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Experimental evolution, behavior and genetics: Associative learning as a case study

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Abstract The evolutionary dynamics of behavioral traits reflect phenotypic and genetic changes. Methodological difficulties in analyzing the genetic dynamics of complex traits have left open questions on the mechanisms that have shaped complex behaviors and cognitive abilities. A strategy to investigate the change of behavior across generations is to assume that genetic constraints have a negligible role in evolution (the phenotypic gambit) and focus on the phenotype as a proxy for genetic evolution. Empirical evidence and technologic advances in genomics question the choice of neglecting the genetic underlying the dynamics of behavioral evolution. I first discuss the relevance of genetic factors – e.g. genetic variability, genetic linkage, gene interactions – in shaping evolution, showing the importance of taking genetic factors into account when dealing with evolutionary dynamics. I subsequently describe the recent advancements in genetics and genomics that make the investigation of the ongoing evolutionary process of behavioral traits finally attainable. In particular, by applying genomic resequencing to experimental evolution – a method called Evolve & Resequence – it is possible to monitor at the same time phenotypic and genomic changes in populations exposed to controlled selective pressures. Experimental evolution of associative learning, a well-known trait that promptly responds to selection, is a convenient model to illustrate this approach applied to behavior and cognition. Taking into account the recent achievements of the field, I discuss how to design and conduct an effective Evolve & Resequence study on associative learning in *Drosophila*. By integrating phenotypic and genomic data in the investigation of evolutionary dynamics, new insights can be gained on longstanding questions such as the modularity of mind and its evolution [*Current Zoology* 61 (2): 226–241, 2015].

Keywords Experimental evolution, Behavior, Genomics, Evolve and Resequence, Learning, *Drosophila*.

1 Introduction: Evolutionary Dynamics of Behavioral Traits

Reconstructing the evolution of behavioral traits can be a major challenge, especially when phenotypic variability is high. This variability is due to genetic factors originating from evolutionary processes and environmental effects. In the last decades behavior genetics has investigated the role of genetic and environmental variables in determining individual differences but the processes that eventually lead to different behaviors and cognitive abilities remain to a large extent elusive. Genetic data can provide hints about evolutionary processes but until recently limitations in analyzing the genetics of complex traits and their evolutionary dynamics have constrained this investigation. Grafen (1984) has even theorized the notion of "**phenotypic gambit**" [see Glossary for words in bold], according to which genetic mechanisms do not significantly constrain the trajectories of phenotypic evolution. Following this idea, phenotypic evolution can be considered a proxy for genetic evolution, and integration between phenotypic and genetic studies is not necessary.

Advances in genomics such as the development of **high-throughput sequencing** technologies (Metzker, 2010) have open new possibilities for investigating behavioral traits and go beyond the phenotypic gambit approach. Rittschof and Robinson (2014) have shown how gene expression profiles can be used in the study of behavioral adaptation to describe behavioral strategies, predict behavior and investigate plasticity. In the next sections I will show that genomic investigation can be applied also to the ongoing evolutionary change observed during **experimental evolution** (see Kawecki et al., 2012) through the **Evolve and Resequence** method (E&R) (Turner et al., 2011). Beside complementing other methods in the investigation of the **genetic architecture** of a trait, this approach can help clarifying the evolutionary basis of behavior and its dynamics, the role of genetic constrains and the genetic modularity of different traits. Since recent literature already provides a comprehensive review of experimental evolution and

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genomics (Kawecki et al., 2012; Schlötterer et al., 2014; Schlötterer et al., 2014) the goal of this paper is to elucidate the necessity and possibilities to use experimental evolution coupled with genomics on behavioral traits.

In my analysis I will focus on **associative learning**, a convenient candidate to study the connection between behavior, genomics and evolution. Seminal research on associative learning in rats (Krechevsky, 1933; Garcia and Koelling, 1966) and imprintability to different stimuli in chickens (Goodwin and Hess, 1969) and quail (Kovach, 1990) has revealed that animals are endowed with species-specific predispositions that facilitate or orient learning towards specific stimuli. These predispositions are associated with **segregating variants** that respond to selection (Tryon, 1940; Graves and Siegel, 1969; Mery, 2013; Dunlap and Stephens, 2014), thus showing a great potential for experimental evolution.

In the last decades geneticists have investigated single genes that affect learning by using mutational analysis (Wen et al., 1997; Busto et al., 2010 provide a review for fruit flies), and have only recently started more systemic studies that address the interactions and multiple effects of specific genes (Cressy et al., 2014). So far though, a comprehensive analysis of the evolutionary dynamics of learning, which includes genetic dynamics of natural populations, has not been pursued. This prevents us to fully understand the mechanisms that have shaped learning abilities. I will show the importance of incorporating genetic data into evolutionary analysis and how to take advantage of the E&R methods to investigate the evolutionary dynamics of learning and other behavioral traits. This approach is important to clarify longstanding issues on the evolution of the mind, such has the modularity of its architecture and evolution.

2 Experimental Evolution and the Heritable Component of Learning

Investigating the heritable component of behavior and its evolution has intrigued researchers for centuries. Given that behavioral phenotypes are often influenced by the interactions between a large number of genes and the environment, unraveling these traits poses more than a challenge. The case of learning is representative because this trait is central to ensure behavioral flexibility in different domains and it is widespread among different species and taxa, from nematodes (Qin and Wheeler, 2007) to arthropods (Giurfa, 2013), birds and mammals (Shettleworth, 1998). Moreover, learning abilities are an important component of individual plasticity and can constrain adaptation within an individual's lifespan (Fawcett et al., 2013). In model species such as *Drosophila melanogaster*, much has been done to identify molecular mechanisms, pathways and neural networks of associative learning.

Well before genetic dissection tools became available, experimental evolution studies have been used to unveil the portion of learning that is under genetic control. This method is based on the observation of evolution in populations exposed to an experimentally controlled environment for many subsequent generations (McGuire and Hirsch, 1977; Dykhuizen and Hartl, 1983; Travisano and Lenski, 1996) and can be used to test evolutionary theories and to provide novel insights into the phenotypic and genetic outcomes of evolution (Adams, 2004; Bull and Wang, 2010). The different approaches used for experimental evolution (**artificial selection**, mass selection and **laboratory natural selection**) work by imposing selection for specific traits and observing changes across subsequent generations. In 1909 Galton had already suggested a breeding experiment to obtain a line of "superior" dogs. Few decades later Tolman (1924) bred lines of rats good and bad in solving learning tasks, and observed an increase of their behavioral specificities in the next generation. Carrying on this line of research, Tryon (1940) selected lines of rats with enhanced *vs.* decreased capability to learn how to navigate through a maze. He used the total number of errors on a maze as selection criterion with the aim of creating "bright" and "dull" lines rats. Few generations after the beginning of artificial selection, the so-called bright lines solved the maze with significantly fewer errors than the so-called dull lines.

Krechevsky (1933) investigated whether the selected lines of rats used different strategies to solve/navigate through a maze. He found that the few-errors line typically used spatial strategies (e.g. right\left), whereas the many-errors line adopted visual strategies (e.g. dark/light). Hence selective pressures can modify learning capabilities and have a specific effect at the level of the strategies/mechanisms used to solve a task. Searle (1949) has also found several differences in the behavioral profile of rats selected for different performance in maze-learning (see also Rosenzweig, 1998 for a review of the role of motivation vs. learning in these experiments). These biases/predispositions have been called **constraints on** or **preparedness for learning** (see Domjan and Galef, 1983; Dunlap and Stephens, 2014). Evidence of preparedness for learning has been documented also

in different associative learning tasks in rats (Garcia and Koelling, 1966), in **filial imprinting** in precocial birds (Graves and Siegel, 1969 for imprintability in chicks of domestic fowl; Kovach, 1990 for color imprintability in quails), in different cognitive domains in young domestic chicks (Johnson et al., 1992; Vallortigara, 2012), in the preferential use of "win-shift" over "win-stay" strategies during foraging in birds (Gill and Wolf, 1977; Kamil, 1978; Cole et al., 1982), among other traits. Recently, Dunlap and Stephens (2014) have used laboratory natural selection on *Drosophila* to investigate the role of the environment in shaping predispositions for learning by manipulating the reliability of olfactory and visual cues as predictors. In populations where the visual-taste association was maintained reliable across generations, after 40 generations of selection individuals significantly increased their promptness in learning the association between visual and olfactory cues compared to controls. These results provide direct evidence that preparedness for learning can be shaped by evolutionary pressures.

In the last decades, experimental evolution studies on learning have been mainly conducted in insects. Brandes (1988, 1991) has used artificial selection on the honeybee *Apis mellifera* to identify the portion of phenotypic variability of the proboscis extension reflex (PER) that is controlled by genetic factors. He found an intermediate heritability (between 0.39 and 0.54) of this trait. Ferguson et al. (2001) used artificial selection on drones, showing that also individual variation in reversal learning performance has a heritable component. While these studies used breeding in single generations, more extended selection has been conducted in the fruit fly *Drosophila melanogaster*: by using artificial selection for 25 generations, Lofdahl et al. (1992) showed that fruit flies can be selected for conditioning (and extinction), whereas Mery and Kawecki (2002) and Dunlap and Stephens (2009, 2014) have used the **oviposition paradigm** to propagate flies under laboratory natural selection. In this method female flies are first exposed to olfactory (or visual) stimuli associated to palatable or aversive food (e.g. orange juice smell associated with palatable food, apple juice smell associated with aversive food), and then tested for oviposition preference guided by odor (or vision). Flies that remember the association presented during the exposure phase are expected to lay more eggs in the substrate whose smell (or color) was never associated with aversive food than flies with worse learning capabilities. To induce selection for enhanced learning, Mery & Kawecki (2002) used only the eggs laid in the substrate whose smell was never associated with aversive food as founders of subsequent generations. As effect of this selection regime, in about 15 generations the proportion of good learners significantly increased in selected populations (but not in control populations not exposed to selection). Dunlap and collaborators (Dunlap and Stephens, 2009, 2014) have used this method to test specific hypotheses on evolution, showing that the environmental pressures can shape learning capabilities.

Artificial selection studies confirm the flexibility of learning capabilities in response to environmental pressures also in non-model organisms such as the blow fly *Phormia regina* (McGuire and Hirsch, 1977) and the parasitic wasps *Cotesia rubecula* and *C. glomerata* (van den Berg et al., 2011). In this study experimenters selected *C. glomerata*, that in the wild is able to form long-term memory after a single conditioning trial, to form long-term memory only after repeated spaced conditioning, as *C. rubecola* does. Learning mechanisms are hence sensitive to selective pressures in a variety of species.

As I have illustrated, experimental evolution has been used to select for enhanced or decreased learning abilities. None of these studies though have investigated how selection for a specific learning strategy constrains not only the strategy preferentially used by an individual but also the evolvability of alternative learning strategies. **Reverse selection** studies (Teotonio and Rose, 2001; Estes and Teotonio, 2009) – in which populations selected for phenotype P_1 are then exposed to selection for the ancestral phenotype P_0 – have shown that the evolutionary history of a population can influence subsequent evolution. In the case of learning, **directional selection** for a specific strategy might remove allelic variants associated with the ancestral phenotype, thus preventing or slowing down the evolution towards the ancestral state.

Overall, experimental evolution studies have showed a prompt response to selection for learning in different species and taxa (e.g. Tryon, 1940 in rats; Brandes, 1988 in honeybees; Mery and Kawecki, 2002 in fruit flies). This indicates that natural populations host a high degree of **genetic variability** for learning. The aforementioned studies though have investigated the genetic component of learning only indirectly, without identifying allelic variants underlying differences in behavior. A recent study used a genetically simplified *D. melanogaster* population to investigate the evolutionary dynamics of associative learning in an experimentally de-

fined set of polymorphic loci (Cressy et al., 2014). Researchers used experimental evolution on an experimentally built population that contained only few segregating loci beside a mutation that disrupted olfactory learning (null mutation in the *rutabaga* adenylyl cyclase). In this genetically simplified population, after few generations of selection the olfactory learning phenotype was rescued through the action of eight out of 23 variable loci. The single locus which provided a detectable phenotypic effect could explain only a small fraction of the population-level response. On this basis, the authors suggested that the effect of combinations among several alleles is the most likely explanation for the rescue of the *rutabaga* defect. Moreover, because each of the three selected replicate populations was heterogeneous and no single genotype was prevalent, data indicate the presence of multiple genetic solutions (see also Nepoux et al., 2015). Although this experiment suggests that a null mutation in the *rutabaga* adenylyl cyclase can be rescued through the interaction of several genes, little is known on the role of genetic constraints in the preferential evolutionary trajectories. In the next section I will discuss the issue of genetic constraints on evolution.

3 Genetic Constraints on Evolution

The necessary components of evolution are (i) variation between individuals, (ii) heritability of that variation and (iii) differential reproductive success of different variants. In the case of direct competition between populations or species and in the case of environmental change, (iv) the rate of evolution is crucial to determine the differential success or even the survival/extinction of a lineage. In this section I will discuss how genetic factors affect evolution either as prerequisites for evolution and in influencing evolutionary rate and evolutionary trajectories.

Genetic factors such as mutations and **recombination** are the primary source of variability and absence/low levels of genetic variation are a limit for evolution. As pointed out by Hoffmann (2014), populations and species may lack (functional) genes or variants necessary to adapt to new environmental conditions. This is the case of the Antarctic fish *Trematomus bernachii* (Hofmann et al., 2000) and some fruit fly species (Hoffmann et al., 2003) that lack copies of genes necessary for surviving in hot conditions. In such situations, the absence of the appropriate functional genes can pose fundamental evolutionary limits. Although many traits are influenced by several genes, related networks and regulatory mechanisms, in some circumstances – e.g. small sized populations – it is reasonable to expect lack or reduced variability. This condition can be modified only when, if the population survives, new variants have arisen, which in turn depends on other genetic factors such as mutation and recombination rate.

Absence of genetic variation can be detected through lack of response to selection or extremely low or absent heritability (Hoffmann, 2014). An indirect evidence of genetic constraints on evolution comes from the comparison of narrow- and widespread *Drosophila* species (Kellermann et al., 2009). Narrowly distributed tropical species consistently exhibit/display lower means and genetic variation for desiccation and cold resistance, suggesting that specialist species may simply lack genetic variation in key traits, limiting their ability to adapt to conditions beyond their current range. Or, given that two species of rainforest-restricted *Drosophila* respond to mild but not to harsh selection pressures (Heerwaarden and Sgrò, 2014), it is possible that reduced genetic variation can slow down the rate of evolutionary responses. Further empirical studies should clarify to which extent the mechanisms and strategies of learning are constrained by genetic variability.

The evolutionary history of a population can impose genetic constraints on evolution by modifying the patterns of genetic variation and producing population-specific **fitness landscapes** (de Visser and Krug, 2014). This is particularly clear in reverse selection studies (Bull and Charnov, 1985), where the duration and strength of selective pressures influence the convergence to ancestral genotypes (see Desai, 2009 for a discussion). The influence of the past and its duration in turn depend on population genetic parameters, such as population size, mutation and recombination rates, breeding system.

Even in the presence of genetic variation for traits under selection, adaptive evolution can be limited or slowed-down by factors connected to the genetic architecture of a trait. This is the case studied in populations of prairie plants exposed to warmer and drier climates (Etterson and Shaw, 2001). In spite of the genetic variability harbored in these populations for three selected traits, antagonistic interactions slow-down the evolutionary change expected in response to specific rates of environmental change.

The main systemic effects at the level of genetic architecture are **pleiotropy** and **epistasis**. Pleiotropic effects occur when one allele affects multiple traits. For instance, the *D. melanogaster* learning mutant *dunce*, initially identified as affecting associative learning

(Dudai et al., 1976), has been shown to cause also female sterility (Bellen et al., 1987). Pleiotropy is a frequent condition, because regulatory factors and proteins encoded by genes participate in networks that can influence the expression or regulation of multiple traits and other networks. Due to pleiotropy, a mutation that positively affects one trait can affect another trait in a similar direction, thus contributing to a genetic correlation between traits. But one allele can also determine a trade-off when it increases the fitness relative to one trait while decreasing the fitness of another trait. For instance, a trade-off between (increased) learning ability and (decreased) larval competitiveness (Mery and Kawecki, 2003) and longevity (Burger et al., 2008) has been reported in fruit flies after experimental evolution. In these studies experimenters observed that populations selected for increased learning abilities were associated with fitness costs in other traits. These effects can be explained also by epistasis, namely the interaction between different loci. Epistatic effects have been shown to strongly depend on environmental conditions, thus multiplying the complexity of the interactions (Anholt and Mackay, 2004). Epistasis among variants relevant for the fitness of an individual can constrain the temporal order in which mutations are favored by selection, and influence the spectrum of mutations entering populations and how the evolution of mutational effects constrains the genetic architecture of complex traits at the population level (Jones et al., 2014), thus binding the evolutionary process. An increasing number of studies is pointing at the role of epistasis to explain the genetic complexity of quantitative behavioral variation (Mackay, 2001; Manolio et al., 2009; Huang et al., 2012; Anholt and Mackay, 2015), although this matter is still debated (Hill et al., 2008; Yang et al., 2010; Turner et al., 2013).

Trade-offs refer also to phenomena such as **heterozygote advantage**, that can impose constraints on evolutionary patterns based on the features of genetic inheritance. In diploid organisms, when the heterozygotes at one locus are fitter than either homozygotes, a part of the population will carry the less advantageous homozygous genotypes. In wild Soay sheep, large horns confer an advantage in intra-sexual competition, but variation within the population is still present for horn size. Johnston et al. (2013) showed that most genetic variation in this trait is maintained by a trade-off between natural and sexual selection at a single gene, whose allele conferring larger horns is associated with higher reproductive success, but the alternative allele confers increased survival, resulting in an heterozygote advantage effect.

The spatial arrangement along the chromosome determines further constraints, given that variants closely located tend to be passed together to the next generation, a phenomenon called genetic linkage. Genetic linkage varies between species (e.g. on average it is extremely low in *Drosophila melanogaster* compared to humans) and chromosomal regions. Interestingly, segregating variation has been documented in natural populations for **chromosomal inversions**, and empirical data show that the frequency of inversion polymorphisms is affect by selection (Kennington and Hoffmann, 2013; Kapun et al., 2013). Within these regions genetic linkage is incremented, thus increasing the probability of genetic correlations that can affect evolutionary trajectories.

In the light of the genetic factors discussed in this section – standing variation, pleiotropy, epistasis, genetic linkage – blindly assuming the absence of genetic constraints on phenotypic evolution looks like a risky strategy. In fact, although correlations between phenotypic and genetic traits have been documented (e.g. Cheverud, 1988; Reusch and Blanckenhorn, 1998 and references therein), genetic constraints can prevent or reduce the probability of some trajectories, and penalize evolutionary change. In some circumstances substantial differences have been shown between genetic and environmental relationships and inferring genetic correlations from phenotypic data can be unreliable (see for instance Willis et al., 1991 for a thorough discussion of this topic). Hadfield et al. (2007) showed that in bird coloration important genetic patterns can be obscured at the level of phenotypes even in individuals reared in the same brood. For example, the relationship between nestling tarsus length (a health index) and color patch is very different at the genetic, natal and phenotypic levels. While a negative relationship between tarsus length and back color has a genetic basis, a positive relationship arises at the level of the nest, and there is no phenotypic correlation between these two traits. Considering phenotypes a proxy for underlying genotypes is particularly hazardous for behavioral traits, that have a low or moderate heritability. In fact, the lower the heritability of a trait, the larger the difference between phenotypic and genetic correlations is expected (Hadfield et al., 2007).

An integration between behavioral and genetic/genomic studies will be important to understand the origins of different behavioral mechanisms, the evolutionary potential and mechanisms. In the next section I will discuss the issues connected to this enterprise by referring to traditional and more recent methods to investi-

gate behavioral traits, learning included.

4 Genetic and Genomic Investigation of Behavioral Traits

Phenotypic variation in behavioral traits derives from different sources: environmental variables including previous experience, developmental changes and epigenetic effects[①], genetic variables, and interactions between these factors. As already mentioned, the level of complexity underlying phenotypic variation can be particularly high in the presence of traits with a complex genetic architecture, epistatic and pleiotropic effects. For these reasons identifying the causative variants relevant for a complex trait such as learning can be a challenge.

Historically, researchers have adopted mutational analysis to infer the function of specific genes by looking at the phenotypic changes induced by genes experimentally mutated – e.g. *amnesiac* (Quinn et al., 1979), *dunce* (Dudai et al., 1976) and *rutabaga* (Quinn et al., 1974) mutants for learning (see also Folkers, 1982); *period* mutants on circadian rhythms (Konopka and Benzer, 1971); *paralitic* mutants for deficits in locomotion (Siddiqi and Benzer, 1976). Large phenotypic effects determined by the damage of a single gene can have a crucial role in revealing pathways or neural circuits connected to specific behaviors. This method has been particularly fruitful in the study of circadian clocks (Hardin, 2011; Zheng and Sehgal, 2012) and olfactory learning, a model system which has close similarities in insects and mammals (Davis, 2004). In the fruit fly *Drosophila melanogaster* researchers have successfully identified variants that impair different memory stages of olfactory learning (Tully, 1996; Davis, 2005; Busto et al., 2010). However most of these variants are so detrimental that have not been found in natural populations (Mery, 2013), providing little insight into the evolutionary history of the trait.

Although studies based on mutagenesis cannot clarify the evolutionary history of a trait, as Anholt et al. (2015) noticed they can clarify some of the cellular pathways and mechanisms underlying behavioral traits, and contribute to the dissection of the genetic architecture of behavioral phenotypes. This approach though can hardly identify connectivity and synergistic effects between genes that simultaneously contribute to a phenotype. Several studies have documented that epistasis and pleiotropy are determinant features of the genetic architecture of quantitative traits (Swarup et al., 2012; e.g. Huang et al., 2012). Methods that allow a more systemic approach in investigating complex traits are **quantitative trait loci** (QTL) **mapping** (Mackay et al., 2009) and **genome-wide association studies** (GWAS) (Manolio et al., 2009).

QTL analysis focuses on quantitative trait loci, namely regions of the genome that influence traits that vary in a continuous way, depending on multiple genes. QTL mapping is based on linkage-based analyses (in which individuals with known relationships are used to identify segregating markers that predict the organismal phenotype after few generations of recombination) or association mapping (in which causal loci are identified using **linkage disequilibrium**, namely the association of linked polymorphisms). Traditional QTL studies suffer from very low genetic resolution. For instance Steinberger et al. (2003) could not go beyond the chromosome level while trying to identify genomic regions relevant for individual variation in spatial learning in mice. By choosing the appropriate experimental design (e.g. extreme QTL analysis or linkage group selection, in which frequencies of genetic markers are estimated by comparing large groups selected for the trait of interest with unselected control groups) and applying high-throughput sequencing technologies it is now possible to increase the genetic resolution.

In some cases QTL studies have revealed that complex behaviors can have an unexpectedly simple and modular genetic architecture. Consider the example of the burrowing behavior of *Peromyscus mice* (Weber et al., 2013). In nature *Peromyscus maniculatus*, which lives in unexposed habitats, builds small and simple burrows with no escape tunnels. On the contrary, the sister species *Peromyscus polyonotus* builds complex burrows with a long entrance and an escape tunnel, that are particularly convenient in the exposed habitats where this species lives (Weber and Hoekstra, 2009). Genetic crosses between these species showed that the complex burrows are dominant over simple burrows and evolved as derived traits through the addition of multiple genetic changes. The length of the entrance tunnel and the presence of the escape tunnel are uncoupled, thus indicating the modularity and evolutionary independence of these traits. Quantitative trait loci analysis showed that tunnel length is affected by at least three independent genetic regions, whereas the presence

① See Fitzsimons and Scott (2011) for learning, and Peixoto and Abel (2013) for learning and high-throughput sequencing.

of an escape tunnel is associated with a single locus. The relative simplicity of the genetic basis of these traits suggests the possibility to directly investigate whether genetic factors, such as the presence of specific genetic variants, have influenced the evolution of long burrows with secondary tunnels in species such as *Peromyscus aztecus* (Weber and Hoekstra, 2009), that lives in environments with sparse cover. It would be interesting to study whether, for relatively simple traits such as the burrowing behavior of *Peromyscus* mice, reverse selection can identify any influence of recent evolutionary history at the phenotypic level.

In most cases though, QTL analysis has revealed a complex genetic architecture for behavioral traits, and pervasive presence of epistasis without identifying causative variants replicated across different experimental replicates and studies (e.g. Huang et al., 2012; Turner et al., 2013).

Genome-wide association studies (GWAS) are another technique used to uncover, and in some cases map, the genetic basis of phenotypic variation present in natural populations. This approach has mainly focused on phenotypes of biomedical importance, trying to detect associations between genotype frequency and specific phenotypes (McCarthy et al., 2008; Marigorta and Navarro, 2013). Papassotiropoulos et al. (2011) have applied this method to short-term memory in human beings, identifying a polymorphism of the *SCN1A* gene that is important to short-term memory. In the last years a large number of GWAS studies has been conducted on behavioral traits, but this effort has not provided the advancement originally expected in the understanding of the genetic architecture of the investigated traits. It has been claimed that few genetic associations reported are well established (Munafò, 2009).

Limitations of QTL mapping and GWAS include risk of false-positive results, lack of sensitivity to rare variants, difficulties in identifying loci with small, polygenic and non-additive effects. These studies can typically explain only a small fraction of heritability (Manolio et al., 2009) and result in low predictability or low resolution in the identification of causative variants. Although high-throughput sequencing permits investigation of whole genomes, connected methodological and statistical issues complicate the interpretation of results (e.g. Vilhjálmsson and Nordborg, 2013; Broer et al., 2013). The large amount of data processed is accompanied with an inflation of potentially spurious associations and scarcely replicated results (Huang et al., 2012).

QTL mapping and GWAS typically take a static picture of the investigated trait. Combining experimental evolution and high-throughput sequencing adds information about the consistency of evolutionary trajectories in time. This method, originally proposed by Huang et al. (2009), is known as Evolve & Resequence (E&R), after Turner et al. (Turner et al., 2011). In the last years the E&R approach has become more and more popular to investigate complex eukaryotes such as *Drosophila* (Schlötterer et al., 2014; Schlötterer et al., 2014) for either artificial selection (Turner et al., 2011; Remolina et al., 2012; Turner and Miller, 2012) or laboratory selection studies (e.g. Burke et al., 2010; Orozco-ter Wengel et al., 2012; Tobler et al., 2014). A crucial advancement that allows geneticists to investigate whole genome population dynamics of diploid organisms is the use of Pool-seq, namely the simultaneous analysis of the DNA of multiple individuals from a population sequenced together (Futschik and Schlötterer, 2010; Schlötterer et al., 2014). This method has been recently extended to RNA data (see Konczal et al., 2014), and to GWAS, revealing to be a convenient and relatively cheap technique to identify causative loci that determine natural variation, at least for traits with a relatively simple genetic architecture such as abdominal pigmentation in fruit flies (Bastide et al., 2013).

E&R is based on estimating the allelic frequencies[②] of a starting population not exposed to selection, and resampling the populations at subsequent generations. Technological limitation of current sequencing techniques prevent the study of **copy-number variation**, so that only single-nucleotide polymorphism (SNP) variants can be analyzed. Since copy-number variants may be subject to selection (e.g. Sebat et al., 2004; Emerson et al., 2008; Huang et al., 2014) this limitation has to be taken into account. By comparing the change in allele frequencies between selected and control populations, and taking into account the consistency across experimental replicates, it is possible to identify genomic regions that respond to selection, track allelic changes and eventually identify causative variants. Applied to behavioral traits, such as the duration of the inter-pulse interval of the *Drosophila* male courtship song, this me-

[②]Although I focus on the study of allele frequency changes, other approaches that can be applied to an E&R include exome sequencing and high-throughput RNA sequencing (see Schlötterer et al., 2014).

thod revealed to be more powerful than GWAS in identifying causative variants (Turner et al., 2013). In spite of this, technical and theoretical difficulties/limitations should be taken into account when deciding to apply this method and designing an experiment, in order to maximize the benefits/costs ratio and reduce the number of false positives (Kofler and Schlötterer, 2014). Potential pitfalls of this approach have been analyzed elsewhere (Baldwin-Brown et al., 2014; Schlötterer et al., 2014; Schlötterer et al., 2014; Kofler and Schlötterer, 2014). This work has produced a set of suggestions to increase the power, accuracy and resolution of E&R studies that can be summarized as follows: have sufficient genomic coverage (number of **reads** aligned under different genomic regions), large population size, low genetic linkage in the starting population, high number of replicate populations, and many generations of selection. In the next paragraph I will discuss in more detail the most important issues to take into account to design and conduct an E&R study on behavioral traits, using the well-studied example of associative learning as a case study.

5 Evolve and Resequence: Associative Learning

E&R is a direct way to investigate the evolutionary origins of behavioral traits at the phenotypic and genetic level. Theoretical and simulation work (see Kessner and Novembre, 2014; Schlötterer, et al., 2014; Kofler and Schlötterer, 2014) has recently clarified the importance of experimental design and statistical approaches for E&R. While discussing the crucial aspects to design and conduct an E&R study on behavior, I will outline the issues that need to be considered focusing on associative learning in *Drosophila* as a case study.

5.1 Model system

In the choice of the model system for an E&R study, many issues must be taken into account beside ease of propagation and generation time: variability in the investigated phenotype and its responsiveness to selection, availability and quality of the reference genome, genomic features that can facilitate/made genomic analyses difficult (e.g. linkage disequilibrium), availability of genetic tools to investigate the pathways involved in a trait. Studying different species can provide comparative evidence on the evolution of associative learning and species-specific features. I will show why at present *Drosophila* appears a particularly suitable candidate for E&R.

Drosophila melanogaster is one of the leading model systems for the study of associative learning, a trait that has already been shown to promptly respond to selection in this species (Mery and Kawecki, 2002; Dunlap and Stephens, 2014) but that has not been tested for the genomic correlates of phenotypic evolution. In addition to the convenience in laboratory propagation and the presence of documented complex behaviors, several advantages are connected with the use of this species: a compact and high-quality reference genome, short generation time, genetic-tools such as transgenic and mutant lines that can help validate candidate genes. For instance Jenett et al. (2012) have made available almost 7,000 GAL4 enhancer *Drosophila* lines whose neural activity has been mapped in the brain and associated with a defined genomic region. On the other hand *D. melanogaster*'s genome harbors many segregating inversions that increase the genetic linkage, thus decreasing the power and resolution of the study. A convenient substitute of *D. melanogaster*, which presents similar advantages, is the close relative species *D. simulans*, whose genome is almost free from chromosomal inversions (Aulard et al., 2004). Another possibility would be to use *D. melanogaster* and take advantage of the further information available for fully-sequenced individual genomes (Franssen et al., 2015). This could be achieved either by sequencing individuals from the sequenced replicates or by using as starting population an amplified population derived from sequenced lines, such as the *Drosophila* Genetic Reference Panel (Mackay et al., 2012) or the *Drosophila* Synthetic Population Resource (King et al., 2012). At present equivalent genetic and genomic resources are not available for other species that have been shown to respond to selection for learning, such as *Phormia regina* (McGuire and Hirsch, 1977). Species that have been effectively used to correlate gene expression patterns with behavioral phenotypes, such as the honeybee (Robinson et al., 2005; Rittschof and Robinson, 2014) are often not suitable for experimental evolution due to slow generation time and limitations in maintenance of large numbers of colonies/lines.

5.2 Starting population, maintenance and propagation

Depending on the phenotype and organism investigated it is feasible to purse an E&R study for a different number of generations, with important implications for the choice and composition of the starting population. Differently from experimental evolution studies in bacteria, that can be conducted for thousands of generations (Wiser et al., 2013), insects and vertebrates can be studied only for a limited number of generations. For in-

stance, about ten years are required to propagate 300 subsequent generations in standard conditions using *Drosophila*, while in the same amount of time less than 50 generations of rodents can be propagated. Hence it is no surprise that while it is possible to observe the emergence and evolutionary dynamics of new mutations in microorganisms, much of the evolutionary change observed in E&R for insects and vertebrates reflects frequency changes in alleles already present in the starting population. For this reason when applying E&R to insects or vertebrates it is often recommendable to start an experiment using wild-derived populations as diverse and large as possible.

In the case of fruit flies, two main strategies are possible to establish a starting population: use cages derived from hundreds (or even thousands) of individuals at a census size large enough to prevent inbreeding, or isolate founder fertilized females in dedicated culture vials – what is called an **isofemale line** (David et al., 2005) – to be subsequently amplified and divided in independent experimental replicates. If the number of founder lines that survive the full-sib mating is large enough, the isofemale line regime could reduce the loss of alleles due **genetic drift** compared to medium/large maintenance cages. However, the net loss of lines/alleles associated with the inbreeding and maintenance procedure requires a large starting population size. Moreover, as noted by a reviewer, if the lost alleles exhibit antagonistic pleiotropy, their loss could alter responses and conclusions compared to the original population. Relevant advantages of the isofemale line design include the possibility to start new experimental replicates at further stages using a nearly identical genetic pool, take advantage of individually sequenced lines (Mackay et al., 2012; King et al., 2012) and precisely manipulate the genetic variation in the population, whereas large cage designs allow to reduce the effect of laboratory adaptation before the beginning of the experimental procedure and can more faithfully reproduce the behavior of a natural population.

It has been noticed that suboptimal and variable rearing conditions reduce genetic repeatability (Gibert et al., 1998; David et al., 2005), with the risk of undermining the speed and power of E&R studies. Behavioral traits such as learning are known to be particularly prone to environmental effects (Anholt and Mackay, 2004), hence attention has to be paid on constant rearing conditions. In the case of fruit flies environmental variables that should be keep as constant as possible are: temperature, relative humidity, light/dark cycle, presence of high nutrient food with (David et al., 2005), larval and adult population density. Selective pressures other than learning can be spuriously introduced by the propagation procedure hence particular attention should be paid at this level.

5.3 Selection and experimental paradigm

In E&R studies researchers can impose selection by explicitly allowing a defined subset of individuals to breed (e.g. scoring individuals for learning abilities and propagating only the best learners, similarly to what Turner and Miller (Turner and Miller, 2012) did, selecting the most extreme phenotypes for the inter-pulse song interval in male fruit flies). Unfortunately phenotyping can be too time-consuming to be pursued on a large number of individuals and replicates, thus forcing experimenters to reduce the population size and increasing inbreeding/genetic linkage at the expenses of genetic resolution and statistical power. Moreover, by dismissing the fitness of many traits but the selected one, artificial selection can produce evolved populations that would not be produced by natural selection. A more convenient approach to E&R on learning should allow assessing learning abilities in large number of flies in parallel in multiple replicates, while flies breed freely during the experiment.

When using experimental evolution, the impact of unintended selective pressures should be reduced. In the aforementioned oviposition paradigm (Mery and Kawecki, 2002), in each generation the eggs laid during the test phase were rinsed and then transferred to a neutral medium for the subsequent stages of the experiment [alternatively, Dunlap and Stevens (2009) moved eggs individually with a needle], introducing selection for surviving the egg washing procedure. Postponing the moment in which the parental individuals lay eggs after the learning assessment would be sufficient to remove this selective pressure, and to impose selection to both male and female individuals. Fig. 1 shows an alternative paradigm used to investigate associative learning in fruit flies (Versace and Reisenberger, 2015). This method, implemented in a simple **T-maze**, does not require individual handling of the eggs and can be used on both sexes. After starvation, hundreds of flies are exposed to odor A associated with aversive food A- (Fig. 1.1 Exposure A). Only flies that have sampled aversive food A- (i.e. food A supplemented with an odorless aversive substance such as quinine) are passed to the next stage. Collected flies are exposed to odor B associated with appetitive food B+ (Fig. 1, 2, Exposure B). Flies trapped in the vials during the second exposure phase are then

tested (Fig. 1.3, Test). In the test phase experimenters expose flies to odor A and B (no food is supplemented with quinine at this stage. Only flies that during the test phase choose the odor never associated with quinine are propagated to the next generation. In subsequent generations the odor associated with the aversive stimulus is swapped, to prevent selection for perceptual preference. In this paradigm compulsory experience with both odors and flavors helps reducing random choices in the test phase, compared to the standard oviposition paradigm.

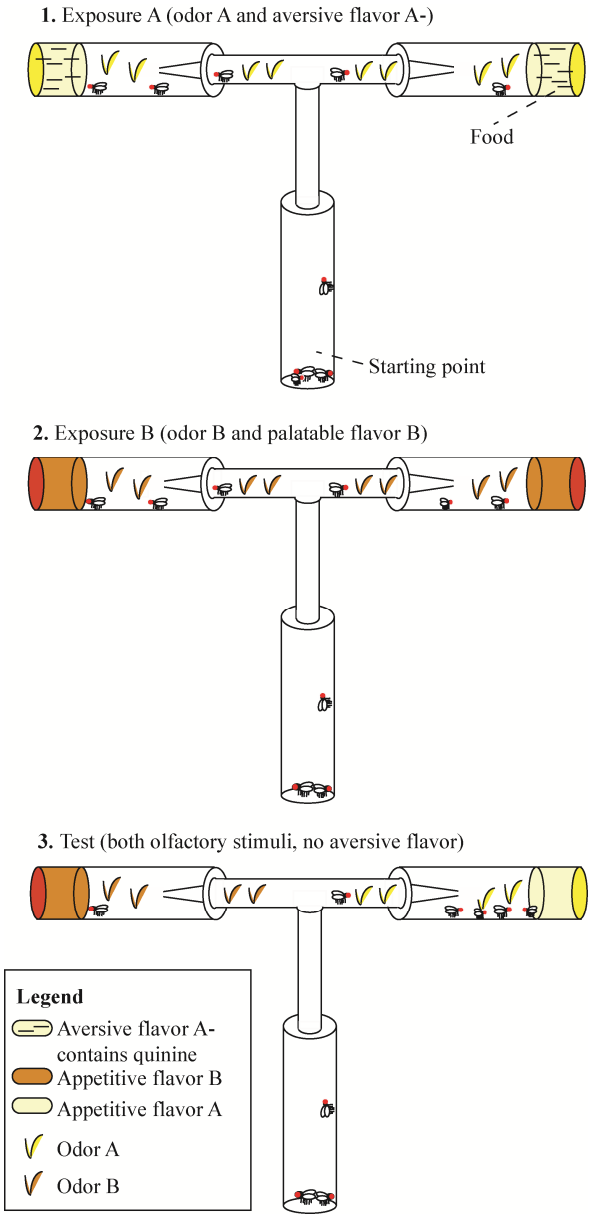

Fig. 1 Olfactory learning paradigm

During exposure A flies experience the conditioned stimulus A (odor A) and the unconditioned aversive stimulus A (the bitter flavor A). During exposure B flies experience the conditioned stimulus B (odor B) and the unconditioned palatable stimulus B (the palatable flavor B). After a certain delay, in the subsequent test phase, flies can approach the food chamber associated with odor A or B.

Moreover, both males and females can be selected for learning, with the possibility to maximize the ratio between population size selected and population size propagated. The presence of social partners is known to influence learning and information transmission in *Drosophila* (Kohn et al., 2013; Battesti et al., 2015), and this method could be used also to investigate the differences in learning in the presence of one or both sexes.

5.4 Genomics and validation of candidate loci

Computer simulations (Baldwin-Brown et al., 2014; Kofler and Schlötterer, 2014) have clarified to which extent large population size, high number of replicates and many generations of selection can increase the power of E&R studies. In particular, increasing the number of replicates can be more cost-effective than increasing the population size or the number of generations. Theoretical work (Futschik and Schlötterer, 2010) has shown how expanding the size of the group used for sequencing can increase the precision in the estimate of allele frequencies, thanks to a reduction of sampling error and unbalanced representation of individuals in the pool. Compared to individual sequencing, **Pool-seq** can currently provide whole-genome coverage sufficient to make precise estimates of alleles present in a large population at a reduced cost. This cost-effective method is therefore convenient to sequence and analyze multiple time points during evolution and track evolutionary trajectories (rate of change, plateauing, fluctuating trajectories etc.) of alleles present in multicellular organisms.

A successful E&R study can shed light into the evolutionary dynamics of a trait and identify candidate genes that can be compared to those obtained in GWAS (Turner et al., 2013) and QTL mapping studies (Weber et al., 2013; Kessner and Novembre, 2014), or used to investigate their functional role. Variants related to traits with a simple genetic basis, such as thorax pigmentation (Bastide et al., 2013), can be studied using RNA interference, quantitative complementation tests or allelic replacements: all these methods have been extensively used in *Drosophila*. In the case of behavioral traits with complex genetic architecture and affected by environmental noise, such as learning, the validation of candidates variants can be more challenging (Rockman, 2012; Schlötterer, Tobler, et al., 2014).

Further insight on the genetic architecture of a trait and its dynamics can be obtained by investigating the trajectories of the genomic change during E&R (e.g. Orozco-terWengel et al., 2012; Tobler et al., 2014). But only a parallel investigation of phenotypic and genomic

trajectories will produce a comprehensive understanding of the trait of interest: for instance, to which extent faster/more extended phenotypic change correlates with faster/more extended genomic change during evolution and reverse selection of associative learning abilities?

6 Final Remark

In spite of individual and species-specific differences, the evolutionary dynamics of behavioral traits and cognition have remained to a large extent elusive. While the evolution of morphological traits can be tracked using fossil records, little is known on the historical changes of behavior, even for widespread capabilities such as learning. Genetic and genomic data carry important traces of evolutionary processes but until recently limitations in analyzing the genetics of complex traits and their evolutionary dynamics have constrained this investigation. Advances in genomics represent a great opportunity for geneticists, behavioral ecologists and neuroscientists to investigate not only the underlying basis of complex behavioral traits but also their evolutionary dynamics. I have reviewed theoretical and empirical evidence that shows reasons to move beyond the assumption, known as "phenotypic gambit", that adaptation is not constrained by the underlying genetics (Grafen, 1984). In fact, factors such as lack of genetic variability, trade-offs and **genetic linkage** can influence the trajectories and rate of adaptation, and even survival of taxa. This is particularly true for behavioral traits that in many cases have a complex genetic architecture and are affected by environmental variables. As recently summarized by Wray and Hoekstra (2014): "Changes in the hereditary material are an essential part of adaptation and speciation". Hence a decision not to include genetics into phenotypic models of evolution will be mainly driven by practical and technical reasons.

The improvement of high-throughput sequencing technologies and related bioinformatics and statistical tools has progressively reduced limitations in analyzing the genetics underlying behavioral traits. A particularly promising approach to investigate the genetic architecture and evolutionary dynamics of behavioral traits is the Evolve and Resequence (E&R) method (reviewed in Schlötterer et al., 2014). In E&R studies researchers sequence the genomic composition of populations exposed to selective pressures imposed by the experimenters at subsequent time points, while monitoring the phenotypic change. By comparing evolutionary changes between replicates exposed to similar/different selection regimes it is possible to identify genetic variants that are associated with the phenotypic change, and investigate the ongoing process of evolution.

My analysis has focused on associative learning, a widespread trait extensively studied at the behavioral and genetic level, that has been shown to respond to selection in species with a generation time suitable for experimental evolution (see McGuire and Hirsch, 1977 for blow flies; Mery and Kawecki, 2002 for fruit flies; van den Berg et al., 2011 for parasitic wasps; Dunlap and Stephens, 2014 for fruit flies). At present *Drosophila* represents a very convenient model to investigate associative learning using the E&R: a wide amount of literature on the molecular and neurobiological basis of learning is available in fruit flies and extraordinary dedicated genetic (e.g. Jenett et al., 2012) and genomic (e.g. Mackay et al., 2012; King et al., 2012) tools have been developed for this model. Moreover, researchers have developed different paradigms suitable for experimental evolution of learning in fruit flies (Mery and Kawecki, 2002; Versace and Reisenberger, 2015) and conducted E&R with this model on a number of different trait (Burke et al., 2010; Turner et al., 2011; Remolina et al., 2012; Tobler et al., 2014).

E&R has been used to track the evolutionary responses of known and putative causative variants and to identify new candidates suitable for functional validation. At the moment it is not clear whether this approach can have the power to identify most of genetic variation that underlies individual variability or only a limited portion of it (see for instance Turner et al., 2011, 2013; Turner and Miller, 2012). In spite of this E&R is the most promising method to address long standing issues in the study of the evolution of cognition and behavior about the repeatability of evolution at the phenotypic and genetic level, the modular architecture and evolution of the mind, the pleiotropic origin of coevolving traits, the role of trade-offs, the presence of genetic constraints in behavioral and cognitive traits.

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Glossary

Artificial selection: Method of propagation of a population in which the target phenotype is measured in the parental generation and experimenters allow to reproduce only individuals that show the desired phenotype. For instance, the 10% fastest developing flies are mated with other flies of the same extreme, and the 10% slowest developing flies are mated with other flies of the same extreme.

Associative learning: Modification of behavior that derives from the association of different events. The association of two stimuli leads to classical conditioning (e.g. when the association between the neutral odor A with the aversive flavor A- produces avoidance of odor A); the association between a response and an event produces operant conditioning (e.g. when the association between pressing a lever and obtaining a food reward produces an increase in the number of pressures on the lever).

Chromosomal inversion: 180 degrees rotation of a chromosomal segment along the chromosome.

Constraints on learning: Limitations on the capability to learn specific associations; these limitations can be species-specific or more general.

Copy-number variation: interindividual variation in the number of copies of a certain DNA fragment.

Directional selection: Selective pressure that favors one allele over another, or that favors increased/decreased values of a continuous trait.

Epistasis: Interaction between different alleles that determines an effect on a trait that is different from the sum of each individual effect.

Evolve and Resequence (E&R): Experimental evolution research design coupled with resequencing of the investigated populations at different time points to investigate the underlying genomic/transcriptomic evolutionary change.

Experimental evolution: Research based on the investigation of evolutionary change that occurs across generations in response to experimentally controlled selective pressures.

Filial imprinting: Learning process in which newborn animals develop attachment to the first conspicuous objects they experience.

Fitness landscape: Set of relative fitness values associated to different genotypes present in a population.

Genetic architecture: The genetic basis underlying a phenotypic trait. It is simple when only one or few loci determine the phenotype, and complex when multiple loci and interactions between loci contribute in determining the phenotype.

Genetic drift: Random change in allele frequency due to random variation in reproductive success.

Genetic linkage: Association between variants located at different loci along the genome.

Genetic variability: Genetic differences among individuals of a population/species.

Genome-wide association studies (GWAS): Approach used to identify the phenotype-genotype relation based on associations between genetic markers located on the whole genome and specific phenotypes.

Heterozygote advantage: Condition in which heterozygote individuals have higher fitness than the correspondent homozygotes.

High-throughput sequencing: Set of methods that produce large volumes of data used to determine the order of nucleotides within a DNA/RNA molecule.

Isofemale line: In insects, a wild fertilized female isolated in a dedicated culture vial, and propagated by full-sib mating for multiple generations.

Laboratory natural selection: Selection regime in which selective pressure is indirectly controlled by the experimenters by manipulating the environment and not directly allowing only a portion of the population to be propagated.

Linkage disequilibrium: See genetic linkage.

Mapping: Method used to localize a genomic segment on the genome.

Oviposition paradigm: Experimental paradigm used to assess the preference for specific media through the number of eggs laid in different substrates.

Phenotypic gambit: Exclusive use of phenotypes to measure evolutionary changes based on the assumption that evolutionary change driven by natural selection is not constrained by genetics.

Pleiotropy: Effect of one genetic variant on multiple traits.

Pool-seq: Sequencing method in which the DNA/RNA from a group of individuals is mixed and then sequenced simultaneously.

Preparedness for learning: Bias that enhances learning of specific associations.

Quantitative trait locus (*QTL*): Set of methods to establish the connection between a continuous trait and its underlying genetics using information on the phenotype, the pedigree of the investigated individuals and the genetic linkage of associated markers.

Quantitative trait locus (QTL) mapping: Region of the genome that influences a trait which varies in a continuous way.

Read: String of nucleotides produced by sequencing devices from fragments of DNA/RNA molecules.

Recombination: Exchange of genetic regions between chromosomes that happens during meiosis and that can produce genetic combinations different from the initial ones.

Reverse selection: Selection procedure in which, after exposing the target population to a selective pressure, the population is selected in the direction of the ancestral phenotype.

*Segregating variant*s: Different alleles present in a population.

T-maze: Experimental apparatus in the shape of a T. The two arms of the maze can be associated with different stimuli or outcomes.