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Experimental Evolution of *rutabaga* Suppression

A Dissertation Presented

by

Michael Jason Cressy

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Abstract of the Dissertation

Experimental Evolution of *rutabaga* Suppression

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In

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One of the challenges to understanding the relationship between genotype and phenotype is that complex phenotypes, such as learning and memory, emerge from interactions amongst groups of genes. Despite its widespread relevance, the nature of multi-gene interactions are ill understood, in part, because most genetic studies are limited to pair-wise studies. To investigate this question, a novel approach was developed and implemented in *Drosophila*, using the biologically important and clinically relevant cAMP pathway as a model. I used selective breeding to evolve combinations of alleles capable of suppressing the learning defect of mutations in the *rutabaga* adenylyl cyclase gene. Unlike a classical suppressor screen, the use of experimental evolution allowed me to explore the potential impact of gene interactions among more than two loci. And unlike a classical selective breeding experiment, the genetic

variability was constrained to a set of 23 known loci, providing access to the underlying causal alleles. After 41 generations a clear response to selection was observed. Remarkably, selected groups had performance at levels approaching that of wild-type despite the fact that all animals were homozygous for a null allele of *rutabaga*. High throughput genotyping and multivariate analyses lead to identification of loci underlying the selection response. Using independent genetic experiments, I then exhaustively tested the effects of each of the identified loci as well as of all di-allele combinations. One of the 8 loci partially but significantly suppressed *rutabaga* on its own. Simulations of the lab evolution experiment indicate that combinations of up to 5 loci could feasibly have been selected. Interactions involving 6 or more loci likely could not. Taken together, the findings in this thesis support the idea that multiple combinations among even a limited set of loci are capable of bypassing the requirement for a central player such as *rutabaga*. This speaks to the remarkable flexibility of gene networks. Understanding how gene networks are modified in response to a selective pressure can help to model complex phenotypes, including those associated with human disease.

Dedication

I would like to dedicate this thesis to my father Michael Edwin Cressy. His memory gives me the strength to accomplish my dreams.

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Publications

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Introduction

Historical Perspective on genetics of traits

Understanding phenotype to genotype relationships is a fundamental goal in the study of genetics. Gregor Mendel's studies on pea plants established the laws of segregation and independent assortment, and helped to model a basic understanding of the patterns of gene inheritance (Hartwell et al. 2000).

In the early 1900's Thomas Morgan Hunt established the fruit fly *Drosophila melanogastor* as a model to study the Mendelian model of inheritance. The first mutation (*white,w*), was discovered serendipitously, and was later found to be a spontaneous sex-linked mutation in the *w* gene giving the flies white eyes (Hartwell et al. 2000). Studying the pattern of inheritance in *w* mutants emphasized how mutations could give us the necessary insight on how a single gene can affect a phenotype. Over the next hundred years the large-scale induction of mutations would be the standard procedure for identifying genotype to phenotype relationships.

Forward mutagenesis to identify genotype to phenotype relationships

Forward mutagenesis has been the workhorse for identifying large numbers mutations is genes that have substantial phenotypic effects. The first mutagen that was widely used was X-rays. H.J. Muller, a student in Morgan's lab was the first to show that X-rays can cause mutations in genes (Muller 1927). Mutations that had a large phenotypic effect were uncovered using this method. This approach of mutating the genome is effective, however a drawback of this approach is that it causes large

deletions and chromosome rearrangements, making it difficult to identify the causal gene. It wasn't until much later that chemical mutagenesis was perfected, giving scientists the ability to lesion single genes. Saturation mutagenesis screens were able to identify mutations every gene in the genome at least once. In practice, it is unlikely that for a given screen all genes were uncovered that affect the phenotype of interest, but it was a huge step forward in high through-put identification of mutations with importance to a given phenotype.

The early chemical mutagenesis screens were done using Ethyl methane suplhonate (EMS), an alkylating agent that induces point mutations (Lewis 1968). In the early 1980's Christiane Nusslein-Volhard and Eric Wieschaus performed the most exhaustive and informative saturation forward mutagenesis screen using EMS. This screen was remarkable because it attempted to identify all genes necessary for the development of the *Drosophila* embryo (Nusslein-Volhard and Wieschaus 1980). By looking at segmentation defects in the larvae of *Drosophila,* genes were identified that could be parsed into a distinct hierarchical pathway, reviewed in (St Johnston and Nusslein-Volhard 1992). In the fertilized *Drosophila* egg the maternal genes *bicoid* and *hunchback* express mRNA's in a gradient from anterior to posterior and mRNA's from the genes *caudal* and *nanos* are expressed in a gradient from posterior to anterior. The expression pattern of these maternal effect genes activates gap genes that have expression patterns in broad segments in the developing embryo. The broad pattern of the gap genes set up the more specific expression pattern of the pair-rule genes. The pair-rule genes express in a 7-stripe pattern in the embryo. Finally, the segment polarity genes are the last genes to be expressed in segmentation, and have a 14-stripe

pattern. Importantly, this screen highlighted that the gene requirement for this intricate process is relatively small, and it is possible to construct this process using forward mutagenesis to identify the necessary components.

In parallel with the above saturation screen for developmental biolgy, a similar approach was used to dissect behavior. An EMS screen was done in the lab of Seymour Benzer, using an olfactory learning paradigm where flies were conditioned to associate an odor with an electric shock. Known as the Quinn Harris Benzer (QHB) assay, it was used to uncover X chromosome mutations that had learning defects (Quinn et al. 1974). This screen isolated mutations in genes of the cAMP cascade, the canonical pathway to memory in *Drosophila* (discussed below). Central components of this pathway, *dunce(dnc)* and *rutabaga(rut),* later identified as a type IV phospo-diesterase and a Ca++ responsive adenlyl cyclase respectively, were the first genes identified (Dudai et al. 1976; Livingstone et al. 1984). This was the first screen for individual genes that have a role in learning and memory in *Drosophila*. One limitation to EMS mutagenesis screens is that molecular identification of the causal loci is very labor intensive.

Subsequently, transposon insertion mutagenesis was developed in part to facilitate cloning of the affected loci. The p-element is a transposable element that has been engineered to lack mobilization in our strains. When transposase, the protein that induces mobilization is introduced, the p-element can mobilize "jump" randomly into the genome. This method of mutagenesis is elegant because unlike EMS, the engineered p-element has a mini-white gene that when inserted into the genome of a white-eyed fly will be expressed and easily tracked. Therefore only novel mutants will be tested for the

phenotype of interest. Another advantage of this approach is that with the sequencing of the *Drosophila* genome the precise location of the insertion of the P-element can be identified, simplifying the ability to track the allele (Searles et al. 1982).

Over the next 20 years several additional screens were done to uncover genes involved in learning and memory (Boynton and Tully 1992; Dura et al. 1993; Kamyshev et al. 2002; Dubnau et al. 2003). The largest, (the Hartford screen) was a near saturation mutagenesis screen performed in the lab of Tim Tully in the 1990's to identify autosomal mutations that have a role in memory. The screen used a modified version of the QHB assay (see below), and identified over 55 new mutations (Dubnau et al. 2003). A subset of these mutations will form the core of this thesis.

The first screens identified the cAMP pathway as the canonical pathway for learning and memory in *Drosophila*, however, most of the alleles identified in the Hartford screen are not part of this pathway. This makes it difficult to place this network of alleles important for learning and memory, into a mechanistic context. Typically, scientists have relied on epistasis to discover how genes interact.

Uncovering genotype to phenotype relationships has long been the motivation for geneticists from Mendel to Benzer. Over the course of the last 100 years many genetic tools have been used to tease apart these relationships. Genetic screens have proven to be the one of the most influential tools in modern genetics, and have identified many genes important in phenotypes ranging from genes involved in memory to pathways important for human disease.

Forward genetic screens have been essential for identifying genes important for a phenotype, but a limitation of this approach is that it can only identify individual genes.

Understanding the role of individual genes for simple traits is helpful, but understanding the role of a gene in a mechanistic sense by definition involves understanding gene interaction. Uncovering gene interactions can provide us with an understanding of the mechanisms of both biochemical pathways and the inheritance of complex traits.

Enhancer suppressor screens and epistasis

Enhancer/Suppressor screens have been essential in finding interacting genes and also in identifying biochemical pathways in *Drosophila.* One classic example is the set of experiments done in the lab of Gerry Rubin to identify genes in the Ras pathway (Gaul et al. 1993; Karim et al. 1996; Chang and Rubin 1997; Therrien et al. 1998; Karim and Rubin 1999; Rebay et al. 2000; Therrien et al. 2000). The *Drosophila* compound eye has a stereotypical ordered ommatidial structure. Each ommatidium consists of eight photoreceptors, four cone cells, and a bristle. The last photoreceptor to differentiate is dependent on the receptor tyrosine kinase (RTK) sevenless (*sev*), as well as the ligand bride of sevenless (*boss*) (Simon 1994). Studies have shown signaling cascades to function downstream of RTK's (see (Margolis and Skolnik 1994) for review). A mutation in *sev* or in *boss* results in flies that a rough eye phenotype. The rough eyed phenotype was utilized to identify genes that interact downstream of *sev*. This approach would identify alleles that interact with *sev* by affecting the roughness of the eye. Using a gain of function allele of *sev* as the background, a second site mutagenesis screen was done to identify alleles that interact with *sev*. Alleles that suppress the rough-eyed phenotype would yield eyes that revert to a more wild-type appearance. Alleles that enhance the phenotype would give the eyes a more rough

appearance (Simon 1994). There were several downstream genes that were identified in this screen including the *Ras1*, a gene that encodes for a small GTPase. The Ras pathway plays a pivotal role in many biological processes in organisms across phyla. Disruptions in the Ras pathway can lead to many different diseases including cancer in humans (Aoki et al. 2008)

Subsequently the group took an activated form of *Ras1* expressed in the eye, and performed a series of screens to identify dominant downstream components of the pathway (Karim et al. 1996; Chang and Rubin 1997; Therrien et al. 1998). The identified genes included the downstream components *Raf,* the first kinase in the MAP kinase cascade as well as other genes in this pathway. Using pair-wise interactions, and the effect these interactions had on the roughness of the eye, a biochemical pathway was assembled. This technique was remarkable in identifying the constituents of this linear pathway, yet some complex traits are likely to involve many genes that interact in networks of activity, and not necessarily in a linear fashion. Identifying interactions in a network of genes may be difficult to obtain by studying pair-wise interactions. A classic modifier screen approach does not fully model the complexity of the gene interactions.

Epistasis is a genetic feature that has historically had several definitions, but has been difficult to study in the context of population genetics. What role does epistasis play in natural selection and evolution? The idea of epistasis goes back over 100 years when the term was first defined by Bateson in his book describing Mendel's work (Bateson 1909), and since then the term has been used generally to explain interactions between genes. The various definitions have been categorized into three main 'types' of

epistasis reviewed by (Phillips 2008). Functional epistasis explains protein-protein interactions, and compositional epistasis explains the effect genetic background has on a given allele. The final type of epistasis is statistical epistasis, this is the type of epistasis that was first described by (Fisher 1918) which describes any interaction that is non-additive. This statistical definition of epistasis is the form that I will discuss and apply in this thesis. An important aspect of epistasis for the purpose of this thesis is that non-additive interactions can skew the expected phenotypic output outside the expected range (Dubnau and Tully 1998).

A study by Bruno van Swinderin and Ralph Greenspan helped to further the understanding of how epistatic multi gene interactions can affect a phenotype. They tested multi gene interactions in the context of a specific behavior (van Swinderen and Greenspan 2005). A mutation in the gene *syntaxin1A* causes flies to display a bottom dwelling phenotype when heated to 39 degrees (Littleton et al. 1998). A collection of EP lines was then crossed to the *syx1A* mutant to measure how trans heterozygous interactions affect the bottom dwelling phenotype. EP lines contain a P-element transposon with a constitutive promoter that expresses the immediate downstream gene of where it inserts. From this small scale screen a network of 16 genes were identified to interact with *syx1A*. This network was then tested in the presence and absence of *syx1A*. The results from this experiment suggest that even from a small network of 16 genes, epistasis is prevalent. All possible effects on the stability of a given gene interaction were observed. There are interactions that are unchanged in the presence or absence of *syx1A* and there are also interactions that completely change sign. Negative interactions become positive and vice versa. These experiments suggest that

networks are extremely flexible. Perturbations cause the network to shift and compensate for the absence of an important gene. What non-additivity says about the underlying variation is that interactions of genes in different contexts can result in profound phenotypic changes. This ‘cryptic variation’ may underlie the flexibility in gene networks.

In the lab we artificially substitute alleles and measure phenotypes, however in natural populations there may be thousands of allelic variants in a population and how these different alleles interact may have an impact on evolution. If an epistatic interaction gives an organism a selective advantage it is likely that it would become pervasive in populations. In this thesis, I exploited variation and the flexibility of gene networks to compensate for the loss of a canonical learning and memory mutant.

Artificial bidirectional selection for quantitative traits

Another method scientists have used to identify genotype to phenotype relationships is by performing artificial selection experiments. Selection has been used for tens of thousands of years to domesticate plants and animals. In a Ralph Greenspan review on selection, he makes a compelling argument that almost any quantitative trait can be altered in either direction (Greenspan 2003). In one of the first screens using *Drosophila*, F.E. Lutz used selection to screen for differences in wing vein morphology (Lutz 1911). In 1927, Robert Tryon started his landmark experiments on maze learning in rats (Tryon 1940). This was one of the first experiments that used bi-directional selection for learning. Tryon used a food reward assay to measure maze “bright” and maze “dull” pure breeding lines. Rats were placed in a seventeen choice

blind T-maze and each rat was tested in the maze 19 times. Rats were scored on the amount of wrong choices made until they eventually traversed the maze to get to the food reward. After each of the trials “bright” rats were mated with “bright” rats and “dull” rats the same. The less times a rat entered the blind alley the “brighter” the rat. The selection was carried on for 18 generations and at the end, the two strains of rats had a score consistent with what their respective “genotype” inferred.

In the 1950’s, Jerry Hirsch would start one of the longest selection experiments to date. *Drosophila melanogastor* are negatively geotactic, having a tendency to run against the force of gravity. Hirsch and a constant rotation of undergraduate students took a heterogeneous population of flies and selected for high and low geotactic behavior for over 500 generations, spanning over 35 years (Ricker and Hirsch 1985). During the first 50 generations of selection the two lines started to deviate from the mean of the ancestral population. Between generations 50 and 100 the two lines reached asymptotic levels. The levels were at the extremes of the geotactic assay. During the course of the experiment there were periods of time where selection was relaxed, and during these times the scores of the lines would start to regress back to the score of the founding population. When selection was restarted the lines quickly returned to asymptotic levels. These results indicate that even though the separate lines had already reached asymptote there was enough heterogeneity present to bring the populations back to wild-type, and also that this heterogeneity is likely involving interactions amongst heterozygotes.

These observations imply that after hundreds of generations the necessary genetic elements for geotaxis had not yet reached fixation, and for what may seem to

be a simple behavior, the genetic requirement is complex. For example, the fact that it required approximately 100 generations to reach fixation indicates that multiple loci are involved. But because the underlying variants are not known one cannot identify the structure of the gene interactions.

There are important observations that can be made from pre-genomic selection experiments. First, genetics underlies complex behavioral phenotypes, and multiple gene interactions are driving the selection response. It also seems to be that epistasis is not the exception but more likely the rule. An important limitation should also be mentioned. One cannot even start to understand the mechanisms of gene interactions because these experiments cannot identify the causal genes.

In the era of genomics two methods have been used to attempt to identify the causal genes in multi-generation selection experiments. The first is quantitative trait loci (QTL) mapping. In QTL analysis a reference strain with many genetic markers is established. A series of crosses are then made between the strains that have the phenotype of interest to the reference strain. Markers of interest are identified that segregate with the phenotype. It must be noted that the resolution of this method is limited by recombination to narrow down the region that is statistically associated with the trait. In practice, this can identify chromosomal regions, but rarely leads to identification of the causal gene. This method was used in a study by Trudy Mackay to identify loci important for longevity. The results of this study identified no less than 11 QTL's as important for longevity. This indicates that longevity is a complex quantitative trait, but there is still difficulty in identifying single causal genes (Mackay 2002).

With the advent of genome sequencing, a second method attempting to identify genes in multi-generation selection studies was used. Micro-arrays are a platform that can measure expression differences in individual genes covering most of the genome. RNA is isolated from tissue and hybridized to a cDNA platform that contains most if not all genes in the genome. This method of detecting expression differences could be used to determine differential expression patterns in selected lines (Toma et al. 2002; Dierick and Greenspan 2006; Edwards et al. 2006).

Simultaneously, in two labs (Dierick and Greenspan 2006; Edwards et al. 2006) this method of identifying genes was implemented in a selection experiment done looking at aggression. I will detail one of them, done in the lab of Ralph Greenspan. *Drosophila* like many organisms display aggression when competing for a food source or a mate (Baker 1983). Defending these scarce resources in the wild gives the aggressor a better chance of passing on its genes to the next generation.

The selection was done much the same way as classical selection experiments. Four populations of flies were used in this selection, two controls and two populations selected for increased aggression. Males that displayed increased aggression in the two experimental populations were the flies used to seed the next generation. In 21 generations the flies in the experimental populations showed a significantly higher level of aggression than the unselected flies. This was measured with a variety of aggression assays, including frequency of fighting, the latency of fighting, and also the intensity of fighting. These results are not surprising given the history of selection experiments. What was exciting about this selection was with the advent of sequencing and micro-array technology these populations could be assayed to determine if there are gene

expression differences comparing the aggressive populations to the control populations. This is precisely what was done in this selection. RNA from the heads from control and experimental populations were isolated and assayed for expression levels. One gene Cyp6a20, a cytochrome gene, was found to have an increased expression level in the selected populations. When this gene was tested on its own the flies had an increased level of fighting frequency. Unlike past selection experiments, this selection was important because it was one of the first attempts to identify what gene or genes were contributing to the response to selection. However, an important limitation is that this identified transcriptional changes, but not genotype changes. Therefore, the identified genes could be and likely are responses to the genotype, not the selected alleles themselves. These results are very important in the search for gene interactions contributing to selection. Identifying individual genes that have a clear role in the selected behavior is important but identifying how multi-gene interactions evolve to do this is still not well understood.

Selection experiments have had a profound impact on the way we think about phenotypes. The fact that almost any trait can be bi-directionally selected lends credence to the flexibility of gene networks. Also, phenotypic extremes outside of the normal range hint at the vast genetic variability in populations. Uncovering these variations has been a difficult task. In this thesis I attempted to identify gene interactions in a constrained network of genes important for learning and memory.

Learning and Behavior in drosophila

Seymour Benzer showed great insight in believing that by mutating individual

genes we can determine the genetic requirements for behavior. His use of mutagenesis (see above) was an important step in building a foundation for the modern study of learning and memory in *Drosophila*. In 1985, in the lab of Chip Quinn, Tim Tully and Quinn developed the T-maze (TQ), a Pavlovian associative conditioning assay that is a modification, but much more robust version of the QHB assay (see above)(Tully and Quinn 1985). In the TQ t-maze, flies are tested 100 at a time. They are presented with an odor, the conditioned stimulus (CS+) paired with an electric shock, the unconditioned stimulus (US). After a brief period of rest flies are presented with a second odor in the absence of an electric shock (CS-). Flies that learn this association when given a choice between the two odors will avoid the CS+.

Genetic dissection has suggested the existence of four mechanistically distinct stages of memory consolidation. There are two phases that are measured after 1 training session. Short-term memory (STM) and Middle term memory (MTM), which are tested 2 minutes and 3 hours after training respectively. There are also two forms of consolidated memory measured 24 hours after training. The protocol for this training is slightly different than what is used for STM and MTM. Long-term memory training (LTM) and anesthesia resistant memory (ARM) (Quinn and Dudai 1976; Folkers et al. 1993; Tully et al. 1994a; Tully et al. 1994b; Dubnau et al. 2003; Chen et al. 2008), makes use of automated training machines.

The first two STM mutants uncovered were *dnc* and *rut,* both of these mutants display defects when tested in the T-maze at two minutes after training. It is worth noting that neither of these mutants is totally devoid of STM. They perform in the TQ assay at a level about 50% of wild-type. For review see (Dubnau and Tully 1998).

LTM is protein synthesis dependent and is only induced with repetitive spaced training. Spaced training consists of 10 repeated training sessions with a 15-minute rest interval between trainings, and lasts for up to 7 days. LTM can be disrupted by feeding flies cyclohexamide, a protein synthesis inhibitor (Tully et al. 1994b). Several mutations that affect learning and memory can be parsed into one or more of these mechanistically distinct pathways.

Consolidated memory has 3 phases, acquisition, consolidation, and retrieval. When considering the Pavlovian conditioning paradigm using the T-maze, acquisition is the phase in which the flies learn an association of the odor with a foot shock. During the consolidation phase the flies are storing this associative memory and it is protein synthesis dependent. Retrieval is important when recalling the memory after training.

The role of the cAMP pathway in learning and memory

Work in several labs over the past 3 decades has assembled a canonical pathway to learning and memory in *Drosophila*. The first mutation characterized in the Benzer screen was *dnc,* a mutation that had a defect in cAMP phosphodiesterase activity (Byers et al. 1981; Davis and Kiger 1981). The cAMP signaling pathway was established in mammals and reverse genetics could be used to identify the homologous *Drosophila* proteins. This is precisely what was done to identify *rut* as being defective in the cAMP pathway. The experiments done on *rut* revealed that it was defective in adenylyl cyclase activity(Livingstone et al. 1984). Moreover, *rut* is the only calmodulin/ Ca++ stimulated adenlyl cyclase in *Drosophila.* Further work showed that this mutation was caused by a point mutation in the catalytic portion of the protein (Levin et al. 1992).

These discoveries of cAMP components in *Drosophila* being necessary for learning and memory are convergent with work in other model organisms. The sea slug *Aplysia californica* served as one of the organisms that would further our understanding of the importance of cAMP as it pertains to learning and memory. Non-associative and associative forms of memory, as well as both short and long term plasticity, have been thoroughly studied in *Aplysia* (reviewed in (Alberini 1999)). This work in *Aplysia* identified important components of the cAMP pathway. Because of the importance of the cAMP pathway, subsequent reverse genetic manipulations were used to establish roles for each additional components of this pathway.

Putting together the findings from *Aplysia* with those from *Drosophila*, the following model emerges. The pathway starts with a g-protein coupled receptor, in the aversive Pavlovian assay in flies, this is thought to be the dopamine receptor (Han et al. 1996; Kim et al. 2003). Upon activation of the DA1 receptor and a simultaneous influx of calcium into the cell (driven by odors), the adenlylyl cyclase (*rut*, in *Drosophila*) is activated. *Rut* will sequester ATP and convert it to cAMP, a secondary messenger. cAMP can either be degraded by *dnc* (phosphodiesterase), or signal downstream. cAMP and PKA can have local signaling consequences resulting in short term plasticity/memory. Or cAMP signaling can activate transcription in the nucleus, thought to be involved in LTM. An elevated level of cAMP in the cell causes a localization of the catalytic sub unit of protein kinase A (PKA) into the nucleus. This translocation of the PKA catalytic sub unit activates the transcription factor cAMP response element (CREB). CREB has a well documented role in memory in animals across phyla (Lonze and Ginty 2002).

Work in several labs has identified a role for many of these proteins in learning and or memory. For this thesis I will focus on the adenylyl cyclase *rut*. The role of *rut* in learning and memory has been well characterized (Margulies et al. 2005; Blum et al. 2009). However, *rut* animals in the T-maze have a performance index (PI) that is about 50% of wild-type. *Rut* is a null mutant, but it does not abolish STM. This indicates that there is a component to STM that is *rut* independent. Finding components of the *rut* independent pathway has been difficult, although recent work in the lab of Ron Davis has identified Gilgamesh (*gish*), which encodes a casein kinase, as a required component (Tan et al. 2010**)** and work in the Dubnau lab suggests that the NR1 subunit of the NMDA-receptor also supports *rut* independent memory (Qin and Dubnau, 2010). The cAMP signaling pathway has been localized anatomically to the mushroom bodies (MB), a neuropil structure located in the dorsal posterior region of the *Drosophila* brain.

Neural Circuits for Olfactory Memory

The corpora pedunculata, or Mushroom Bodies (MB) are the anatomical structure essential for olfactory learning and memory. This role was first identified in honeybees, (for review see (Menzel et al. 1996). The importance of the MB in *Drosophila* for learning and memory has been extensively studied. In response to an odor, two sensory organs, the antennae and maxillary palps send a signal through sensory neurons to the antennal lobes. Projection neurons are than relayed from the antennal lobes to the mushroom bodies. Projection neurons synapse onto a region of the mushroom body called the calyx. Each hemisphere of the calyx contains about 2500 neurons referred to as Kenyon cells (Strausfeld et al. 1998; Jefferis et al. 2002).

Kenyon cells send axonal projections into 5 lobes consisting of 3 cell types. All axonal projections are tightly bundled in the peduncle. Gamma neurons form gamma lobes that project horizontally. Alpha/beta neurons have two branches, the alpha branch forms the alpha lobes, and the beta branch forms the beta lobes. The alpha and beta prime lobes wrap around the alpha and beta lobes but are developmentally distinct from the alpha beta lobes (Lee et al. 1999). Some of the initial experiments in *Drosophila* identified mutants that have structural defects in the MB (Heisenberg et al. 1985). In that study, two mutants were identified *mushroom body miniature* (*mbm*) and *mushroom body deranged* (*mbd*). These structural mutants had severe axonal projection defects, and failed to associate an odor with a shock. Although learning is abolished, flies can discriminate odors normally. The results from this study implicate the MB as the site for learning and memory, however these mutants also have defects on other brain regions. Therefore, a more specific manipulation of the MB is necessary.

This was accomplished first by chemical ablation. During development feeding the flies hydroxyurea at a specific developmental time when only mushroom body neuroblasts are dividing, causes defects in the formation of the MB. Flies that have been fed hydroxyurea are devoid of learning and memory when measured using the T-maze (de Belle and Heisenberg 1994). These two studies pinpoint the MB as the learning and memory center of the *Drosophila* brain.

The two previous sections have described the cAMP pathway as being necessary for learning and memory as well as the MB as the anatomical site of those behaviors. The following set of studies used manipulations of individual genes in the MB required for normal cAMP signaling.

The cAMP pathway relies on the activation of a G-coupled protein receptor (discussed above). In (Connolly et al. 1996) they took a constitutively activated form of the GαS subunit of the G-coupled protein receptor and expressed it specifically in the MB using the Gal4 UAS system. Expressing GαS in the MB completely abolishes learning. These experiments led to testing the downstream components of GαS One of the downstream components in the pathway is *rut*. The requirement of *rut* has been localized to specific regions of the MB (Zars et al. 2000). In that study, a panel of Gal4 strains with specific lobular expression of *rut* was used, and the requirement for *rut* for STM was localized to the gamma lobes of the MB. Further manipulations or *rut* identified that the specificity changes from gamma lobes in STM to the alpha beta lobes in LTM (Blum et al. 2009). These experiments were extremely insightful in the understanding of the specificity of individual genes requirement for memory.

The MB have a distinct role in learning and memory. A large number of single gene mutants have been identified. A few of these have been assembled into a pathway -- the cAMP-signaling pathway. But the majority of genes are not assigned to a network or pathway.

Summary

Over the past 150 years understanding genotype to phenotype relationships have been at the core of genetic studies. Forward mutagenesis has uncovered tens of thousands of genes that have distinct roles in every imaginable phenotype. Pathways have been dissected using enhancer/suppressor screens, and selection experiments

have been used to select extremes for a myriad of phenotypes including every measurable quantitative trait. Yet our understanding of how multi-gene higher order interactions contribute to phenotype is poorly understood. This is especially the case when it comes to human disease.

To investigate the role of multi gene interactions in learning and memory I developed and implemented a novel approach in *Drosophila* using the cAMP pathway as a model. I modeled an enhancer/suppressor screen but unlike a classic screen I used selective breeding to evolve combinations of alleles capable of suppressing the *rut* adenlyl cyclase gene. And unlike a classical selective breeding experiment, the genetic variability was constrained to a set of 23 molecularly characterized loci with known involvement in memory. This strategy models the multi-gene interactions that influence naturally occurring variation in complex phenotypes, but also makes it feasible to fully genotype the causative loci across multiple animals.

Using this experimental design, a large-scale artificial selection was completed that spanned 41 generations. A robust response to selection was observed, resulting in dramatic improvement in learning performance despite the fact that all animals remain null mutant for *rut*. It is demonstrated using control populations that the selection response requires the presence of the characterized genetic variants indicating that heterogeneity at these 23 loci drive the response, highlighting the flexibility of gene networks.

I also genotyped 288 animals from each replicate population at two different generation time-points. Multivariate analysis of the high dimensional genotype data set identified alleles at 8 loci that explain much of the phenotypic effect. Using

independent genetic experiments, I tested the effects on the *rut* learning defect with each of these 8 alleles in isolation, as well as each of the 28 di-allele combinations and several three-way combinations among them. Remarkably, only one of the identified loci significantly suppresses the *rutabaga* learning defect on its own, and none of the possible 2-way combinations yields significant suppression. I also tested a permissive allele hypothesis (Chapter 3) that did not yield suppression.

Chapter 2

Suppressor screening by selective breeding: the evolution of adenylyl cyclase independent learning in *Drosophila*

This chapter is very similar to a manuscript in preparation for submission for publication. Partha Mitra and Dan Valente made contributions to all sections, but made large contributions specifically in the sections containing statistical and multi-variate analysis.

Introduction

Natural populations of animals exhibit remarkably narrow ranges of phenotypic variation relative to the extent of underlying genetic heterogeneity. The phenotypic effects of this hidden genetic variation can be revealed, however, in response to selective pressure, environmental stress or the presence of strong deleterious mutations. The clinical severity of Mendelian genetic disorders, for example, can be modulated by variation at additional loci that on their own would have little clinical consequence. To investigate the modulatory impact of such cryptic genetic variation on a Mendelian trait, we used selective breeding over the course of more than 40

generations to evolve nearly normal levels of Pavlovian learning in fruit flies that carry null mutations in the *rutabaga* adenylyl cyclase. We constrained the starting genetic variability to a set of 23 loci with known impact in the learning assay, which provided a means to track the underlying genotypic response. We identified 8 out of 23 loci that appear to drive the selection response. By testing the effects of each of the 8 loci and all di-allele combinations among them, we demonstrate that at least one locus can partially suppress the *rutabaga* learning defect on its own. Our findings also support the conclusions that multiple genetic solutions underlie the selected suppression of *rutabaga* and that typical solutions involve interactions among several genes.

The cAMP pathway is a conserved signaling mechanism known to underlie many forms of memory and learning (Alberini 1999; Heisenberg 2003; Davis 2005). Mutations in *rut,* the *Drosophila* calcium responsive adenylyl cyclase, result in severely reduced olfactory memory (Heisenberg 2003; Davis 2005; Margulies, Tully et al. 2005; Keene and Waddell 2007). We first established a selection procedure based on this learning assay that is capable of fractionating higher performing individuals from a genetically heterogeneous population (Fig. 2.1). We next applied this fractionation procedure to a multi-generational selection experiment to suppress the *rut*^{1} memory defect with combinations of alleles that had been identified in a forward screen (Dubnau, Chiang et al. 2003).

Materials and Methods

Construction of starting populations:

As a starting point for this selection experiment, we created a founding population of animals in which the genetic variation was constrained to 23 transposon insertion derived alleles identified in a screen for mutants with memory defects (Dubnau, Chiang et al. 2003). The construction of the founder populations was guided by a theoretical analysis of the population heterozygosity. The number of possible genotypes with *N* loci grows exponentially with the number of loci. Therefore, the entire genotype space cannot be realistically tested for as many loci as are examined in this study (*N*=23). With a properly chosen founder population, however, it is possible to sample a large volume of genotype space in a realistic number of generations. Because the number of generations in our study is small compared to the effective population size, our calculations show that use of founding animals that are homozygous for the mutant alleles at between 3 and 4 of the 23 loci gives a marked increase in diversity over the case where each animal has just 1, while remaining within tractable experimental population sizes.

Based on this analysis, we first created a series of 7 input strains in which each animal is homozygous for the rut^1 null allele and homozygous for between 3 and 4 of the 23 transposon alleles. To minimize the effects of linkage between loci in the initial population, we selected the transposon-populated loci in each founding fly to be as far apart as possible (Dubnau, Chiang et al. 2003), maximizing the rate at which equilibration is expected to occur during the course of the experiment. All 7 input strains were generated on the same inbred isolate (Wiso[CJ1]) from the standard

Canton S strain (Dubnau et al., 2003). For construction of the 7 “3-mer” and “4-mer” strains (Table 2.1) each of the 23 P-element mutations (Dubnau et al., 2003) first were backcrossed for 5 generations to our wild type reference strain (w^{1118}*(isoCJ7)* (Dubnau, Chiang et al. 2003). This strain was derived as an isogenic isolate from Canton Special (Tully, Cambiazo et al. 1994). The 3-mer and 4-mer combinations (Table 2.1) then were assembled along with the rut^1 homozygous mutant using standard genetic approaches. Unlinked loci were selected for each of these 7 strains (each was confirmed with PCR, data not shown).

The 8 populations were established as follows: The two isogenic rut^1 mutant populations were constructed by placing the X-linked rut^1 allele into the w^{1118}*(isoCJ7)* strain using Chromosome II and III balancers. The six populations with genetic heterogeneity were established by mixing the 7 rut^1 mutant 3-mer and 4-mer strains (Table 2.1) in equal proportions. These were interbred for one generation with no selection. After one generation of inter-breeding, six replicate populations were established from 200 breeding pairs in custom designed cressy population cages (CPCs). Each was homozygous for rut^1 and heterozygous for up to 8 of the 23 alleles. These populations were further divided into two experimental groups. Three (Morgan, Muller and Lewis) were subjected to experimental selection, and 3 (Sturtevant, Bridges and Dobzhansky) served as unselected experimental controls.

Prediction of relaxation to linkage equilibrium

The disequilibrium D_{ij} between any pair of loci i and j is expected to exponentially decrease according to

$$D_{ij}\left[t\right]=\left(1-r_{ij}\right)^{t}D_{ij}[0],$$

where t is the generation number and r_{ij} is the recombination rate between the two loci (Mackay and Falconer 1996). In addition, if one defines the homozygosity as the complement of the heterozygosity, $\hat{H}_E = 1 - H_E$, it can be shown that the two-loci homozygosity (in which an organism is considered homozygous only if each of two loci are homozygous themselves), also exhibits exponentially decaying behavior according to the equation

$$\frac{d\hat{H}_E}{dt}=(1-r_{ij})\hat{H}_E^0+r_{ij}\hat{H}_E^i\hat{H}_E^j,$$

where $\hat{H}_E^0$ denotes the initial two-loci homozygosity, and $\hat{H}_E^i$ and $\hat{H}_E^j$ denote the homozygosity for individual loci. These measures taken together suggest that as generational time proceeds, equilibrium between any two loci is approached according to r_{ij} and heterozygosity between these two loci will increase according to the recombination rate as well. Figure 2.2 shows a histogram of the relaxation times to equilibrium for all pairs of alleles on chromosomes II and III for the mutants which are of interest to this study. The unselected lines will reach equilibrium in approximately 20 generations. Selection will, of course, modify this outcome.

Experimental Design: Theory

The creation of the founder populations was guided by a theoretical analysis of the population heterozygosity. The number of possible genotypes with N loci grows exponentially with the number of loci. Therefore, the entire genotype space cannot be realistically tested for as many loci as are examined in this study (N=23). With a properly chosen founder population, however, it is possible to sample a large volume of genotype space in a realistic number of generations. Because the number of generations in our study is small compared to the effective population size (and therefore small compared to the expected times to fixation), we can estimate the diversity of the population for the unselected case assuming Hardy- Weinberg equilibrium. Note that selection can in general be assumed to reduce this diversity, so the unselected or neutral considerations provide an upper bound for the expected genotypic diversity in the population. In the absence of linkage, two measures of population diversity can be defined: heterozygosity and entropy. The heterozygosity, H_E, is the expected probability that a given individual will be heterozygous at any given locus and is given by

$$H_E = 1 - \frac{1}{N}\sum_{i=1}^{N}(p_i^2 + q_i^2), \qquad (1)$$

where ($p_1, p_2, \ldots, p_N$) is the allelic frequency distribution over the loci and $q_i = 1 - p_i$. The entropy of the genotype distribution, S, can be written as

$$S = -\sum_{i=1}^{N}\left[p_i \log_2(p_i) + q_i \log_2(q_i)\right]. \qquad (2)$$

Using the notion of 'typicality' in an information theoretical sense, the entropy can be used to describe the size of the population in which a 'typical' fly is represented once on average; this size is given by 2^S.

In the founding population, let each fly have *k* loci populated with transposons, in a homozygous or heterozygous manner. It follows that $p_i=k/N$ for homozygotes and $p_i=k/2N$ for heterozygotes. Both measures quickly increase for low values of *k*, suggesting that a more diverse population can be obtained with a founding population in which each fly has greater number of populated loci. Our calculations show that choice of founding members with *k*=3 or *k*=4 gives a marked increase in diversity over *k*=1, while remaining within tractable experimental population sizes (*S*=4.17, *H*= 0.017 for k=1; *S*=10.12, *H*=0.049 for *k*=3 ; *S*=12.65, *H*=0.064 for *k*=4). Note however that this design introduces linkages between loci in the initial population; we selected the transposon-populated loci in each founding fly to be as far apart as possible, maximizing the rate at which equilibration is expected to occur during the course of the experiment.

Selection

Each population consisted of approximately 200 breeding pairs, enough to give rise to > 5000 progeny. During the course of 41 generations, approximately 2000 females and 2000 males were collected from the each of 4 selected populations (Mor, Mul, Lew and the rut^1 isogenic selected population). These flies were trained in groups of 100 in a standard Pavlovian olfactory learning assay, and then fractionated in a T-

maze choice between the paired (CS+) and unpaired (CS-) odor ((Tully and Quinn 1985); see below). With each group of 100, flies that chose correctly were collected and pooled. These pooled flies were iteratively trained and tested until approximately 200 males and 200 females that consistently chose correctly had been selected from the original 4000. These flies then were used to seed the next generation in the CPCs. For unselected populations (Bri, Dob, Stu and the *rut*^{1} isogenic unselected control), approximately 200 males and 200 females were randomly chosen to seed the next generation. During generations 1-20, all females that were processed through selection were collected as virgins. After generation 20, this was relaxed (approximately 0-2 day old) animals were used to bias towards virgin females. In order to control for odor bias selection, the odors used as CS+ and CS- were alternated from one generation to the next. For all groups, 3-octanol (OCT) was used as the CS+ during even generations, and 4-methyl-cyclohexanol (MCH) for odd numbered generations. At each generation, an additional 800 animals from each of the 8 populations were used to quantify the mean learning performance for each population at each generation time point (see Pavlovian learning procedure below). In addition, approximately 288 animals per population were frozen for genotyping after each generation (see genotyping below).

Pavlovian learning procedure:

2-3 day old flies were trained and tested for 2-minute memory performance using a standard Pavlovian olfactory conditioning paradigm(Tully and Quinn 1985). The animals were trained and tested in groups of 100. Each group was sequentially exposed to one

odor (the conditioned stimulus (CS+); either 3-octanol or 4-methyl-cyclohexanol) which was paired with a 60-volt electric shock and then a second odor (either 3-octanol or 4-methyl-cyclohexanol) without shock. Within 2 minutes after training, animals were transferred to the choice point in a radial T-maze, where they were given 2 minutes to choose between the CS+ and CS- odors. For all cases where performance indices were calculated, the learning protocol was repeated with a separate group of flies using the reciprocal odor as the CS+. A half performance index was calculated for each of the two groups by dividing the number of flies that chose correctly, minus the flies that chose incorrectly by the total number of flies in the experiment. A final performance index then was calculated by averaging both reciprocal half-performance indexes..

Single allele effects on rutabaga performance

Effects on rut^1 performance of individual alleles was tested by crossing homozygous mutant rut^1 virgin females to homozygous mutant males. Male progeny were hemizygous for rut^1 and heterozygous for one mutant allele. Females were heterozygous for rut^1 and heterozygous for one mutant allele.

Multi-allele tests

Di-allele heterozygous combinations were generated by crossing flies that were homozygous both for rut^1 and for one additional allele with flies that were homozygous

for *rut*^{1} and for a second allele. All progeny were homozygous (or hemizygous) for *rut*^{1} and heterozygous for each of the 2 mutant alleles. Tri-allele heterozygous combinations were generated by crossing flies that were homozygous for *rut*^{1} and for one mutant allele with flies that were *rut*^{1} mutant and homozygous for each of 2 alleles. Male progeny were hemizygous for *rut*^{1} and heterozygous for all three mutant alleles. Conceptually similar crossing schemes were used to generate animals that had combinations of homozygosity and heterezygosity at more than one locus. In each case, memory performance was measured using the Pavlovian conditioning assay(Tully and Quinn 1985).

Statistical analyses of behavioral experiments:

The behavioral data from this paradigm were normally distributed and thus could be analyzed by analysis of variance (ANOVA). JMP software was utilized to perform Tukey-Kramer honestly significant difference tests, with comparisons made between all genotypes. Statistical significance in the figures represents a difference in performance in comparison to mutant male control levels with $p < 0.05$. Error bars represent standard error of the mean.

High-Throughput Genotyping

Each of the 23 alleles corresponds to an insertion of a P-element transposon at a defined chromosomal locus. Using the BioTrove OpenArray platform, the two alleles for a given locus were detected by ‘taqman probes’ carrying different fluorescent tags (FAM

for wild type, VIC for mutant) in a duplex PCR reaction. 288 flies from each of the six experimental populations (Selected Lines: MOR, MUL, LEW; Unselected Lines: BRI, DOB, STU) were genotyped at the 23 loci in duplicate runs. Genotyping was performed at two generations: generation 11 and generation 25. Design of primer and probe sequences used for PCR genotyping are shown (Table 2.2). A proof of concept for the fidelity of this genotyping platform was developed using control DNAs of known genotypes and mixtures of such DNA samples (Table 2.3).

For each probe, a scatter plot was produced from the intensities of the FAM and VIC channels in each sample (not shown). The genotype at the probe-defined locus was determined by the presence of clusters in the scatter plot corresponding to homozygous wildtype (FF), homozygous mutant (VV), and heterozygous (VF) flies. The clusters were conservatively and visually determined at BioTrove in comparison to a cluster depicting the no template controls, and each sample was assigned a genotype ('called') according to which cluster it belonged. Because the experiments were done in duplicate, a genotype was only verified if the calls of each replicate were in agreement. The analysis described henceforth is based only on this quality-checked consensus data.

The Genotype Matrix

The genotype-calling phase resulted in a 3456 x 23 matrix (288 flies in 6 populations at 2 generations; Table 2.4) containing the P-element dosages: 0 for wild-type homozygous, 1 for heterozygous, 2 for mutant homozygous, and NC for No Call. Each row of this matrix corresponds to the full genotype of a single fly. Each column

can be considered an ‘allelic profile’, since it depicts the allele dosages present at this loci under all experimental conditions.

Approximately 1% of the entries in this matrix were ‘No Call’ (NC). The quality control step determined that the largest source of NC was from failed samples and the occasional failed probe (not shown). The rest of the failed reactions appeared to be randomly distributed. For the analysis, samples with more than 30% failed reactions were discarded. Values for the remaining missing data were assigned by calculating empirical genotype distributions of the valid calls at each locus in each population and drawing from these distributions to ‘impute’ the matrix. This procedure (imputation) was repeated 100 times to ensure an accurate depiction of the allele frequencies. The allele frequencies showed little variance over the 100 imputations (data not shown), as did the results from the SVD/LDA analysis (described below). Note that the imputation changes the number of samples analyzed in each population due to the 30% criteria; that is, each population does not contain an equal number of samples. Nevertheless, this does not significantly change results—the sample sizes remained large enough to accurately depict the allele frequencies.

Singular Value Decomposition

The imputed genotype matrix describes the data in a 23-dimensional ‘genotype space’. To reduce the dimensionality and look for any higher-order structure attributable to the different experimental conditions, the matrix was de-meaned and a singular value decomposition (SVD) was performed.

SVD is a technique commonly used for dimensional reduction and can be considered a generalized version of Principal Components Analysis (PCA) (Strang 2006); however, the SVD is a matrix factorization technique that need not be interpreted in statistical terms (as in PCA). The SVD provides orthonormal bases for both the column space and the row space of the original data matrix. In our case, we used SVD as a signal finder to obtain a 'genotype space' effectively describing the genotypes of typical fly and an 'allele space', effectively describing the relevant allelic contributions.

The row and column means were removed from the data matrix. First, the column means were subtracted from each entry, giving a matrix whose entries described the deviation of each locus in each fly from the average dosage at that locus across all flies in the experiment. Then, the row means of this deviation matrix (the average deviation of each fly's genotype) were subtracted from the subsequent entries. Thus, the final matrix is centered around the average allelic profiles and around each fly's average allelic deviation. The removal of the mean allele frequency across all populations highlights the loci where allele frequencies showed significant variation across populations (as opposed to loci which did not vary between selected and unselected lines).

The singular values of the first two modes showed a deviation from an otherwise linear decay (Fig. 2.3), suggesting that these two modes would be descriptive in a dimensional reduction. There was little variation in the singular values over the 100 imputations, as can be seen by the spread of points at each mode. Although the first two modes accounted for only ~29% of the variance in the original matrix, projection of the data onto these modes in the sample space showed a clear separation between

selected and unselected groups in generation 25 (Fig 2.3C), in agreement with the behavioral separation measured (Fig 2.4A). While two modes appeared to be sufficient for discriminant analyses, the third mode was also used in discriminant analyses to account for slightly more variation in the data. Together, the first three modes explained ~38% of the variance in the original matrix. The choice of three modes was conservatively made to avoid the risk of overfitting in the subsequent discrimination analysis and to ensure that the results were not corrupted by noise in the original matrix.

Discrimination Analysis

Although the SVD provides orthogonal directions that span both the genotype space and the allele space that explain the variance in the data, the direction of separation between experimental groups need not be along one of these axes, and in fact, it was not (Fig 2.3C). Having obtained the descriptive vector spaces from SVD, linear discriminant analysis (LDA;(Duda 2000) was used to find the direction of separation between selected and unselected populations in the genotype space in both generation 11 and generation 25. This vector, **w**, pointed in the same direction between generations (a 16.7° mean difference across imputations), although was on average 1.7 times longer in generation 25 than generation 11. This suggests that the genotypic difference relevant to the phenotype was already present in the populations at generation 11 and increased in magnitude by generation 25.

Because the discrimination direction was so similar between generations, an LDA between selected and unselected groups over the *entire* experiment (i.e. on both

generations together) was ultimately used to discover which alleles were responsible for the group separation. Projection of **w** onto allele space resulted in a vector that could be interpreted as a list of weights describing the contribution of each allele to the phenotypic effect (Fig 2.3D). Positive weights suggest alleles beneficial to the memory task, negative weights suggest alleles detrimental to memory. Neither the SVD nor LDA showed significant differences over the 100 imputations (data not shown), lending support to the robustness of the method. The top alleles were then experimentally tested for epistasis with *rutabaga* and for epistasis amongst themselves.

Cross-validation

In order to assess the stability of the discrimination vector (the direction between populations in each generation, as well as the order of the loci sorted by contribution), a number of cross-validation procedures were performed.

First, the selection and generational labels were shuffled (Table 2.5) and the LDA was repeated between selected and unselected groups. A second shuffling method was performed in which the generational structure was maintained, but the selection labels were randomized. In each case, with 1000 random shuffling, the discrimination vectors in generations 11 and 25 were, approximately, perpendicular to each other (as would be expected for randomly drawn vectors from a high dimension space), rather than being approximately parallel, and were also an order of magnitude smaller than in the unshuffled case (Table 2.5) These results support the conclusion that in the unshuffled case, the identified loci are contributing to the separation between selected and unselected groups and not to some other grouping of the data.

As a second test of the robustness of the discrimination vector, a fixed percentage of randomly chosen samples were dropped from the data set. The angle between vectors, relative magnitudes, and order of contribution were remarkably stable to this procedure. Even with as many as 90% of the points discarded, the variation in the discrimination vector was minimal, suggesting a high degree of within-group similarity, and of across-group dissimilarity (Fig. 2.5).

We then assessed the impact of individual populations on the discrimination by discarding one population at a time from the analysis as well as by analyzing single populations against all opposing populations (e.g. a single selected population against the three unselected populations). With each of these tests, the angle between vectors, the relative magnitudes, and the order of contribution were stable, suggesting that no single population was overtly responsible for our observations (Table 2.6). This lends further support to the hypothesis that the increase in amplitude of the positively contributing alleles is a property of the selection procedure and not solely due to drift or other random effects.

Selection Simulation

Independent populations representing both selected and unselected experiments were simulated. Populations were initiated with genotypes containing transposon counts at 23 independently segregating loci, randomly distributed amongst individuals, each at a frequency of 1/7. At each generation, 10,000 genotypes were created by the union of ‘gametes’ from a randomly selected pair of parents. To constitute the first generation, parents were randomly selected in both types of experiment. Thereafter, in unselected

populations, 400 of the 10,000 starting genotypes were randomly selected to seed the next generation and in selected populations 4,000 were chosen for iterative selection. A single round of the iterative selection process consisted of assigning to each fly a random choice, either correct or incorrect, pulled from a binomial distribution with probability determined by the fly's genotype. The flies that chose correctly were passed to the next round of selection. This processed was repeated until 400 or fewer flies remained, and these flies were randomly paired and mated to produce the next generation. The probability of a correct choice by an individual fly was calculated as 0.7 + sC, the sum of 0.7 (the probability of a *rut* fly choosing correctly) and the rescue provided by the fly's genotype (product of the rescue size, s, and the binary variable C indicating presence or absence of a rescuing genotype). We used s=0.28 to approximate full rescue for all simulations.

Results

We chose to use a standard Pavlovian learning assay (Tully and Quinn, 1985) as a behavioral trait for several reasons. First, performance of wild type flies in this assay is quite robust (Heisenberg 2003; Davis 2005; Margulies, Tully et al. 2005; Keene and Waddell 2007). Second, this learning procedure has been used for high-throughput forward mutagenesis screens (Dudai, Jan et al. 1976; Duerr and Quinn 1982; Boynton and Tully 1992; Dura, Preat et al. 1993; DeZazzo, Sandstrom et al. 2000; Dubnau, Chiang et al. 2003; Ryder, Blows et al. 2004). As a result, many single gene mutations have been identified that have strong impact on performance after this conditioning procedure. Memory performance in this assay is normally measured by allowing a

population of genetically homogeneous animals to choose between two odors, one of which has been previously paired with an electric shock reinforcement. We established a selection procedure based on this learning assay that is capable of fractionating higher performing individuals from a genetically heterogeneous population (Fig. 2.1). We next applied this fractionation procedure to a multi-generational selection experiment to suppress the *rut*[1] memory defect with combinations of alleles that had been identified in a forward screen (Dubnau, Chiang et al. 2003).

Design of starting populations:

As a starting point for this selection experiment, we created a founding population of animals in which the genetic variation was constrained to 23 transposon insertion derived alleles identified in a screen for mutants with memory defects (Dubnau, Chiang et al. 2003). The construction of the founder populations was guided by a theoretical analysis of the population heterozygosity (see additional methods). The number of possible genotypes with N loci grows exponentially with the number of loci. Therefore, the entire genotype space cannot be realistically tested for as many loci as are examined in this study (N=23). With a properly chosen founder population, however, it is possible to sample a large volume of genotype space in a realistic number of generations. Because the number of generations in our study is small compared to the effective population size, our calculations show (see additional methods) that use of founding animals that are homozygous for the mutant alleles at between 3 and 4 of the 23 loci gives a marked increase in diversity over the case where each animal has just 1, while remaining within tractable experimental population sizes.

Based on this analysis, we first created a series of 7 input strains in which each animal is homozygous for the *rut*[1] null allele and homozygous for between 3 and 4 of the 23 transposon alleles. To minimize the effects of linkage between loci in the initial population, we selected the transposon-populated loci in each founding fly to be as far apart as possible (Dubnau, Chiang et al. 2003), maximizing the rate at which equilibration is expected to occur during the course of the experiment (See Additional Methods; Fig. 2.2). All 7 input strains were generated on the same inbred isolate (Wiso[CJ1]) from the standard Canton S strain (Dubnau et al., 2003). To create a founding population for artificial selection, each of the 7 input strains were mixed in equal proportions. The 7 strains were allowed to inter-breed for one generation and the progeny were used to seed population cages. At this stage, each animal is heterozygous for between 6 and 8 of the 23 alleles.

Experimental evolution of* nearly normal learning in *rut* mutants*:

Using the above design, we created 6 identical populations with heterogeneity at the 23 defined loci of interest. Three of these (Morgan, Muller and Lewis) were subjected to selection and 3 (Dobzhansky, Sturtevant and Bridges) were not selected for learning ability. These served as controls for effects of drift, natural selection for fecundity, viability, developmental time, etc. In addition to the above 6 populations, two additional control populations were established that did not contain any of the 23 transposon alleles and were homozygous for the *rut*[1] null allele in the same inbred background as the other populations. One of these “isogenic controls” underwent selection, the other was not selected for learning ability. These last two populations were designed to control for contribution of any unknown or *de-novo* alleles segregating in the inbred background. Importantly, these

control strains did not respond to selection over 41 generations (see below).

For the first 14 generations, the performance levels of the selected and unselected populations were indistinguishable. In fact, seasonable and other experimental variation was well controlled because the week-to-week fluctuation in performance was highly correlated across populations, which were always trained and tested in parallel in an experimenter blind manner. Between the 14th and 41st generations, a clear response to selection was observed in all three selected groups that contained genetic diversity. By generation 41, the performance of Morgan, Muller and Lewis (selected groups) approached the maximal levels of short-term memory normally seen only in wild type animals (Fig. 2.4 A,B, D). No effects were seen on levels of long-term memory fecundity, longevity, generation time (Fig. 2.5). The suppression of the learning defect is striking given the fact that all animals in these populations are homozygous for a *rut*^{1} null allele (confirmed by PCR genotyping of the *rut*^{1} allele with multiple animals, not shown). In contrast, we did not observe a response to selection in the control isogenic strain that lacked variability at the 23 loci. The fact that learning performance did increase in each of the replicate populations that contained variability at the 23 loci (Fig. 2.4 A,B, D), but not in the inbred control population (Fig. 2.4C), strongly supports the conclusion that the 23 loci are causal of the selection response. We cannot rule out contributions of trans-generational epigenetics or of *de-novo* mutations [which have been estimated to occur in *drosophila* at a rate of 8.0×10^{-6} per locus per generation] (Drake, Charlesworth et al. 1998). To the extent that such rare events impact our selection, however, they nevertheless would need to act in concert with the 23 controlled loci.

Genotyping and multivariate analysis:

288 flies in each of 6 populations at generations 11 and 25 were genotyped at the 23 loci (Table 2.4). Genotyping resulted in a 3456 x 23 matrix containing the P-element dosages: 0 for wild-type homozygous, 1 for heterozygous, 2 for mutant homozygous (Fig. 2.3A). Each row of this matrix corresponds to the full genotype of a single fly, and thus, the genotype matrix defines flies in a 23-dimensional space. Each column can be considered an 'allelic profile,' since it depicts the allele dosages present at this locus under all experimental conditions.

What genotypic differences between selected and unselected lines are responsible for the observed phenotypic response to selection? In statistical terms, this corresponds to characterizing the differences between the corresponding genotype distributions. There are 3^23 possible genotypes, whereas each population is sampled only 288 times (assuming each fly presents an independent sample). Therefore, it is not possible to characterize the full distributions (curse of dimensionality). Following standard practice, we can characterize the first and second moments of the distributions, and characterize the differences between the distributions using discriminant analysis.

The first moments, or means, are given by the average P-element dosages as a function of loci. These form 6 vectors of length 23 each (three selected and three unselected), corresponding to the means of each population. These are displayed in (Figs. S4,S5), and show significant differences in loci (Fig. 2.6, Tables 2.7,2.8). Note however that in this analysis, each locus is treated independently and the covariance structure is not accounted for. There are 23x22/2 = 253 entries to the covariance matrix,

and only 288 samples – in addition there are missing data. Thus there are not enough degrees of freedom to estimate each element of the covariance matrix independently. We therefore use standard dimensionality reduction methods by performing an SVD of the imputed data matrix containing all 6 populations

The three leading components capture 38% of the variance, and we restrict our subsequent analysis to projections onto these components (eigen genotypes). For ease of visualization, the genotype data projected onto the first two components (29% of the variance) is shown in Fig. 2.3C. Direct visual examination shows that the distributions corresponding to selected and unselected lines are displaced from each other in a systematic way. Note that although this is a “linear” technique, there is no strong evidence in the figure that a non-linear separation surface is required, and given the paucity of data we keep to linear separating hyperplanes. Nonlinear regression methods did not significantly change the results (data not shown).

We performed linear discriminant analysis (LDA) in this reduced three-dimensional space to determine the direction of strongest separation between selected and unselected lines in genotype space. This vector is found to be similar to the difference in the means (first moments), but shows some differences for the loci with smaller weights (Fig. 2.8). The robustness of the discrimination vector was confirmed through a number of bootstrap cross-validation procedures (Fig 2.9). We projected this discrimination vector into allele space, resulting in a list of weights describing the relative contribution of each allele to the phenotypic effect. The top 8 alleles identified by the SVD/LDA were D0077, E3272, E3945, C0113, E1023, E4299, E3145, and D0940.

One of the 8 identified loci significantly suppresses rut:

We tested the effects of each of the 8 individual loci as heterozygotes on the *rut*^{1} mutant learning levels as well as on learning in rut+ animals (Figs. 2.10, 2.11). One of these, E3272 (nord), which contains a P-element transposon insertion in a gene (CG30418) with fibronectin type III domain homology, is capable of partially but significantly (N = 17, Tukey HSD) suppressing the learning performance of *rut*^{1}- on its own when present as a heterozygote (Figs. 2.10, 2.11). It is worth note that this effect is non-linear with respect to the dosage of E3272 because it only is observed when E3272 is heterozygous. In contrast, E3272 had no effect on the *rut* defect when E3272 homozygous (Fig. 2.12). We also tested the effects of E1847, which was identified in the SVD/LDA as opposing the selection response. Performance of *rut*^{1}; E1847/+ animals was indistinguishable from that of *rut*^{1} mutants (Fig. 2.11D).

To our knowledge, E3272 is the first case of a genetic suppressor of any learning mutant, although *rut* was shown to be a suppresser of *dnc* in a measure of terminal varicosities and branches in larval motor neurons (Zhong, Budnik et al. 1992). The identification of an individual suppressor gene is gratifying, however, given the E3272 effect size and allele frequency, this effect alone cannot explain the magnitude of the observed population level response to selection (Fig. 2.13). In fact even for the extreme scenario where a suppressing genotype confers maximal performance (equivalent to the wild type rutabaga+ genotype), 55% of the animals in a mixed population would need to contain suppressing genotypes to cause the observed performance at generation 41 (Fig. 2.13). The required fraction of animals with suppressing genotypes

logically increases as the effect size of the typical suppressing genotype decreases (Fig. 2.13). Thus the observed performance improvement would require either few high frequency genotype solutions or many rare solutions.

Two-way interactions among the identified 8 loci do not explain the selected *rut* suppression:

We next tested the effects of each of the double heterozygous combinations (Fig. 2.10B). For each of the 28 possible di-allele combinations among the 8 identified loci, learning performance was measured in males that were rut^1 hemizygous and heterozygous for two of the 8 loci. None of these di-allele combinations yielded significant suppression of *rut*. 9 of the 28 di-allele combinations actually reduce the learning performance of *rut*- (Fig. 2.10D). In order to ask whether interactions between the E3272 suppressor and loci not identified by the SVD/LDA might be involved, we tested for significant co-occurrence in the genotype data of E3272 with each of the other 22 loci segregating in the experiment. For the E3272-D0940 combination, we do observe a significantly higher than expected frequency (Table 2.9), however this double combination does not yield suppression (Fig. 2.10D). Taken together, these findings indicate that two-way heterozygous combinations are not likely to drive the selected suppression. We cannot rule out a role for 2-way interactions among loci in the homozygous state, however, we observe little homozygosity in the genotype data set in any of the replicate populations (Table 2.4). Moreover, in the few cases where we have examined effects in homozygotes we do not observe suppression (Data not shown).

Additive effects of top three alleles:

We wondered whether each of these loci might contribute a small additive effect. In this case, the contribution of each allele in isolation might be below detection threshold in our behavioral assay. To test this, we constructed animals that were heterozygous for all of the three top identified loci (D0077, E3272, E3945; Fig 2.10C). This triple-heterozygote did not significantly suppress the rut^1 defect. Thus small additive effects are not detected among the three loci with highest amplitude contribution to the separation between selected and unselected groups as detected by the SVD/LDA analysis.

Selection response likely involves combinations of between 3 and 5 loci:

In order to explore the genetic landscape more systematically, we used simulations to examine the potential for higher order gene networks to contribute, under known constraints of our experimental design (See methods above). The simulation used a population size of 200 breeding pairs, 23 unlinked loci with 2 alleles each and started with allele frequencies of 1/7. In a model of drift, we observed as expected from theoretical arguments, that on average, alleles remain present at around the starting frequency but also can be randomly lost from the population (Fig. 2.14). We next conferred a selective advantage to the heterozygous state for a single locus. The simulated selective force drives the allele frequency to 0.5 with the time-course dependent on the selective advantage.

We next used simulations to model the involvement of multi-locus genotypic solutions. In order to simplify the simulations, we first considered the case where only a single fully heterozygous allele combination confers selective advantage. When selection is maximally strong, such suppressing combinations appear and reach optimal frequency (0.5 for each of the underlying loci) within 40 generations for combinations of less than 6 loci (Fig. 2.15). With genetic solutions involving 6 loci, the underlying alleles do not reach frequency of 0.5 within the time-course of our experiment and for combinations involving 7 or more loci, solutions are never found within 40 generations (with 10,000 simulated populations). This upper bound on the complexity of possible solutions most likely is due both to the exponential expansion of genotype space for higher order combinations and to the opposing action of drift, which can eliminate individual alleles from a population before selection has a chance to act.

Conclusions

Several key insights stem from this study. First, our selection demonstrates a remarkable flexibility in the gene network underlying memory and learning [see (van Swinderen and Greenspan 2005; Greenspan 2009)]. Even with a relatively small set of 23 loci and two alleles/locus, there is sufficient combinatorial potential to select for high levels of learning that is independent of the canonical rut adenylyl cyclase-mediated cAMP signaling pathway. This by definition invokes non-additive epistasis because each of the alleles that we supplied to the population were identified in screen for reduced memory and learning (Dubnau et al. 2003). Second, our experimental findings

together with the outcome of simulations strongly support the hypothesis that the typical genetic solution involves combinatorial action of several genes. Although we did identify one single-locus suppressor, it's quantitative impact on the phenotype can only explain a small fraction of the population level response. Third, because of the high degree of genotype heterogeneity in all selected populations, even the more prominent genotypes are present in a relatively small fraction of animals. It follows that the observed population level suppression relies on multiple genetic solutions within each replicate population.

Cryptic genetic variation (Gibson and Dworkin 2004) has potentially broad impact on traits with complex inheritance but also influences phenotypic severity (penetrance) of traits with apparently simple inheritance. In the case of Mendelian human genetic disorders, for example, clinical severity can be dramatically modulated by additional loci that have no clinical impact on their own e.g. (Petrij, Giles et al. 1995; Merlo and Boyle 2003; Duclot, Jacquet et al. 2010)

Linkage and GWA studies have successfully identified genetic variants that are associated with human disease, although for GWA, these typically have relatively small effect sizes and explain only a small fraction of the heritability for a given disorder (Manolio, Collins et al. 2009; Eichler, Flint et al. 2010). The sources of this 'missing heritability' are currently unknown, but in principle could involve types of genetic variation that are not sampled by current methods, trans-generational epigenetic effects, over-estimates of heritability, gene by environment interaction or gene epistasis that would be difficult to detect. Because of the highly constrained nature of our selection experiment, we can all but rule out the impact of rare un-sampled variants,

environmental variation and trans-generational epigenetics. We nevertheless observe a high degree of ‘missing heterability’ even though our experiment includes a relatively small number of segregating loci. This outcome seems most compatible with a model in which phenotypic modification of the *rut* -learning defect involves a diverse set of genotypes that typically include several different interacting loci.

Figures and Legends

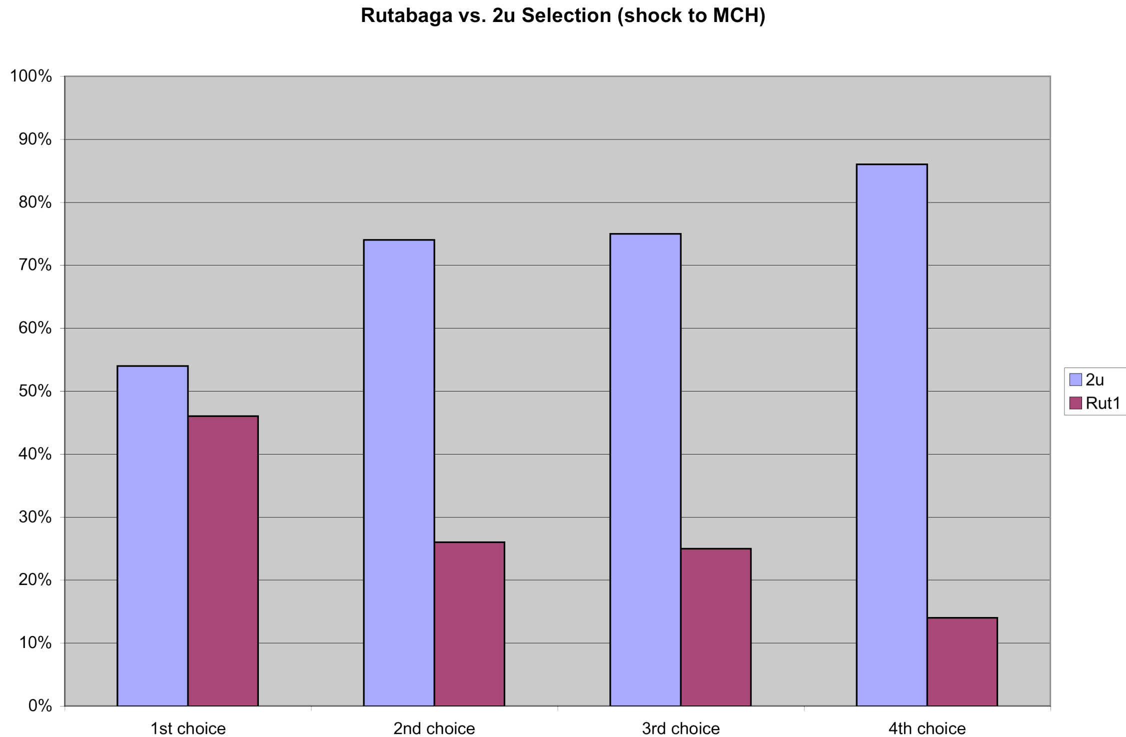


Figure 2.1. Proof of principle for learning selection: Groups of white-eyed rut+ and red-eyed *rut*^{1} mutant animals were mixed in equal proportions and trained in the olfactory learning assay (see Methods). This mixed population was trained and tested in the standard T-maze (Tully and Quinn 1985); see methods). The fraction of animals that avoided the CS+ odor was then re-trained and re-fractionated at the choice point of the T-maze. This fractionation procedure was iterated, and the ratio of white (wild type, high learning) versus red-eyed (*rut*^{1} mutant, low learning) animals was determined after each round. Using this procedure, we are able to enrich for *rut*$^+$ flies so that after just 4 choices, 85% of the animals are *rut*$^+$. Proportions of red- and white-eyed individuals are

shown after one, two, three or four choices. The number of animals at start of each experiment was 200 (1 choice), 400 (2 choices), 800 (3 choices) and 2000 (4 choices).

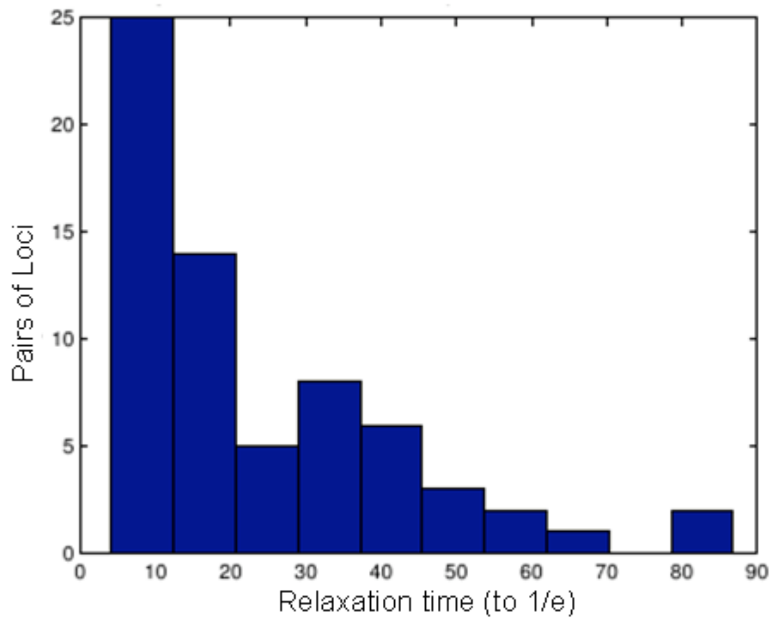
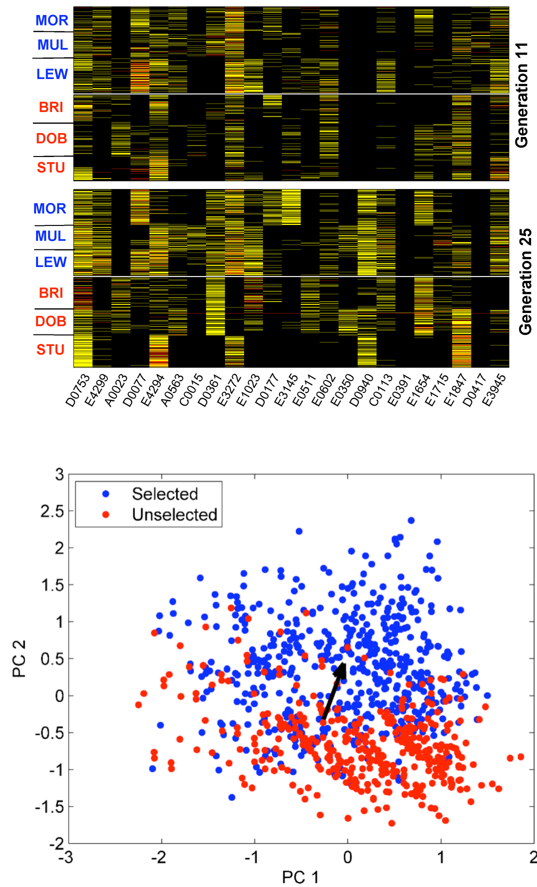


Figure 2.2 Relaxation times (to 1/e) for all pairs of loci on chromosomes II and III in the collection of 60 mutants described in the screen by Dubnau (2003). The median is 20.95 generations. Relaxation times are calculated as $\tau = 1 - r_{ij}$ where $r_{ij} = 4n_f d$, n_f is the proportional fraction of females in the population, and d is the distance between i and j in centimorgans.

A



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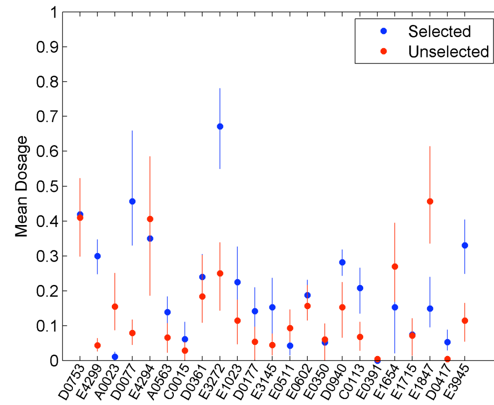

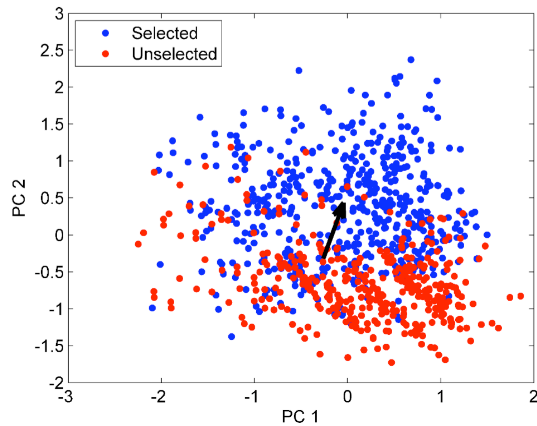

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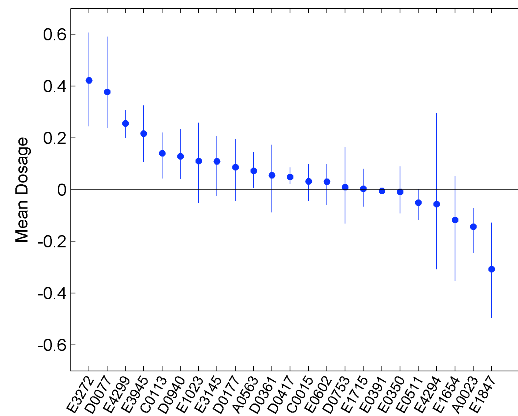


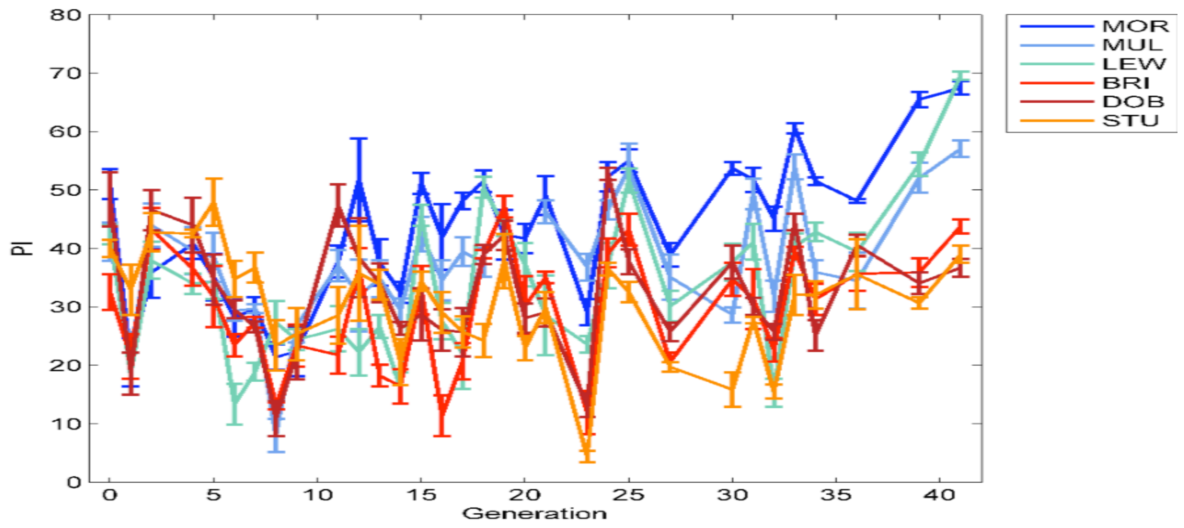
D

Figure 2.3. Genotype data and Multivariate analyses identify 8 loci underlying selection response. (**A**) Heat map of genotypes are shown for flies sampled from all populations at generations 11 and 25. Individual samples are arranged by population on the Y-axis with selected groups (**Mor**, **Mul** and **Lew**) and unselected (**Dob**, **Stu** and **Bri**). Each of the 23 loci are shown on the Y axis. Black ticks denote homozygosity for the wild type allele. Yellow ticks denote heterozygosity. Red denotes homozygosity for the mutant allele. Missing data values were ‘filled in’ by imputation (see methods).

Shown is the result of a single imputation. (**B**) Difference between mean dosages in selected and unselected groups. Error bars are 95% confidence intervals obtained from a combination jackknife/bootstrap procedure to account for group substructure.

(**C**) SVD analysis (see methods) of individual fly genotypes found in (**A**). Selected flies in (blue) and unselected in (red) are plotted by principle component 1 (PC1) on the X-axis, and principle component 2 (PC2) on the Y- axis. Again, data are from a single imputation (corresponding to the matrix in **A**) (**D**) Plot of the discrimination vector with the alleles (X-axis) sorted by contribution (Y- axis). Mean dosages for each loci in each population. Error bars are bootstrapped 95% confidence intervals.

A



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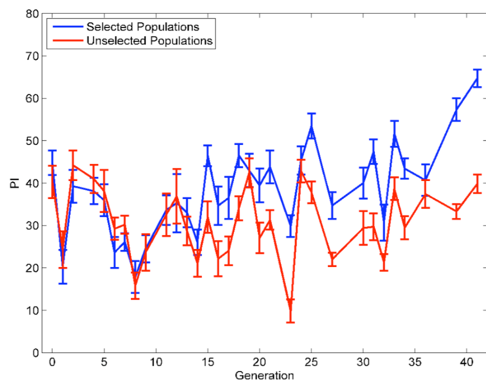

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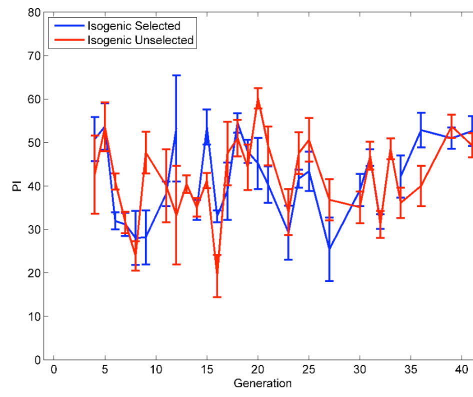

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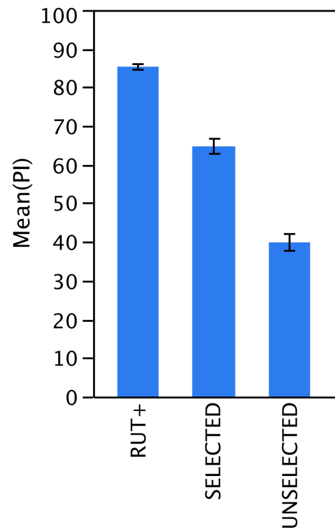


Figure 2.4. Multi-generational selection of improved learning in *rut^1* mutants. 6 populations (**A,B**) are *rut^1 homozygous mutant-* and heterogeneous at 23 loci (see text). Three of these (MOR, MUL, LEW) underwent selection, three (BRI, DOB and STU) were allowed to drift. Two control populations are *rut-* and do not contain any of the 23 transposon insertions (**C**). All populations are in the same inbred bacground (methods). (A) Learning Performance Index (PI) of individual selected populations [Morgan (dark blue), Lewis (teal), Muller (light blue)] and individual unselected populations [Bridges (light red), Dobzhansky (dark red), and Sturtevant (orange)]. N=4 PI measurements per population at each time-point. (B) Mean Performance Index of 3 selected populations Morgan, Muller, Lewis (blue) and 3 unselected populations Dobzhansky, Sturtevant, Bridges (red). (**C**) Performance indices of selected (blue) and unselected (Red) controls on an inbred background. (N = 4 PI measurements per group).

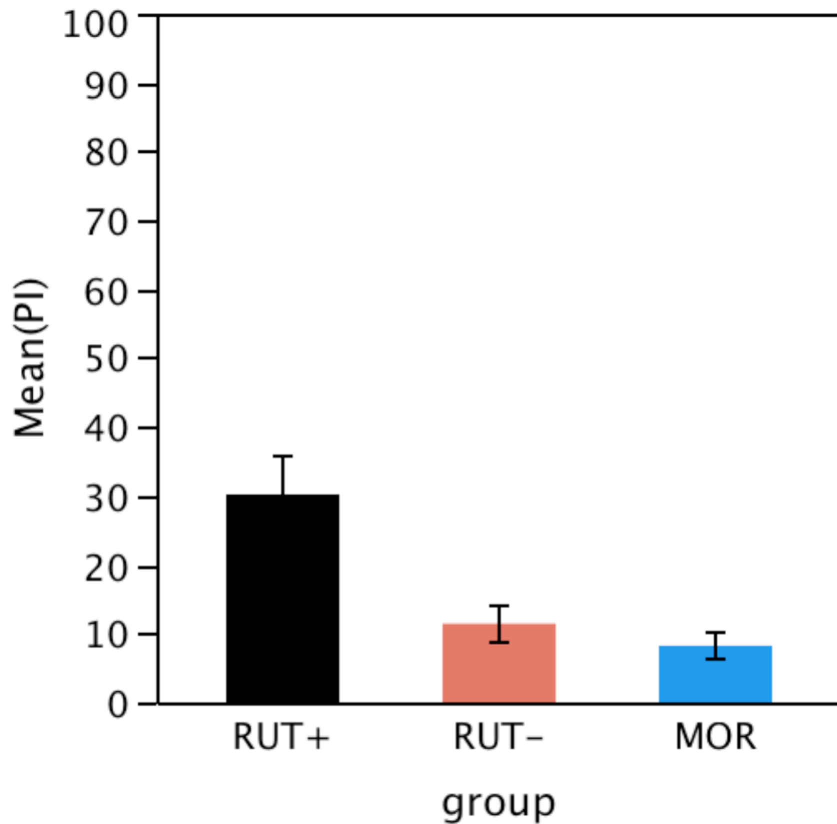
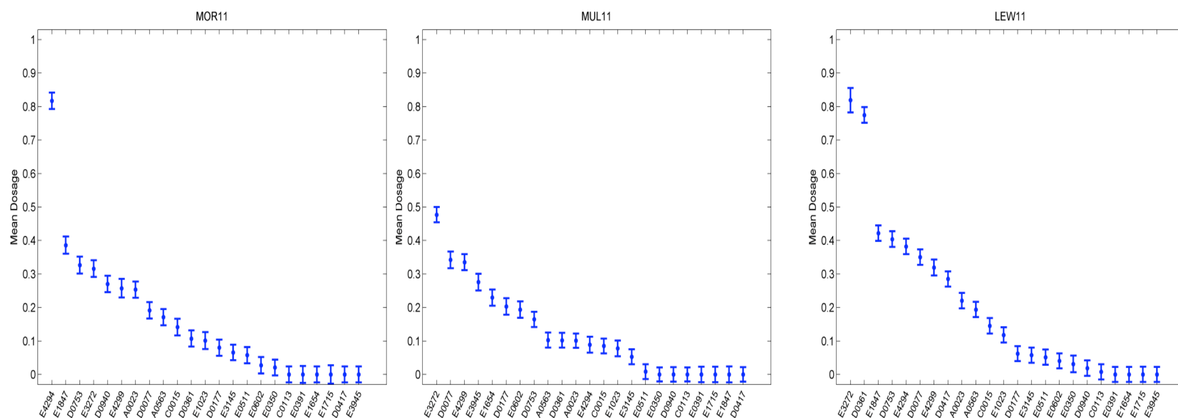


Figure 2.5 Selected improvement in short-term memory does not impact long-term memory performance.

Consolidated long-term memory was tested for one of the selected populations by testing performance 24 hours after a standard 10X spaced training procedure (reference Tully et al., 1994; Dubnau et al., 2003). Memory performance of Morgan was compared with that of *rut*+ wild type control and of rut^1 mutant animals. Performance of Morgan is not significantly different from that of rut^1 (Tukey HSD).

Selected, Gen 11



Unselected, Gen

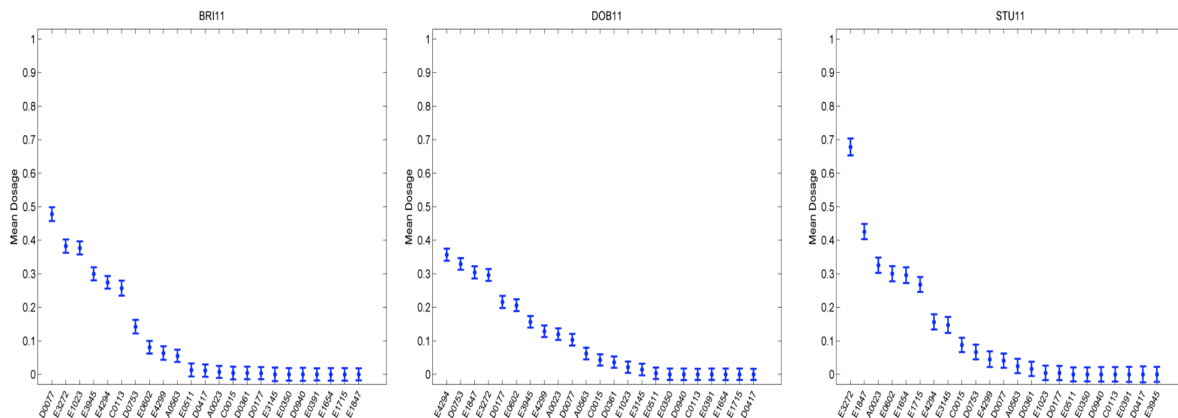
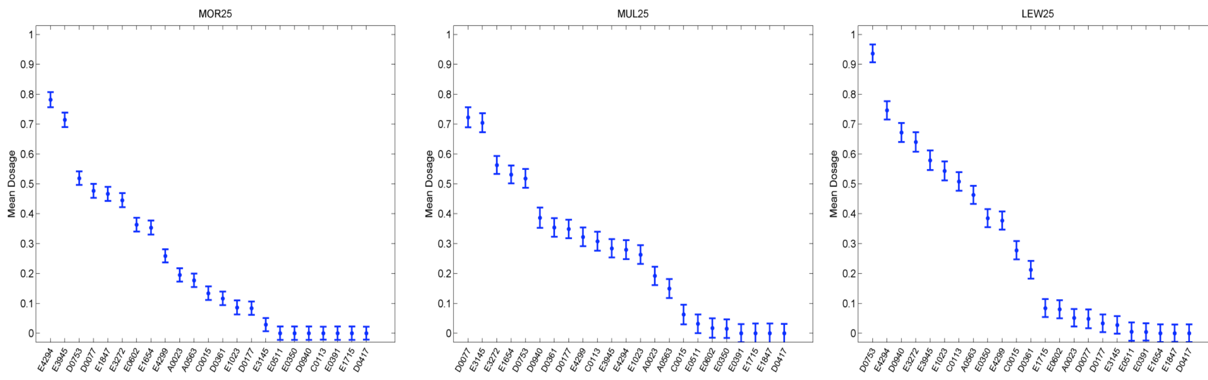


Figure 2.6. Loci Dosage by Populations at Generation 11.

Mean dosage vs. loci, with standard errors, sorted by mean dosage for each of the six populations in generation 11 (Selected - MOR11, MUL11 LEW11, Unselected - BRI11, DOB11, STU11). All populations show significant differences across many loci (see Table 2.7)

Selected, Gen 25



Unselected, Gen 25

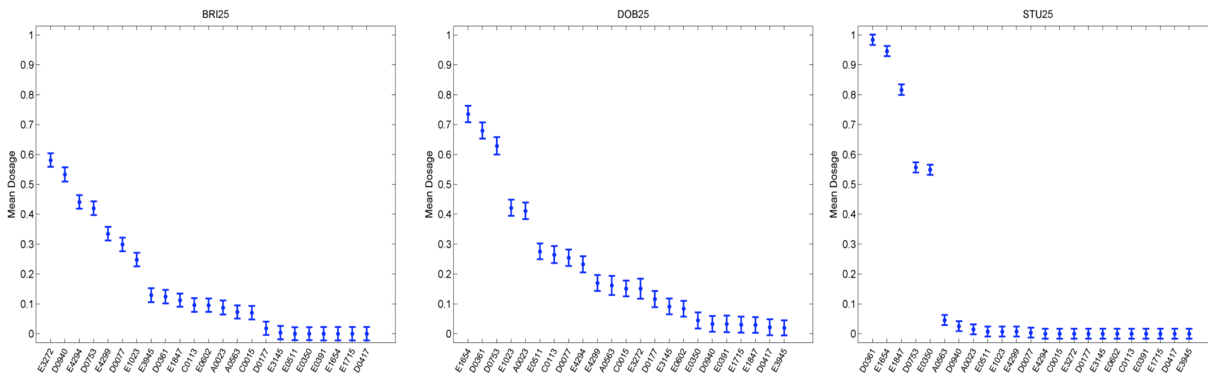


Figure 2.7. Loci Dosage by Populations at Generation 25.

Mean dosage vs. loci, with standard errors, sorted by mean dosage for each of the six populations in generation 25 (Selected - MOR25, MUL25 LEW25, Unselected - BRI25, DOB25, STU25). All populations show significant differences across many loci (see Table 2.8).

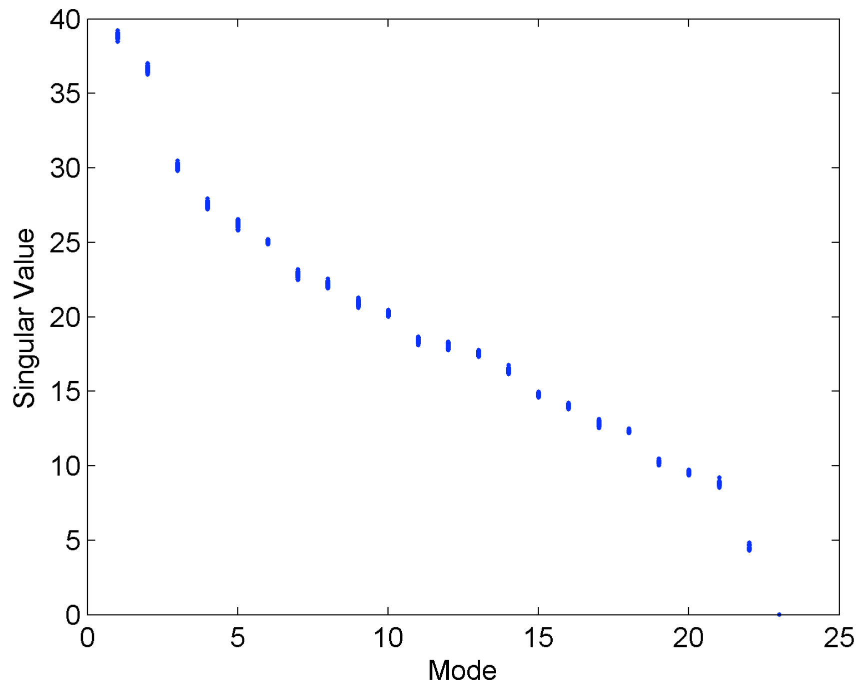
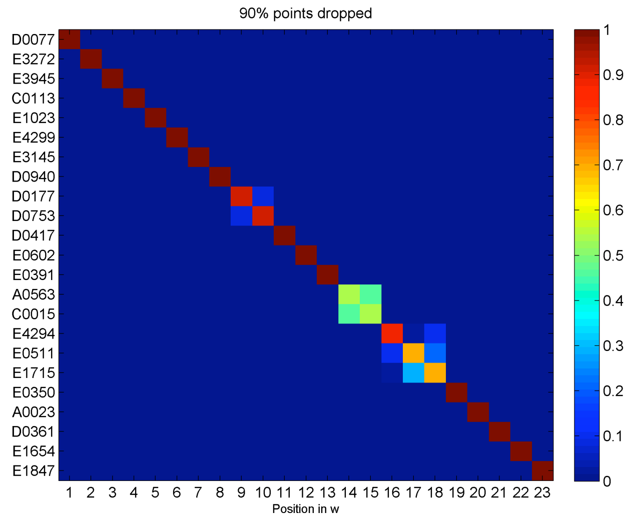


Fig 2.8. Singular values as obtained from the SVD. The spread in data points at each mode are the singular values obtained from each of 100 imputations. The small variation suggests that the imputation method does not significantly affect the decomposition results.

A



B

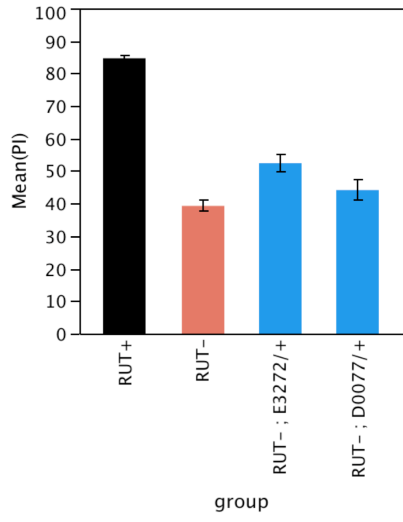
% dropped	$\theta_{11\text{-}25}$	$\|w_{11}\|$	$\|w_{25}\|$
10	16.54	.0015	.0026
50	17.00	.0027	.0046
90	21.4	.0147	.0242

Figure 2.9. Discriminant vector is robust to dropping samples.

(**A**) Heat-map displays the frequency with which the amplitude ranking for each allele is the same is in the original discrimination vector (Fig 2.3**C**) when 90% of the points are

dropped. (**B**) Effect of dropping 10%, 50% or 90% of samples on the direction and length of the discriminant vector. The angle $\theta_{11\text{-}25}$ is the direction between the vector **w** discriminating selected vs. unselected populations in generations 11 and 25 and $|\mathbf{w}_{11}|$, $|\mathbf{w}_{25}|$ are the lengths of the discrimination vectors in generation 11 and 25 respectively. The values in the table are averages over 100 imputations of the genotype matrix.

A



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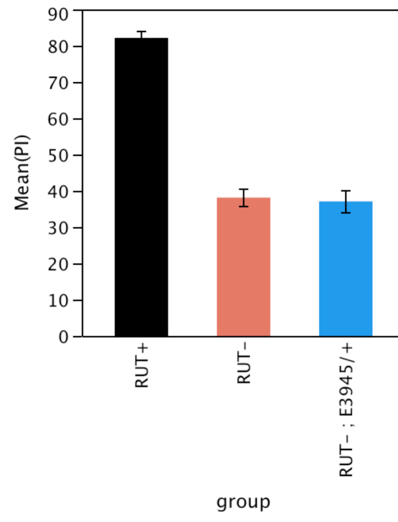

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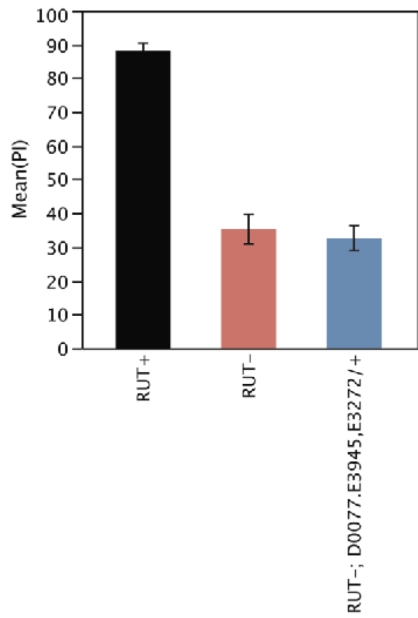

D

	D0077	**E3272**	**E3945**	**C0113**	**E1023**	**E4299**	**E3145**	**D0940**
D0077								
E3272	0.544							
E3945	0.371							
C0113								
E1023								
E4299	0.487	0.628	0.468					
E3145								
D0940			0.488	0.474		0.611	0.56	

Figure 2.10. **Effects on *rut*[1] memory performance single, double and triple heterozygous combinations among alleles identified in by SVD/LDA.** Memory performance of animals that are hemizygous for the *rut*[1] mutation and heterozygous for E3272 or D0077 (A) or E3945 (B) relative to performance of *rut*[1] hemizygous or rut+ males. E3272, but not D0077 or E3945, yields a partial but significant (N = 17, Tukey HSD) suppression of the *rut*[1] memory defect. Animals that were *rut*[1] hemizygous and D0077/+, E3272/+, E3945/+ triple heterozygous exhibit memory performance that is not significantly different from that of *rut*[1] (**C**). Effects on the *rut*[1] memory levels of each of the 28 heterozygous di-allele combinations amongst top 8 alleles also were tested for memory performance (**D**). In 19 di-allele combinations, no significant impact on the memory performance of *rut*[1] was observed (yellow boxes in top half matrix). 9 combinations significantly enhanced the learning defect of *rut*[1] (blue), i.e. they exhibited lower levels of learning [Tukey HSD]. These effect sizes are shown in the bottom half matrix as a fraction of *rut*[1] performance levels.

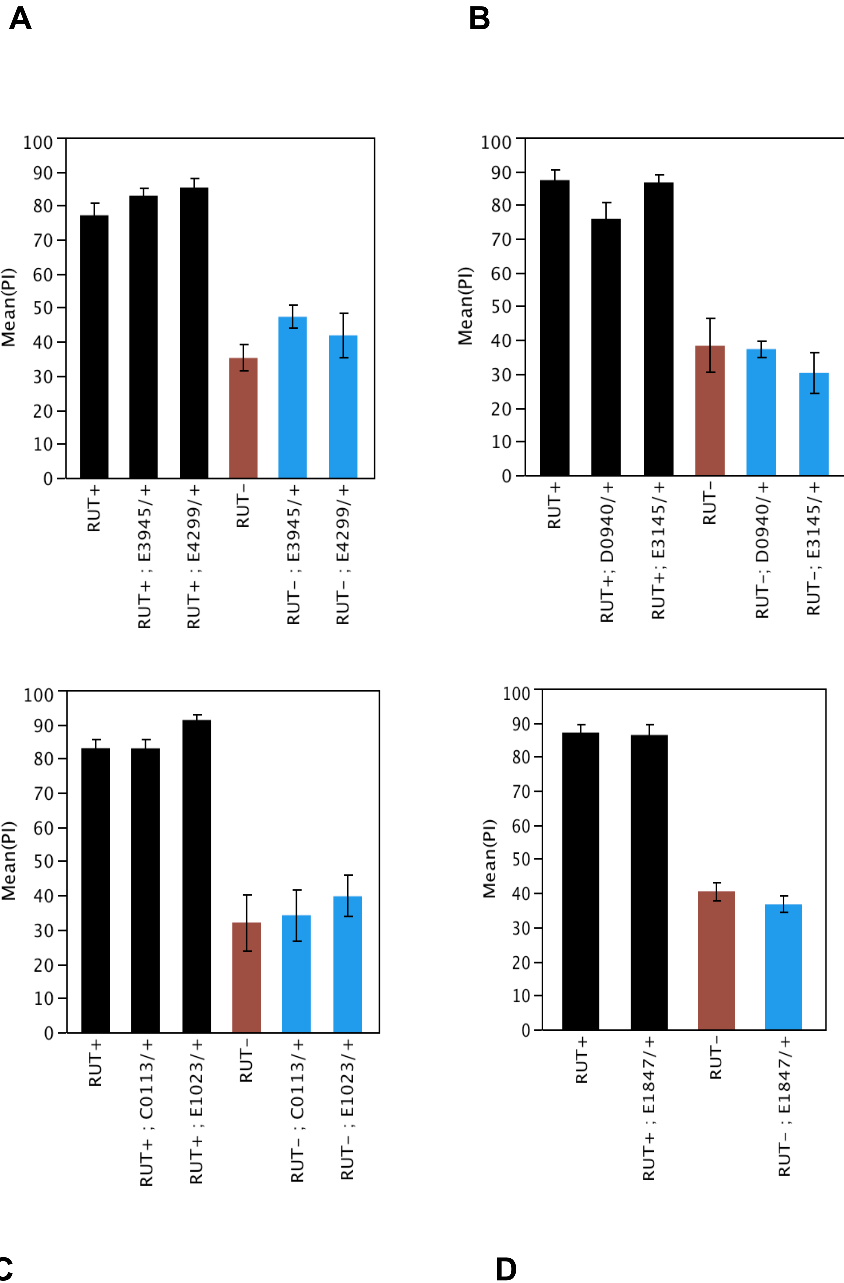

Figure 2.11. Effects on *rut*[1] memory performance of single alleles identified in by SVD/LDA. Effects are shown for the 5 single alleles identified by the SVD/LDA that were not tested in Fig.3, as well as for E1847, whose amplitude opposed the separation between groups (Fig. 2C). These 5 loci and E1847 showed no effects on performance

of rut1 heterozygous females or ***rut¹*** hemizygous males (Tukey HSD). ***rut¹*** hemizygous performance is shown for comparison. N >7 for all groups.

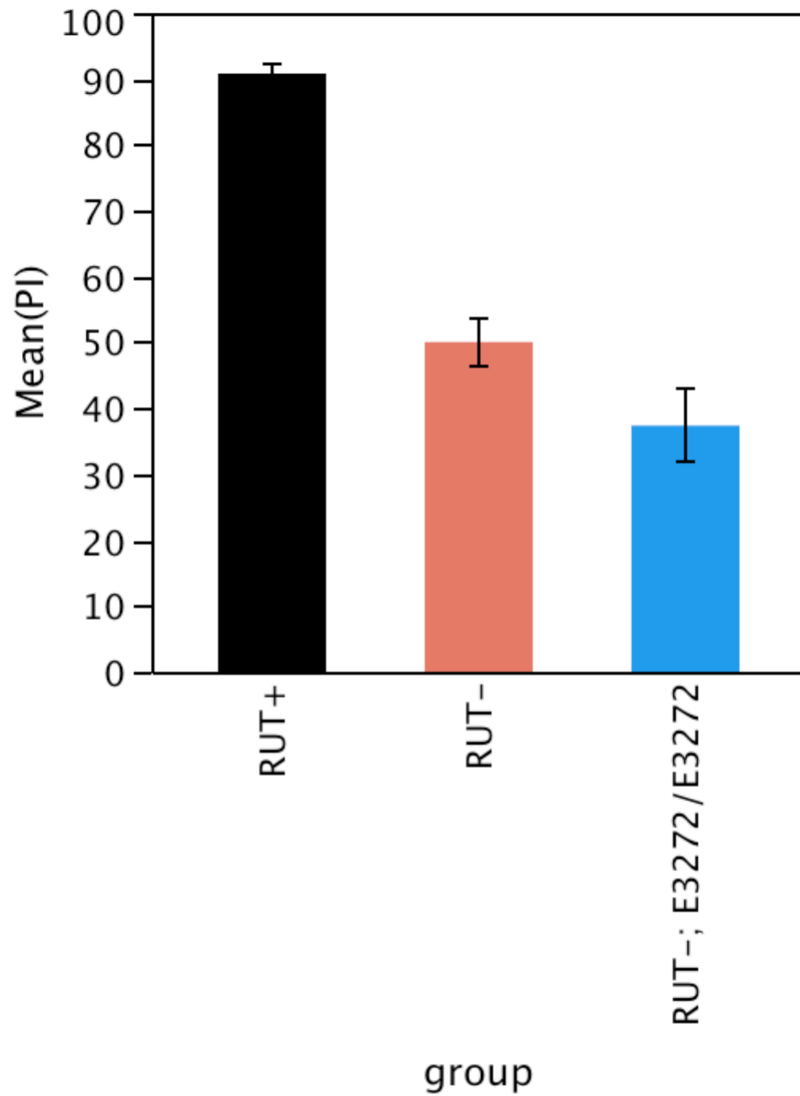

Figure 2.12. Effects on *rut*^{1} memory performance E3272 homozygote. *rut*^{1}; E3272 double homozygous animals exhibit no difference in short term memory performance (Tukey HSD) than *rut*^{1}. N = 6 for all groups.

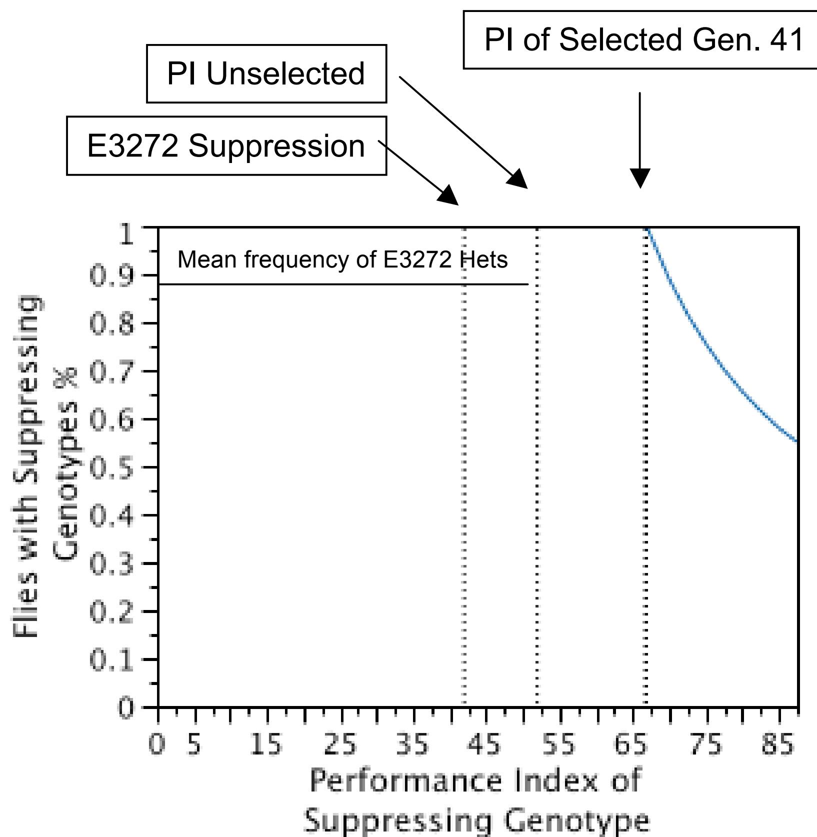

Figure 2.13. Percentage of suppressed genotypes necessary to explain population level response to selection. Percentage of genotypes necessary for observed response to selection at generation 41 shown as a function of the performance of a suppressing genotype (blue curve). Vertical dashed lines indicate the mean performance (42.0) of unselected populations, the mean performance (52.0) of E3272/*rut* trans-heterozygous crosses, and the mean performance (66.9) of selected populations at generation 41. The horizontal line indicates mean frequency of E3272 alleles in selected populations. The blue curve was calculated by keeping mutant performance (X^{mut}) constant at .42, keeping the overall performance level constant at 66.9, and solving for the necessary suppressing genotypes (X^{sup}). This allowed us to determine the necessary suppressing genotypes as X^{sup} ranged from 55% to 100%.

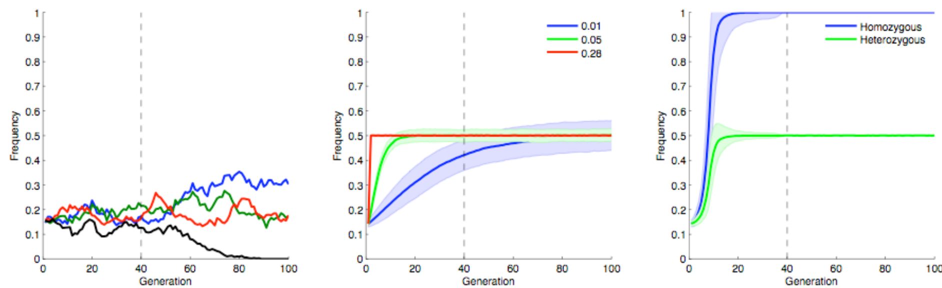

Figure 2.14. Selection model validation. Monte Carlo simulation provides a realistic model of selection experiments. When the model is initialized with no selective pressure, alleles behave as expected for a model of drift (**A**). In some cases, allele frequencies drift to 0 (example shown in **black**), while most alleles fluctuate around the starting frequency 0.14 (examples shown in **red**, **green**, and **blue**). When selective advantage is given to the heterozyote at a single locus (**B**), the allele frequency reaches an optimum 0.5 with a timecourse dependent on selective advantage. With selective advantage of 0.01 (**blue** curve, mean across populations 1 s.d.), 0.05 (**green** curve), or 0.28 (**red** curve), frequency reaches 0.5 in approximately 90, 20, and 2 generations respectively. A combination of 1 homozygous allele with 2 heterozygous alleles given an advantage of 0.28 behaves as expected (**C**). The homozygous allele frequency goes to fixation at 1 (**blue** curve), while the frequency of the heterozygous alleles is maintained around 0.5 (**green** curve). All simulations model population size (200 breeding pairs), number of loci (23), and starting allele frequencies (0.14) of the experimental selection (see methods). N = 10^3 populations for all simulations shown.

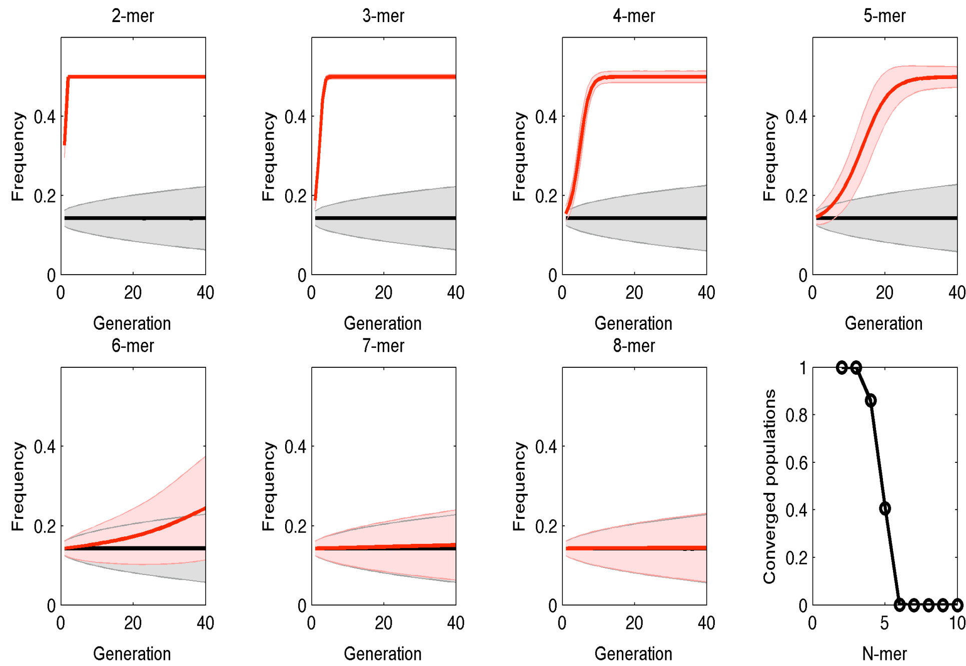

Figure 2.15. Numerous combinations of 3 to 5 loci must contribute to selection response. Simulated selection experiments, using parameters similar to biological selection experiments, estimate that up to 6 heterozygous loci, working in combination, contribute to the observed selection response. (**A**) A clear response to selection in simulated populations (N = 10^4 populations, for each *N*-mer combination) is observed within 40 generations for fully-rescuing heterozygous combinations of less than 7 loci. Selected alleles (**red**, mean frequency 1 s.d. of all *N* selected alleles from all populations) reach a frequency in the population of 0.5 within 40 generations for *N*-mer combinations of $N < 6$. When $N = 6$, selected allele frequencies have not reached 0.5 in most populations, though it is possible. Unselected alleles in the same populations are

subject to random drift (**black**, mean frequency 1 s.d. of all 23 - N alleles). (**B**) The probability of an individual population converging on a solution of N heterozygous loci is estimated as the proportion of simulated populations that reached a mean frequency of selected alleles 0.48 over generations 38 - 40. For N = 2,3, p = 1. For N = 4, p = 0.8605. For N = 5, p = 0.4067. For N = 6, $p = 10^{-4}$. For N = 7…10, $p < 10^{-4}$.

Strain	Allele 1	Allele 2	Allele 3	Allele 4
1	D0177	D0753*	E3947	E4294*
2	D0361	D0417	E1023	E 4299
3	A0023	C0015	D0940	E1715
4	E3945	C0113	E4294*	D0753*
5	E0391	E3145	D0077	
6	E0602	E1847	A0563	
7	E0511	E1654	E3272	

Table 2.1. Starter Strains .1-7 (Column 1), each contain between 3 and 5 of the 23 alleles (Columns 2-5). *these alleles were present in more than one strain. Genomic insertion sites of all 23 transposons are described elsewhere (Dubnau et al., 2003).

Table 2.2. Sequences used to make probes and primers for genotyping of P-element insertion alleles. The locus for which primers and probes were designed are in Column 1 (ABI)_ [Column 2 shows the sequence to which gene specific primers were designed (ABI). The location of the P-element insertion is denoted with a “P” (Table Attached)

Table 2.3. Genotype proof of principle. A two flourophore assay was developed in which FAM (F) detects wild type and VIC (V) detects mutant. DNA was extracted from homozygote mutant animals and from wild-type animals. “Heterozygote” animals were mimicked by mixing wild-type and mutant animals in a 50/50 mixture.

The results for 10 alleles are shown in column 1. Column 2 shows genotype calls for a wild type animal. Wild type FAM fluorphores “FF” are detected at all 10 loci. Odd columns 3-21 show genotype calls for animals that were homozygous for a single allele. For mutant animals the VAM fluorophore “VV” is detected using the allele specific probes and primers and the wild-type FAM fluorophore “FF” at the other 9 loci. Genotype calls for DNA samples from mixed “heterozygous” DNA’s are shown in even numbered columns 4-22. For these simulated heterozygote animals, both fluorophores “VF” are detected for the locus in question only. Genotype calls identified “FF” (Table Attached).

Table 2.4. Raw genotype data. -1: No Call. 0: Homozygous Wildtype. 1: Heterozygous. 2: Homozygous mutant (Table Attached).

Condition	θ_{11-25}	$\|w_{11}\|$	$\|w_{25}\|$
Original	16.5	0.0013	0.0023
All labels shuffled	90.0	9.42e-5	9.14e-5
Maintain generations, shuffle selection labels	90.0	1.11e-4	8.5e-5

Table 2.5. Effect of shuffling labels on the properties of the discrimination vector w. θ_{11-25} is the direction between the vector **w** discriminating selected vs. unselected populations in generations 11 and 25 and $|\mathbf{w}_{11}|$, $|\mathbf{w}_{25}|$ are the lengths of the discrimination vectors in generation 11 and 25 respectively. The values in the table are averages over 100 imputations of the genotype matrix.

Population dropped	**$\theta_{11\text{-}25}$**	**$\lvert w_{11}\rvert$**	**$\lvert w_{25}\rvert$**
MOR	**13.95**	**0.0015**	**0.0027**
MUL	**8.65**	**0.0025**	**0.0030**
LEW	**33.57**	**0.0013**	**0.0028**
BRI	**15.52**	**0.0014**	**0.0032**
DOB	**40.4**	**0.0016**	**0.0025**
STU	**30.4**	**0.0018**	**0.0031**

Table 2.6. The effect of dropping single populations from the data set on the robustness of the discrimination vector w. $\theta_{11\text{-}25}$ is the direction between the vector **w** discriminating selected vs. unselected populations in generations 11 and 25 and **$\lvert w_{11}\rvert$, $\lvert w_{25}\rvert$** are the lengths of the discrimination vectors in generation 11 and 25 respectively. The values in the table are averages over 100 imputations of the genotype matrix

MOR		MUL		LEW	
E3272	22	E3272	22	D0077	21
D0077	17	D0361	18	E3272	21
E4299	16	E1847	18	E1023	16
E3945	15	D0753	15	E3945	16
E1654	14	E4294	15	E4294	15
D0177	13	D0077	10	C0113	15
E0602	13	E4299	9	D0753	14
D0753	10	D0417	8	E0602	13
A0563	8	A0023	0	E4299	11
D0361	6	A0563	0	A0563	11
A0023	0	C0015	0	E0511	6
E4294	0	E1023	0	D0417	4
C0015	0	D0177	0	A0023	0
E1023	0	E3145	0	C0015	0
E3145	0	E0511	0	D0361	0
E0511	0	E0602	0	D0177	0
E0350	0	E0350	0	E3145	0
D0940	0	D0940	0	E0350	0
C0113	0	C0113	0	D0940	0
E0391	0	E0391	0	E0391	0
E1715	0	E1654	0	E1654	0
E1847	0	E1715	0	E1715	0
D0417	0	E3945	0	E1847	0

BRI		DOB		STU	
D0077	21	D0753	20	E3272	22
E3145	21	E4294	20	D0940	19
E3272	17	D0940	18	E4294	17
E1654	16	E3272	17	D0753	16
D0753	15	E3945	17	E4299	15
D0940	15	E1023	10	D0077	15
D0361	14	C0113	10	E1023	13
D0177	14	A0563	9	E3945	13
E4299	12	E0350	9	D0361	12
C0113	8	E4299	8	E1847	12
E3945	8	C0015	8	C0113	11
E4294	7	D0361	8	E0602	9
E1023	7	E1715	8	A0023	0
A0023	0	E0602	7	A0563	0
A0563	0	A0023	0	C0015	0
C0015	0	D0077	0	D0177	0
E0511	0	D0177	0	E3145	0
E0602	0	E3145	0	E0511	0
E0350	0	E0511	0	E0350	0
E0391	0	E0391	0	E0391	0
E1715	0	E1654	0	E1654	0
E1847	0	E1847	0	E1715	0
D0417	0	D0417	0	D0417	0

Table 2.7. Generation 11. Number of loci with a significantly lower mean than the listed allele, as determined from a 1-way ANOVA within each population.

MOR		MUL		LEW	
E4294	20	E3272	19	E4294	22
D0753	19	E1847	19	E3945	20
E1847	19	A0023	18	D0753	17
E3272	17	E0602	18	D0077	17
D0177	17	E1654	15	E1847	17
E0602	17	E1715	14	E3272	16
E3945	13	E4294	13	E0602	12
E4299	0	E3145	11	E1654	11
A0023	0	C0015	10	E4299	0
D0077	0	D0753	9	A0023	0
A0563	0	E4299	0	A0563	0
C0015	0	D0077	0	C0015	0
D0361	0	A0563	0	D0361	0
E1023	0	D0361	0	E1023	0
E3145	0	E1023	0	D0177	0
E0511	0	D0177	0	E3145	0
E0350	0	E0511	0	E0511	0
D0940	0	E0350	0	E0350	0
C0113	0	D0940	0	D0940	0
E0391	0	C0113	0	C0113	0
E1654	0	E0391	0	E0391	0
E1715	0	D0417	0	E1715	0
D0417	0	E3945	0	D0417	0

BRI		DOB		STU	
E1654	21	D0361	20	E4294	21
D0361	19	E1654	20	E1847	21
D0753	18	E1847	20	D0753	20
E1023	18	D0753	18	E3272	18
A0023	16	E0350	17	D0940	18
E0511	16	A0563	10	E4299	0
C0113	16	D0940	10	A0023	0
D0077	7	A0023	9	D0077	0
E4294	7	E0511	9	A0563	0
E4299	0	E1023	5	C0015	0
A0563	0	E4299	0	D0361	0
C0015	0	D0077	0	E1023	0
E3272	0	E4294	0	D0177	0
D0177	0	C0015	0	E3145	0
E3145	0	E3272	0	E0511	0
E0602	0	D0177	0	E0602	0
E0350	0	E3145	0	E0350	0
D0940	0	E0602	0	C0113	0
E0391	0	C0113	0	E0391	0
E1715	0	E0391	0	E1654	0
E1847	0	E1715	0	E1715	0
D0417	0	D0417	0	D0417	0
E3945	0	E3945	0	E3945	0

Table 2.8. Generation 25. Number of loci with a significantly lower mean than the listed allele, as determined from a 1-way ANOVA within each population

Table 2.9. Co-occurrence of E3272 with each of the other 22 loci. The expected frequencies [P(X), row 1) are calculated as the product of E3272 and a given second allele's frequency. The observed frequency [P(X I E3272) is shown in row 2. A chi-square test was used to identify significant effects (chi-squared value assuming chi-squared distribution with 1 degree of freedom shown in row 3), the p-value based on this chi-square value is shown in row 4. To account for multiple (22) comparisons, Bonferonni correction was used. A difference is significant only if it has a p-value of less than .0023. Significantly "Higher" or "Lower" combinations are shown (Row label "SIGNIFICANT'). (Table Attached)

Chapter 3

Genetic tests for interactions with loci not identified by the SVD/LDA

Introduction

There have been numerous attempts to uncover functional gene networks. (Milo et al. 2002; Alon 2007). Often, understanding the components of the network is of main interest in these studies. Less emphasis has been placed on the interactions between multiple genes in these networks. This can attributed to the complexity of the networks, and also the lack of experimental knowledge of gene networks. For example, the cAMP pathway has been identified as the canonical pathway for learning and memory in *Drosophila,* yet there are on the order of 100 genes not implicated in the cAMP pathway that also have a role in learning and memory. It has been difficult placing these 100 genes into a functional gene network (Davis 2005; Margulies et al. 2005; McGuire et al. 2005).

In an attempt to identify complex gene interactions capable of suppressing the learning and memory mutant *rutabaga*, I used a novel strategy to uncover and model higher order gene interaction in *Drosophila* (Chapter 2). Genotyping, followed by multi-variate analysis comparing selected and unselected populations identified 8 alleles from a collection of 23, which appear to be contributing to the response to selection. The 8 alleles were identified and hypothesized to have an instructive role in the selection response when comparing selected and unselected populations. These 8 alleles were exhaustively crossed in single and di-allele combinations in an attempt to identify interactions capable of suppressing the mutant phenotype of *rut* (Fig 2.6-2.9). One mild suppressor of rut was identified (E3272), yet could not account for the separation

observed in (Fig 2.6), and none of the di-allele combinations had suppressed the mutant phenotype of *rut*.

These results taken together with computer simulations suggest several different possibilities. The first and most likely are that higher order interactions between 3 and 5 of the 8 identified alleles are likely responsible for the selection response, yet to experimentally test all possible combinations of 3-5 alleles would be a monumental task. A second possibility is that de novo mutations are suppressing the mutant phenotype of *rut*. This seems unlikely unlikely to be the case because a response is not observed in controls that do not have variation at 23 loci (Fig 2.4). A third possibility is that additional alleles from the 23 are interacting with the 8 alleles to suppress the mutant phenotype of *rut*. This chapter describes an attempt to identify these alleles.

Selection for learning can have a fitness cost (Mery and Kawecki 2003). Selected populations in my study likely had decreased fitness as a result of increased learning. Alleles may increase fitness by suppressing pleiotropic effects on fecundity, or other traits not being experimentally selected for. This in turn would increase the frequency of these alleles in selected populations. To test if alleles other than the 8 increased in selected populations, multi-variate analysis comparing generations 11 and 25 was performed. My data shows that two alleles, D0753 and E4294, have frequencies that increase over generational time in both the selected and unselected populations. This is consistent with the idea that they may play a permissive role in the response to selection by improving general aspects of fitness that may be needed for improved memory to be manifested. e.g. maybe *rut* has an adverse effect on the health of flies.

These 2 alleles were tested in heterozygous combinations with 5 of the top 8 alleles identified in the study in Chapter 2. A third allele D0361 was also tested, this allele was present in a large number of flies in both selected and unselected populations (Fig 2.5A). The hypothesis being tested is that these 3 alleles are playing a permissive role. These 3 alleles are being positively selected for in selected populations to curb a decrease in fitness associated with an increase in learning. They in turn are interacting with the 8 alleles identified in the study in Chapter 2 in a permissive way to suppress the learning phenotype in *rut* mutants.

Materials and Methods

Singular Value Decomposition and Linear Discrimination Analysis

See Chapter 2 Data analysis (pp 10-11).

Permissive allele crosses

Permissive allele crosses were generated by crossing flies that were both homozygous for rut^1 and homozygous for 1 of 5 alleles (E3272, D0077, E3945, E4299 and D0940) to flies that were homozygous for D0753, E4294 or D0361. All progeny tested were hemizygous for rut^1 and heterozygous for each of the 2 mutant alleles (only males were tested).

Results

Genotyping and multi-variate analysis:

288 animals from each of 6 populations were fully genotyped at 23 loci for generations 11 and 25. Genotyping resulted in a 3456 x 23 matrix containing the P-element dosages: 0 for wild-type homozygous (black, 1 for heterozygous (yellow), 2 for mutant homozygous (red) (Fig 2.5A). Based on these numerical values a Singular Value Decomposition (SVD) was performed to determine if there are alleles that increase in selected populations from generation 11 to generation 25 (data not shown). The rationale in this experiment is that the frequency of permissive alleles will increase in selected populations over the course of the selection. The next step analysis was to take these two clouds of genotype data and perform a Linear Discriminant Analysis (LDA). The LDA takes the mean vector of these two clouds in genotype space and projects it into allele space to identify alleles contributing to the separation of the clouds. A linear discriminant analysis (LDA) was performed on the (SVD) comparing selected populations from generations 11 and 25. 2 alleles (D0753, and E4294) were identified to be contributing to the separation (Fig 3.1). These alleles were further tested to determine if they were contributing to the selection response.

Test of Permissive role for 3 identified loci

The two alleles (D0753, and E4294) identified in the LDA and a 3rd allele, D0361 identified by visual inspection of Fig 2.5A were crossed (see methods above) to animals

that were homozygous mutant for rut^1 and also homozygous mutant for 1 of 5 from the top 8 alleles identified in the (LDA) comparing selected and unselected (Figs 3.2A,B, 2.5C). None of these crosses had performances that were significantly higher than rut^1.

Conclusions

The genotyping of populations from the selection (Fig2.2A) helped to develop a hypothesis that the response to selection seen was a result of interactions between and or amongst the top 8 alleles along with some combination of 3 proposed permissive alleles. The first two alleles D0753 and E4294 were tested because of their identification by the (LDA) (Fig 3.1). Also, a 3rd allele (D0361) was chosen because of its presence in all populations (selected and unselected). This was an attempt to identify higher order interactions contributing to selection, but these experiments did not provide evidence that these 3 alleles act as permissive alleles in di-allele combinations with 5 of the top 8 alleles. This does not however rule out the possibility that other multi locus effects are contributing to the response to selection. This in fact it seems likely (see chapter 4).

Figures and legends

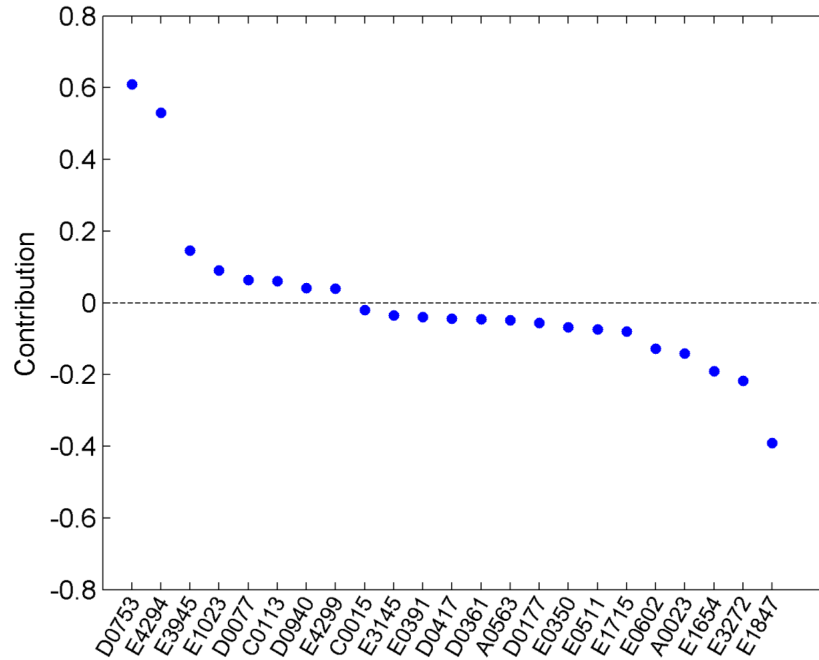

Figure 3.1 Linear Discriminant Analysis (LDA). Plot of the discrimination vector with the alleles (X-axis) sorted by contribution (Y- axis). Mean values of the 100 imputations.

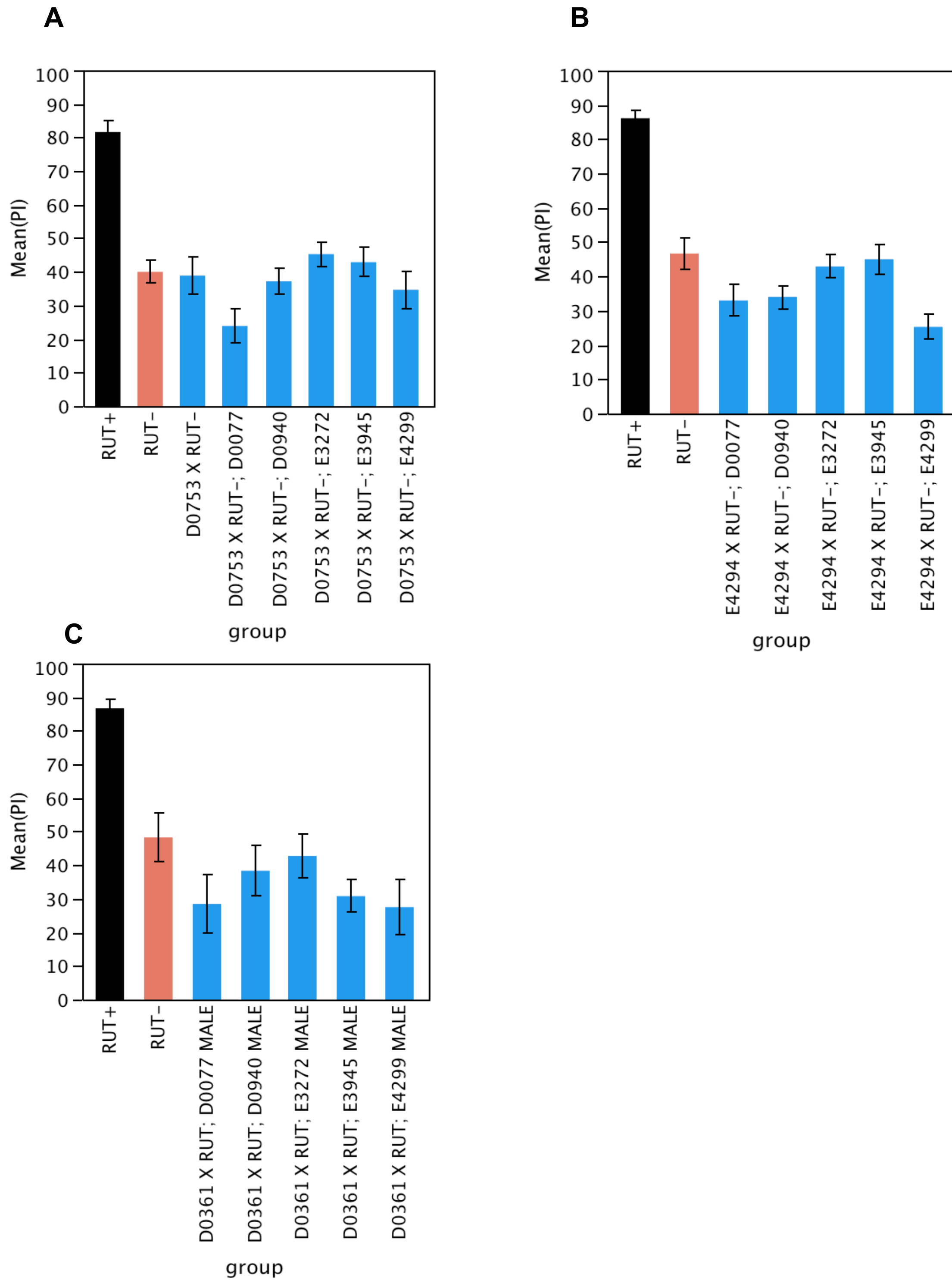

Figure 3.2. Effects on rutabaga (rut) memory performance of permissive alleles crossed to 5 of top 8 single alleles identified in by SVD/LDA. Wild type (rut+) is

shown in (black bars). Effects of each double heterozygote (**A-C**) are shown in (blue bars). rut- mutant performance (red bars) is shown for comparison. In each case, performance of animals that were rut+ and heterozygous for one of the identified hub alleles crossed to rut-; allele(s) was not significantly different from that of rut+ animals (**A-C,** black bars). D0753/+, E4294/+, D0361/+ ; E4299/+, D0940/+, D0077/+, E3945/+ and E3272/+ double heterozygotes have no effect on performance levels relative to rut- (**A**,**B,C**). Performance N of >6 for all groups.

Chapter 4

Conclusions and Perspectives

A fundamental challenge in genetics is identifying genotype to phenotype relationships. In model organisms, this has typically been accomplished using mutagenesis to identify a single locus, then testing the causal relationship between these individual genes and phenotypic traits. Saturation mutagenesis screens make use of this approach to attempt to identify most of the genes whose function is essential to a phenotype. By itself, this approach can reveal a framework for the organizational structure of a biological process, but insight into the underlying molecular and cellular mechanisms usually requires the use interaction screens, fir example, yeast 2 hybrid, or chromosome immuno-precipitation, identifies physically associated proteins. Enhancer suppressor screens can identify second site mutations capable of suppressing or enhancing effects of mutations found in a mutagenesis screen. These approaches are informative for dissecting linear signaling pathways, but components of more complex networks are not easily identified.

Together, molecular and genetic approaches have been informative for dissecting complex biological systems in a few model organisms. This single gene approach also has provided the dominant conceptual basis for attempts to understand gene action in the context of human genetic disease. Linkage studies have successfully discovered common genetic variants underlying many disorders that have a simple Mendelian pattern of inheritance. In the case of complex traits, GWA studies have identified many statistically associated genetic variants, although typically these have relatively small effect sizes and explain only a small fraction of the heritability for a given

disorder. As a result, dissecting the genetic architecture of quantitative traits with complex patterns of inheritance remains challenging, especially for disorders where the population of affected individuals has diverse underlying genetic causes.

In some cases it is thought that the underlying genetic cause will involve single loci with large effect sizes. These cases could be missed by GWA studies either because the causal gene variant is rare or *de-novo* or of a form that is not identified by SNP analyses (e.g. copy number variants). With autism, for example, there is evidence that single locus *de novo* copy number variants may contribute large effect sizes (although many linked genes may be affected). On the other hand, examples where multi-locus epistasis predominates are not readily accessible to these commonly used identification techniques

Although the prevalence of epistasis in both complex traits as well as in human genetic disorders is not yet known, nor is the complexity of the typical gene interaction, several types of studies suggest that higher order epistasis is widespread. In the case of pair-wise epistasis, the evidence is clear both from classical suppressor/enhancer screens as well as from higher-throughput di-allele crossing schemes (Karim et al. 1996; Brem and Kruglyak 2005; Storey et al. 2005). Higher order gene interactions have been experimentally more difficult to study. As a general rule, experimental selection experiments have revealed the existence of multi-locus effects (Tully and Hirsch 1982; Toma et al. 2002; Carlborg et al. 2006; Dierick and Greenspan 2006; Edwards et al. 2006). The vast genetic heterogeneity within the starting outbred populations within most of these studies provides an opportunity to select for higher-order gene interactions, but the contributions of individual loci are not easily identified.

These studies nevertheless have generally supported the view that epistasis is widespread within genetic systems, a view that is parsimonious with evolutionary biologists attempts at modeling the effects of drift, selection and migration on gene networks (Gavrilets and de Jong 1993; Gravner et al. 2007).

The experimental paradigm and data set described in this thesis provide several different levels of insight. First, my data indicate that the gene network governing learning is sufficiently flexible that even a relatively small collection of two alleles at each of 23 loci provides sufficient genetic variation to select for learning in the absence of the canonical *rut* adenylyl cyclase mediated cAMP signaling pathway. This is quite interesting given that each of the alleles that I supplied to the population first were identified in a loss of function screen for reduced memory and learning (Dubnau et al., 2003). This finding in itself speaks to the flexible nature of genetic networks (van Swinderen and Greenspan 2005).

A second novel aspect of this thesis is that E3272 was found to be a mild suppressor of the *rut* adenylyl cyclase. To my knowledge this is the first case of a suppressor of a learning and memory mutant. This finding may establish an entry point to identify *rut* independent learning.

A third novel aspect of this thesis derives from the constrained genetic heterogeneity in the starting populations. This has permitted high-throughput genotyping of multiple animals sampled from each replicate population during the course of selection. This genotype data set identified 8 loci that together contribute to the separation between selected and unselected groups. This feature of this experimental design enabled me to exhaustively test the single and double combinations (di-alleles)

among the 8 loci. The absence of suppression with each of these di-allele combinations strongly argues that the typical selected genotype involves interactions among 3 or more loci.

The landscape of higher order gene interactions underlying complex traits, including disease, have been vastly understudied in mechanistic genetic studies, but have been of fundamental concern to evolutionary biologists and quantitative geneticists for decades. A great deal of theoretical work has dealt with the problem of how fitness can be maintained in a genetically heterogeneous population (Wright 1932; Gravner et al. 2007). This problem is particularly relevant it seems to cases where fit genotypes involve heterozygosity at multiple loci because fit parents have low probability of passing their allele combinations to offspring. This problem is apparent in this data set where it is likely that multiple genotype solutions exist, each of which likely consists of more than 3 loci (mostly heterozygous). These findings appear to be consistent with the notion that high fitness is maintained across many of the possible combinations of a shared core group of alleles (Gavrilets and Gravner 1997; Gravner et al. 2007).

I exhaustively tested all of the single and double-heterozygous combinations among the 8 loci, and also 3 alleles not identified by the SVD/LDA. These 3 alleles appeared to play a permissive role in suppression of *rut* mutant memory. With the exception of E3272, individual loci and simple combinations among them do not explain the suppression of *rut*. Taken together, the findings of this thesis place a lower bound on the complexity of genetic interactions that underlie suppression of the *rut* mutant..

The genotype data also supported the possibility of permissive alleles being necessary for suppression. I failed to find evidence that permissive alleles play a role in suppression, however this cannot be ruled out (Chapter 3).

There are 2 other types of higher order interactions that could possibly be contributing to the response to selection seen in Chapter 2. The first possibility is that there are additional alleles among the 23 that are contributing. This is unlikely given that it is not supported by the raw genotype data or the multi-variate analysis. A second possibility is that *de novo* mutations are contributing to the response. This too is unlikely given that there is no response seen in selected controls. A more viable but also unlikely version of this possibility is that a *de novo* mutation is interacting with a combination of the 23 alleles in selected populations. The reason this is unlikely to be the case is that a response to selection is observed in each of 3 selected populations. This would mean that either the same *de novo* mutation would have to appear in each of the 3 populations, or 3 different new mutations would have to appear, 1 in each population. All of these would have to interact with the 23 alleles in a way that suppresses the mutant phenotype of *rut.* This seems like an improbable set of circumstances to have happened, moreover it would be extremely difficult to identify them if it did.

All of these data combined support the claim that higher-order interactions are likely the cause for the response to selection but such complex interactions are not easily identified because of the huge amount of possible combinations that exist.

In the future, I propose two experiments that would attempt to identify complex genotypes causing suppression of the *rut* mutant phenotype. The first would be a

similar selection as in this thesis, but would constrict the genetic variability even further. One could make 2 strains of flies, each homozygous mutant for *rut,* and homozygous mutant for 4 of the 8 alleles. Therefore, the variability would be restricted to only the 8 alleles that are contributing to the selection response in this thesis. This construction of populations would fully explore the genotype space. Unlike variability at 23 loci, if one had variability at 8 loci there would be 3^8 or (6561) possible genotypes (if you ignore cis/trans phasing). This would dramatically increase the likelihood that genotypes responsible for the selection would be uncovered. Also, unlike the selection in this thesis, a response should be observed much faster.

A second proposed experiment would be to utilize the *Drosophila* toolbox to uncover gene interactions. Balancer chromosomes have been used ubiquitously in *Drosophila* to track mutations. Balancer chromosomes have a series of inversions and rearrangements that prevent recombination. This allows for chromosomes to be maintained and kept in a steady state. The idea would be to take the two individual strains (discussed above). These two strains crossed together will have F1 progeny that are trans-heterozygous for the 8 alleles contributing to the response to selection identified in Chapter 2. These F1's can be subsequently crossed taking the heterogeneous population of F2 and subject them to iterative fractionation in the T-maze as in chapter 2. I would then cross them to balancer strains to "capture" chromosomes for later study. These 'captured' chromosomes can be genotyped, and then further tested to determine the specific effect they have on learning. Individual strains will be crossed to *rut* homozygote animals, these crosses would give rise to strains consisting of male progeny hemi-zygous for *rut* and heterozygous for this

‘captured’ chromosome. The females would be heterozygous for *rut* and therefore learning will be normal. These strains would be tested for learning, only measuring male performance. Presumably if the required genetic architecture necessary for *rut* suppression consists of combinations amongst these 8 alleles, this method would isolate them. These two approaches, although similar have separate advantages and disadvantages, which I will now outline. The first approach has the advantage of mimicking the selection in this thesis, in which a robust response to selection was observed. Another advantage is that the genetic variation will only consist of alleles predicted to be contributing to *rut* suppression. One disadvantage of this approach is the amount of work that a selection entails. This will be decreased as compared to the selection here but remains an arduous task. Another disadvantage is that if there are an intractable amount of solutions to rut mutant suppression consisting of many combinations of the 8 alleles, it will be extremely difficult to test experimentally.

The second approach has the advantage of not having to select for multiple generations. Also, this method would identify individual flies that have the genotypic requirement for *rut* suppression. Unlike a selection where alleles necessary for suppression are inferred from genotype frequencies, this method would be able to identify actual individual genotypes. The disadvantages to this approach are the same as the 1st experiment, and also a disadvantage to any approach looking for these types of interactions. If there are a few solutions necessary for suppression they will be easily identified, however if there are many solutions it will be impossible to identify all of them.

These two approaches combined should sample the entire genotype space necessary for *rut* suppression. If higher order interactions are identified, the next

logical course of study would be to perform a more detailed network analysis. For example, if 5 higher order interactions from amongst the 8 alleles were identified that can suppress *rut* mutant learning, these 5 interactions can be placed into a network. Further analysis of the alleles and how they interact genetically and bio-chemically will give insight into the mechanisms of suppression. An understanding of the flexibility of gene networks will increase our understanding of the inheritance of complex traits, and with hope can help to understand the inheritance of genetic disorders.

In the next few paragraphs I will discuss each of the hypotheses that could explain the observed response to selection. Categorically, there are three possibilities, hypotheses that have been completely ruled out, hypotheses that cannot be ruled out, but are unlikely, and finally a hypothesis that I view as the most likely explanation for the observed response.

Hypotheses that can be ruled out are single locus suppressors with a large effect, pair-wise interactions amongst the top eight alleles, a single "magic" genotype, and also combinations of greater than 5 interacting loci. With the exception of E3272 (which has a minor effect) there are no single loci that can account for the striking difference in performance between the selected and unselected populations. I also can rule out pair-wise combinations among the top 8 alleles identified by the SVD/LDA, because I tested all 28 possible pair-wise combinations behaviorally. None of these pairwise combinations suppressed the mutant effect of *rutabaga*, but 9 of the 28 enhanced the phenotype (worse learning). I can also rule out that the idea that there is a single genotype that has a large effect, because Looking I genotyped 288 animals per population at generations 11 and 25. No single genotype is present in all selected

populations, and no one genotype is detected in more than a few animals within a given population. I also modeled the contribution of suppressing genotype to the mean of the populations as a function of its frequency and effect size, and determined that between 55 and 100% of animals would need to contain a suppressing genotype (Fig 2.13). The simulations shown in (Fig 2.15) demonstrate that the chances of identifying interactions among greater than 5 loci is exceedingly low. Combinations of greater than 5 alleles are never observed in the simulations, because of genetic drift.

There are several types of explanations that I view as unlikely although I cannot formally rule them out. First, epigenetic trans-generational effect is a mechanism that has rarely been demonstrated, and to my knowledge has never been shown for learning and memory. It would nonetheless have to also act in concert with the 23 loci in our populations. The second unlikely explanation that cannot be formally ruled out is *de novo* mutations. In *Drosophila,* these mutations occur at a rate of 8.0×10^{-6} per locus per generation, and over the course of 41 generations it is unlikely that *de novo* mutations occurred in all three populations. And like epigenetic trans-generational effects would need to act in concert with the 23 loci.

The final hypothesis that I view as unlikely but cannot rule out is a pure additive effects model. I tested the additive effects of all pair-wise combinations among the top identified 8 loci, and also the 3-way effect of the top 3. None of these combinations showed suppression (Fig 2.9). Also, combinations of up to 5 loci, including both top loci identified by SVD/LDA as well as loci not identified by SVD/LDA (Fig 3.2).

In the literature there are cases where pure additive effects have been simulated, specifically in (Barton and Keightley 2002). In this review, an additive effects simulation

modeled selection on 10 unlinked large effect loci (effect size = 0.5 of a standard deviation of the behavioral trait). They also varied the starting frequencies of the alleles, from 0.01 to > 0.5. For comparison, the starting frequency of each locus in this thesis was 0.14 (see methods of Chapter 2). Barton and Keightley (2002) found that even when a large effect locus is present at a frequency of just .01 it reaches fixation in less than 20 generations. In the selection experiment reported in this thesis, 0.5 of the standard phenotypic deviation is approximately 5 PI points, and the observed response to selection is approximately 25 points. Thus if the observed response were driven by purely additive effects divided equally amongst 5 alleles, these alleles would have reached fixation in less than 20 generations. This is not what we found in our high-throughput genotyping. Even at generation 25 there are no alleles that have reached fixation in any of the three selected lines. Taken together with the experimental tests that failed to detect additive effects among pair-wise combinations and even some three-way combinations, this argues strongly that a pure additive model is unlikely to explain the observed selection response. However, simulations more closely modeling the population sizes and starting frequencies of my populations could be done to cement this conclusion.

The experimental findings of this thesis together with the outcome of the simulations shown in Chapter 2 as well as the simulations shown in (Barton and Keightley 2002) support the hypothesis that the typical genetic solution involves combinatorial and probably non-additive interactions among between 3 and 5 loci. The observed frequency distribution of genotypes and modeling of the impact on population

mean of individual suppressing genotypes further supports the conclusion that multiple such genetic solutions underlie the observed response to selection.

Single locus suppressors with large effect	**Ruled out**. With the exception of E3272 (which has a minor effect), no other single allele has a large suppressive effect.
Pair-wise combinations among top identified alleles	**Ruled out.** All 28 pair-wise combinations amongst the top 8 alleles were tested behaviorally and none had a suppressive effect.
Additive effects	Additive effects cannot be ruled out, but appear unlikely because the combination of the top 3 loci has no effect.
One genotype with large effect	**Ruled out.** No single genotype is abundant in any population.
Complex genotypes among > 5 loci	**Ruled out.** Combinations of loci greater than 5 are never identified in simulations.
Epigenetic Trans-Generational effect	Cannot be ruled out, but such a mechanism has rarely been demonstrated and would nonetheless need to act in concert with the 23 loci.
de novo mutations	Cannot be ruled out, but unlikely because the mutation rate in *drosophila* is estimated to be just 8.0×10^{-6} per locus per generation, and would also need to act in concert with the 23 loci.
Multiple genetic solutions, each involving 3-5 loci	**The most likely explanation for the observed response to selection.**

Table 4.1 Hypotheses that could explain observed suppression

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