity of animal cognition.