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# Gene Expression and Phenotype Response of *Drosophila melanogaster* to Selection.

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ABSTRACT

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The evolution of phenotypic plasticity is currently a topic of paramount interest in a diverse field of sub-disciplines. Salience is placed by all fields in describing the interaction of selection and phenotypic plasticity and the consequence of this interaction more broadly on evolution. Lacking in the discussion is substantial empirical description of genotype/phenotype interactions that by definition constitute the plastic response to novel and stressful environments. Here, I present empirical observations that bring the interaction of genotype and phenotype into focus. *Drosophila melanogaster* populations subjected to selection for tolerance to low food or high alcohol conditions each exhibited an enhancement of adaptive plasticity consistent with predictions associated broadly with the Baldwin Effect. Furthermore, each appears to have followed different courses of regulatory modification to achieve these ends. Broadly implicit in the results is the observation that previous exposure of the population to the conditions of induction may dictate the course of subsequent evolution of the phenotype.

CONTENTS

LIST OF TABLES

LIST OF FIGURES

# CHAPTER 1

## INTRODUCTION

It has been recently observed that there remains outstanding disjunction that exists between evolutionary biologists, ecologists, and quantitative biologists (1). To some degree this has come about because many of the issues addressed by each field separately are not known, are downplayed, or have been ignored since the time of the Modern Synthesis. It has since been found that one mechanism in particular, regulation of gene expression, can account for much of the phenotypic variation visible to selection (2, 3).

Regulation of gene expression is implicit in the expression of phenotypic plasticity. Phenotypic plasticity is defined as the ability of a genotype to express multiple phenotypes. Though it is now largely recognized as being of significant evolutionary consequence, theoretical debate occurs in the absence of empirical resolution. For instance, many authors define phenotypic plasticity as adaptive; although there is scant evidence plasticity is necessarily adaptive. Further confusing the debate are a plurality of quantitative genetic models that are based on assumptions of causal mechanisms that largely go untested in nature (4).

Another mechanism of non-Mendelian heritable variation that has arisen since the Modern Synthesis is epigenetic inheritance. This phenomenon was first described by C.H. Waddington. Waddington inferred that plastic characters induced and subsequently assimilated could, in response to either drift or selection, be canalized and fixed within populations (5-8). However, absent genetic assimilation his experiments could not explain how plastic characters could otherwise arise independently in nature to expand, rather than canalize, plasticity. Furthermore, the vein-less phenotype was obviously not adaptive and required positive selection in order to drive it to high frequencies within an experimental population. The former observation did not then and still does not today have the benefit of much empirical support. The later observation meant canalization failed the ‘adaptability’ criterion established by later authors as the trait in question persisted even in the absence of induction.

Resolving this difficulty to some degree, subsequent authors have speculated that canalization can be reconciled with an expansion of plasticity via the retention of 'hidden' sources of variation (9). Under this scenario the stress response to induction that is most advantageous can become rapidly fixed within a population. In this model, absent induction the basal profile of gene expression goes unchanged; essentially masking the new phenotypic variant that is now propagated within the population by genetic drift. As stated, this stress response upon fixation constitutes a 'phenotypic memory' of the environment of induction. Authors go on to argue that the only way to observe these 'hidden' sources of variation is to stimulate this phenotypic memory by subjecting the population to the same stressors that favored them initially.

These later observations are very much consistent with the often cited, but more often unread, conceptual reasoning of J. Mark Baldwin (10). Ontogenically oriented, he observed that as a child learns to write through repeated attempts, evolution could favor the child more willing to perfect the ability to write through replication. Baldwin also observed that the capacity for variability in a character was likely an early feature of divergence. This observation has since been rearticulated to state 'all phenotypes were primitively plastic' (11).

This observation is strongly supported by the wealth of empirical evidence where broadly the trends appear to be associated with a linearity of change, a simple change in regulation rather than an expansion of net plasticity, or a divergence associated with the plastic response to different environments of induction (12-29). Given these observations and casting them in the proper context of Baldwin's original observations, the most likely manifestation of the Baldwin Effect is not necessarily a dichotomous alternative to canalization. To state it most simply would be to observe that plasticity, under selection, will exhibit increased regulatory specialization and the realignment of this regulation will resist a broad signature of canalization. Recognizing this possibility, others have since observed that the evolution of phenotypic plasticity is most characterized by the emergence of specialized gene regulation in the place of a formerly generalized response to induction (30). Thus, even where no increase in net plasticity is observed one can argue a Baldwin Effect has been observed if canalization for one phenotype participating

in the response to induction is matched by an increase in plasticity in another likewise participating phenotype.

Assuming plasticity is basal to all other phenotypes, we can further define the evolution of adaptive plasticity as an acquired specific response of gene expression to some condition of induction that displaces a formerly unregulated or non-specific gene expression response to the same inductive conditions. Given this, we can further deduce that selection's most likely contribution to the evolution of adaptive plasticity would be to enhance existing regulation or introduce novel regulation of gene expression that optimizes the organism's response to an inducing environment. In short, we can predict that the first response of plasticity to selection on the level of gene expression would be one of no net change in expression variability but new adaptive plasticity will still emerge via the acquisition of a more specific response to induction. Alternatively, we can predict that where previous regulation of gene expression exists for genes participating in the response to induction selection will, under novel and stressful conditions, inevitably lead to an increase in specificity of those responses through the assimilation of novel gene regulation.

Another possibility somewhat related to the evolution of adaptive plasticity is whether or not net plasticity expands even as existing gene regulation is enhanced or assimilated through selection. This is what is often argued by proponents of the Baldwin Effect and several have proposed this may be accomplished via epigenetic inheritance (2). Mechanistically, it stands to reason that this could indeed be quite possible as a great deal of variable gene regulation is attributable to differences within untranslated regions (14, 15). These regions often contain many transposable elements, repeats, and miiRNAs that have remained under the radar for most evolutionary biologists as they have been relatively inaccessible in unsequenced genomes. However, it has been found that of all elements inherent to a genome, transposable elements are the most lineage-specific of eukaryotes, propagate quickly within species, and exhibit disproportional effects on gene regulation (16).

Should induction and selection influence the propagation or activity of transposable elements or miiRNA it may be there is relatively little cost associated with either cumulative adaptive phenotypic plasticity or a corresponding increase in net

plasticity. This notion is a particularly attractive idea as it liberates existing genotypes from bearing the cost associated with either canalization or expansion in net phenotypic plasticity but still facilitates variably regulated responses that also correspond to increase in net plasticity. It is also attractive as most of the effects of such regulatory, untranslated regions would remain as 'hidden' variation only to be observed when and if the inducing medium most affecting the regulatory activity of these regions is introduced. Hence, there is a mechanistic reconciliation for the second possible manifestation of the Baldwin Effect. Contrasted with the former possibility of no change in net plasticity even while adaptive plasticity is acquired, this can simply be restated as 'a growth in net plasticity, corresponding with the acquisition of new regulation of gene expression'.

The possibility one observes at this juncture is that selection for a phenotypic extreme, the response regulated by 'hidden' variation in untranslated regions and mostly expressed as new regulation upon induction will firstly result in a reduction of variability associated with the gene expression response to induction, broadly. Also, one observes that the inferred expansion of regulatory potential implied by a reduction in variable gene expression will either force a redistribution of net phenotypic plasticity or its expansion. Finally, it is only logical to conclude that if either is the case, a broad signature of the Baldwin Effect has been observed and we can reject canalization as the primary vehicle behind the evolution of adaptive plasticity or even the broader accumulation of net plasticity on a genome-wide scale.

Does selection for a phenotypic extreme indeed reduce variability in gene expression and increase the specificity of the response to induction? Secondly, do changes increasing the specificity of response to one inducing medium cascade via plieotropy to a reduction in specificity among other loci, broadly? Thirdly, if either or both of the former are the case, would it be logical to continue to characterizing canalization as a dichotomous alternative to the evolution of adaptive plasticity? Quite simply, it could be inferred such an observation is nothing more than that of one portion of the genome becoming less specifically regulated as compensation for another region that has simultaneously enhanced or expanded the specificity of gene regulation in response to selection.

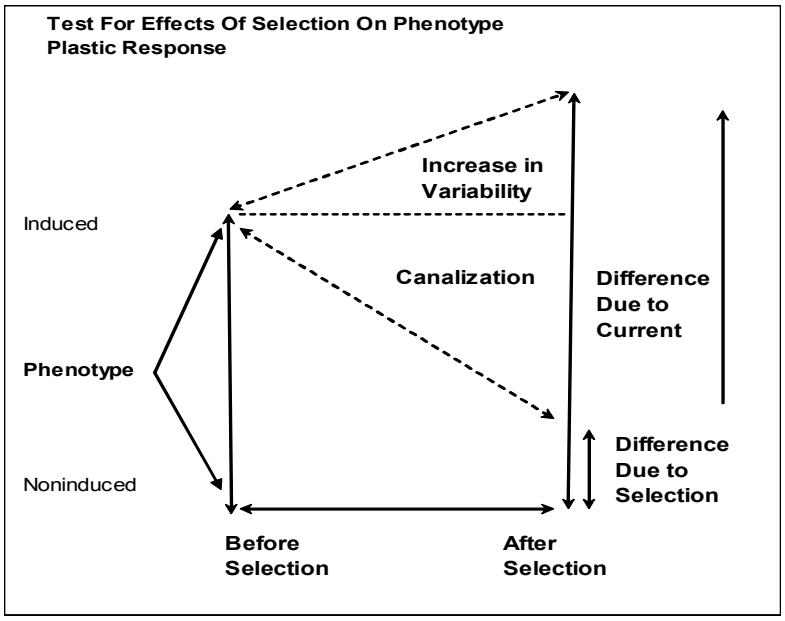


Figure 1 Test for effects of selection on phenotype plastic response- Baldwin Effect is defined as less similarity between inducing and noninducing environments after selection. Waddington Effect (canalization) is defined as more similarity between inducing and non-inducing environments after selection.

The most direct test of whether or not adaptive plasticity has been newly regulated or enhanced is in comparing correlation in expression between environments of induction, before and after selection (Fig. 1). Strictly speaking, if the Baldwin Effect is observed, there should be less similarity between selected lines before and after induction than there is within nonselected controls before and after induction. Otherwise the phenotype (the ability to deviate from a basal rate of gene expression to a plastic response) has been canalized.

If a Baldwin Effect is indicated, it is useful to further characterize the fingerprint of its effect on overall gene expression. If the existing plastic response has been modified or substituted it should be evident in overall gene expression. One such characterization can be performed by establishing a measure of difference in overall gene expression between inducing and noninducing environments. For instance, dividing noninduced gene expression by induced gene expression within selected lines and controls and then analyzing their variance or mean as ‘before selection’ and ‘after selection’, should expose whether or not there has been a significant change in the overall response to induction. A

significant difference before and after selection could indicate there has been an increase in net plasticity. A nonsignificant response indicates there has not been an increase in net plasticity. It is important to note neither result makes any conclusion of whether or not adaptive plasticity has increased. But when this is compared to observations of whether or not a Baldwin Effect has occurred, it should indicate whether or not the response is weighted towards the acquisition of new regulation for the existing plastic response or the substitution of the former plastic response by a new one.

Another way to characterize the acquisition of adaptive plasticity via its fingerprint in over all gene expression is to ask whether or not selected or current environments more strongly predict overall gene expression. Where a Baldwin Effect has been observed and current environment, only, predicts overall gene expression, it can be inferred selection has favored new regulation of the existing plastic response. Where selection, only, predicts overall gene expression, it can be inferred that selection has favored the emergence of a new plastic response. Where the interaction of selection and current is significant, it can be inferred that the acquisition of adaptive plasticity via new regulation has emerged by the loss, gain, or substitution of existing regulation within the existing plastic response by another.

It is also useful to compare the residuals of control noninduced/induced gene expression to selected noninduced/induced gene expression regressions. If the same genes are indeed driving the plastic response to induction after selection as before, there should be a high R-squared value and the data strongly linear. If not, it should be relatively low and the data upon regression will be relatively clumped. This test does not resolve whether or not the disparity is due to new regulation of the existing plastic response or the emergence of a novel assemblage, but it does, when compared with all former data, increase our understanding of specifically how plasticity responds to selection.

Combined, the results of the data should reveal not only whether or not selection has resulted in the acquisition of new adaptive plasticity via variable gene regulation, but should also distinguish whether or not adaptive plasticity takes on the form of a generalized response of change in overall gene expression or a specific response of new gene regulation at induction-specific sites.

Finally, while up until now the ability to deviate from a basal gene expression point has been defined as a phenotype, it is useful to also measure phenotypes that are known to be frequently visible to selection. Such phenotypes should include the measurement of fitness parameters, as they would reveal whether or not the variable regulation is indeed adaptive on a macroscopic scale. Two such variables are egg-to-pupae survival and adult persistence under acute conditions.

In either or both, a progressive increase in survivorship would be an *a priori* test for adaptive evolution. However, the evidence of an increase in plasticity would by definition coincide with the achievement of comparable survivorship in both mediums of induction and non-induction with performance exceeding that of controls on the inducing medium.

# CHAPTER 2

## METHODS

To test whether or not Baldwin expansion of phenotypic plasticity (via the acquisition of new adaptive plasticity) does in fact occur in response to selection, a selection regime was established using 780 wild-caught *Drosophila melanogaster*. Individuals were collected at the Countryside Winery, Blountville, Tennessee. After three generations of laboratory normalization, mated adult females and males were transferred in succession from one treatment medium to the next so that all flies of subsequent generations would share common ancestry. Furthermore, wild flies were chosen over well established laboratory stocks on the grounds that 'hidden' variation, visible only when populations are under stress, would be the target of selection for a phenotypic extreme. Many laboratory lines that have routinely been established from inbred lines notoriously contain less such variation.

The selection regimes were identified from conditions the populations confronted in nature and phenotypes associated with selection in the native environment were identified based on well published responses of *Drosophila* to similar selection pressures exerted in laboratory settings. Among these, exposure to high concentrations of ethanol in the food medium and starvation resistance were ultimately selected. The high alcohol regime consisted of 12% ethanol saturated in the food medium and for starvation resistance low food mediums were prepared with 66% reductions in yeast and sugar. A nonselected regime was also established as a control and consisted of flies on typical laboratory food medium otherwise identical to that used for selected lines minus the additions of alcohol and reductions of nutritional components.

Each selection group and the control line was established in three replicates per regime for a total of nine replicates (three for high alcohol, three for low food, and three for normal food medium). Within each replicate of high alcohol and control lines four bottles were used in which 30 mated females and 30 males were placed. For low food selection it was decided to mimic the annual conditions confronted by the native population in which the annual harvest not only reduced the food available to the

population but created conditions of temporary overcrowding. So, in each of these replicates only two bottles were used and within each were placed 60 mated females and 60 males to simulate the effects of overcrowding on low nutrient substrates. In all lines adult flies were allowed to lay eggs on the food medium for a period of 24 hours and then removed. This has to this point been repeated for 25 generations.

As it has been previously observed, only under natural conditions would some measure of 'hidden' variation (only visible to selection while under stress) be maintained via temporal or spatial heterogeneity in inducing environments. Because of this it was assumed that any advantageous variation upon which later selection would act will have already existed within the populations in question for some time. Additionally, because it has been observed that plasticity is only advantageous insofar as it arrives in the form of a phenotypic variant that could otherwise only be accessed through mutation, these populations would be more likely to have already arrived at mutation/selection balance for the traits in question under natural conditions. Thus, it was predicted that simply increasing the intensity and frequency of former selective bouts to the point they became a new selective norm would lead to an upsetting of this equilibrium and in such a manner any subsequent emergence of adaptive plasticity would be both rapid and visible with an array-based approach to gene expression.

But the true test of adaptive plasticity, as has been also previously observed, is whether or not newly acquired or enhanced gene regulation translates into phenotypes that are visible to selection. To these ends fitness measures indicative of adaptive evolution were identified as the basis upon which such an assessment would be made. Specifically, egg-to-pupae survival under inducing and noninducing environments and adult survival under acute conditions were tested. If Baldwin assumptions were to prove the case, it was predicted that evolution in these 'hidden' traits would not result in a reduction of survivorship relative to noninducing conditions but would increase fitness in the inducing environment.

Correspondingly, relative RNA abundance (a bridge product between transcription and translation) was used as the measure of gene expression in this experiment. RNA is advantageous in that it effectively couples the phenotype with the genotype, but as has been also been observed, may represent products that are transcribed

for regulatory purposes but otherwise go untranslated. If Baldwin effects were to be observed, it was predicted that gene expression (in the form of transcriptional products) would, after selection, exhibit less correlation between induction and non-induction as the emergence of new regulation governing the expression from one relative to the other could be inferred. If Waddington effects were to prove the case, a stronger correlation between expression in inducing and non-inducing environments would exist after selection.

## Phenotypes

To measure egg-to-pupae survival five mated adult females were extracted from the general population of their respective selection lines and control groups and were placed on food medium in standard vials. These females were then allowed to lay eggs until target egg densities were reached. For egg-to-pupae survival in low food medium the target concentration was approximately 200 eggs per vial. For egg-to-pupae survival in high alcohol and standard mediums the target concentration was approximately 100 eggs per vial. Food mediums used in egg-to-pupae survival were identical to those used in the selection regimes and control lines, respectively.

Once females were removed from the food medium, eggs were counted by dissecting microscope in each labeled vial and the number of eggs noted. Ten days after the vials were established the pupae were counted and a percentage of eggs introduced versus pupae that emerged calculated. Comparisons of the data were conducted by nested ANOVA in JMP 5.1 ®.

The second phenotype selected for analysis was that of adult mortality on acute substrates. Under a Baldwin scenario adult survivorship should improve in response to exposure to the inducing environment as larvae. Under a Waddington scenario exposure as larvae to the inducing environment should not be as significant a predictor as being a member of a particular selection regime with regards to the time it takes for 50% of the population within a vial to expire.

Survivorship was assessed by placing adults, segregated by sex, on mediums consisting of extremes of regimes already the subject of selection. To test acute survivorship for low food selection, flies from controls and low-food selected lines were

segregated by sex in empty agar mediums and scored for mortality from the beginning of the introduction until the natural death of the last fly in the vial. To measure acute survivorship in high alcohol flies from high alcohol selected lines and controls were segregated by sex placed on food mediums in standard vials that had been saturated with 30% ethanol and mortality scored from the time of introduction until the natural death of the last fly in the vial. The data were then transformed into mortality curves per vial and then analyzed with nonparametric tests between replicates, lines, maternal condition (normalized or not), sex, and generation in JMP 5.1®.

Both acute survival and egg-to-pupae survivorship measures were taken after the first generation had gone through the selection regime. Because all offspring in generation one were descended from the same collection of females raised under normal conditions no normalization on high food medium prior to the measure was necessary to control for maternal effects. During subsequent trials mated adult females were extracted from the general selection regimes two generations prior to the testing and normalized on standard food mediums to assure phenotype variants being observed would be the products of inheritance rather than maternal stress. Analysis of both acute and egg-to-pupae survival across generations was additionally conducted by trend analysis using Minitab 15 ®, and two-way ANOVA.

## Gene Expression

RNA was extracted from larvae in their first generation of introduction to the selection regime and from larvae collected in generation eight. In generation one RNA comparisons were made between replicates of larvae in alcohol versus replicates of larvae in standard food medium and larvae in low food conditions versus larvae in standard medium. In generation eight the RNA was extracted from each replicate from the same regimes. Additionally, RNA was extracted from selected lines normalized on standard food medium to asses whether or not heritable changes in gene expression were present (as opposed to changes in expression induced by a current environment). RNA was extracted as per the protocols published by Canadian Drosophila Microarray Centre (31).

Eighty larvae were extracted from the food medium and placed in 2mL tubes. These were then snap-frozen with liquid nitrogen. Next, they were homogenized with

Homogenizer and treated with TRIzol and incubated for 5 minutes. After 5 minutes 200 uL of Chloroform was added to each sample and incubated for 3 minutes and subsequently centrifuged for another 15 minutes. Afterwards, the aqueous phase was transferred and RNA precipitated with Isoprobanol for 10 minutes and then centrifuged for another 10 minutes. RNA pellets were then washed in 75% ethanol for 5 minutes twice. RNA pellets were then air-dried and resuspended in Nuclease-free water for 10 minutes and results measured with light by light spectrometry to confirm yields of RNA between 10-100ug. Samples were then shipped to Canadian *Drosophila* Microarray Centre located at the University of Toronto at Mississauga. Once there, they were stored at -70$^0$C until amplification. The following is a summary of the protocol provided by C*D*MC for how that amplification and subsequent hybridization were performed.

Amplification of 14,300 genes per sample provided was performed by using the RNA shipped to the centre as a starting material. Reverse transcription was conducted at 42$^0$C for 2 hours followed by second strand synthesis at 16$^0$C for 2 hours. This was followed by in vitro transcription at 37$^0$C for 4 hours with a reaction mix of 16 uL of dsDNA (obtained from second strand synthesis), 4 uL of T7 ATP soln (75mM), 4uL of T7 CTP soln (75mM), 4uL of T7 GTP soln (75mM), 2uL of T7 UTP soln (75mM), 3uL of AA-UTP (50mM), 4uL of T7 10x Reaction buffer, and 4uL of T7 enzyme mix.

Next amino allyl-aRNA was purified with the MessageAmpII kit using 80% EtOH for a wash buffer. This was then eluted with 100uL nuclease free water and 10uL of 3M NaOAc, 1uL glycogen (20ug/uL), and 120 uL of isopropanol were added and allowed to precipitate at -20$^0$C for at least 75 minutes.

Samples were then centrifuged for 30 minutes until a pellet formed. This was washed with 200uL of 75% EtOH and centrifuged for an additional 5 minutes. EtOH was then pipetted from the tube and allowed to air dry for 5 minutes. The probe was then resuspended in 5uL of water.

Dye conjugation was performed by adding 3uL of .3M $NaHCO_3$ to the resuspended amino allyl-cDNA produced from our samples. 2uL of reactive dye (Alexa647 or Alexa555) were added to each sample and allowed to incubate at room temperature and in the dark for 1 hour. Afterwards, 900uL of dd$H_20$ were added to the conjugated cDNA and purified as before using a column purification kit.

The sample was then washed with 80% EtOH three times and eluted with 3x50ul of water. Samples where then quantified by light spectrometry to determine RNA concentration and equal amounts of each (between 5 and 20ug) were placed into new tubes. Nuclease free water was then added to bring the total volume in each tube to 100uL.

Probe clean up and precipitations were then performed. Samples labeled with Alexa647 and Alexa555 were then combined and 20M NaOAc, 13uL glycogen (20ug/uL), and 340uL isopropanol added. This was then allowed to precipitate at -20$^0$C for at least 30 minutes. Next, this was centrifuged for 30 minutes at top speed. Once a pellet formed it was washed with 200uL of 75% EtOH again and spun for another 5 minutes. All EtOH was then pipetted from the tube and the pellet allowed to air dry for 1 minute before being resuspended in 5uL of water.

Microarray hybridization was next carried out beginning with an addition of 80uL of hybridization buffer to each resuspended probe. The mixture was allowed to incubate at 65$^0$C for 10 minutes. Probes were then placed in the array and the array was placed in a sealed chamber containing a 37$^0$C water bath for 16-18 hours.

The array was then washed 3 times for 15 minutes each in pre-warmed 1xSSC and 0.1%SDS. The array was then washed at room temperature with 1xSSC for an additional minute. Afterwards, the array was scanned and reports generated in QuantArray and GenePix format ®. One data file and two tiff images generated from the hybridization were then returned to our laboratory for analysis.

Data were separated by channel, replicate, and treatment and control genes purged from the dataset. In total 14,300 *Drosophila melanogaster* specific genes were present in the samples analyzed. Total Alexa647 florescence data were normalized via global normalization, log-transformed, and results compared using linear regression, ANOVA, Runs Test, and Pearson's Correlation.

Though gene expression data were collected in both generations one and eight, here analysis will only be of results obtained from a common garden comparison of gene expression between flies from generation eight in inducing and noninducing environments and treated relative to membership in selected or nonselected lines, respectively. Because the question is effectively a question of whether or not selection

has increased regulation and, by inference, plasticity the most direct test for this is to compare gene expression relative to induction between selected and nonselected lines.

# CHAPTER 3

# RESULTS

## Phenotypes

### Egg to Pupae Survival after Selection in Alcohol

Initially, flies exposed to high concentrations of ethanol in the food medium showed increasing fitness consistent with adaptive evolution, but not diagnostic for purposes of ascertaining whether or not plasticity is implicated (Figures 2 &3). However, in by generation 21 alcohol selected lines were outperforming controls (Figure 4) and yet demonstrated a decreased disparity in fitness between both inducing and noninducing environments to a point the difference was no longer statistically different (p=0.288). Over all generations, maternal condition, current environment, and membership in a selected line all independently predicted egg-to-pupae survivorship among alcohol selected lines relative to controls (Table 1).

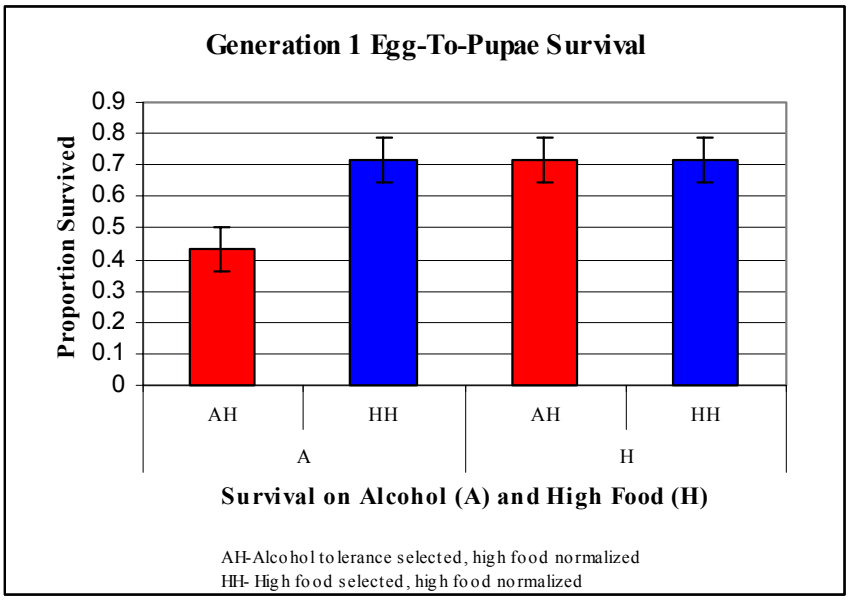


Figure 2 Generation 1: Egg to pupae survival- Alcohol selected lines after one generation of selection perform worse than controls

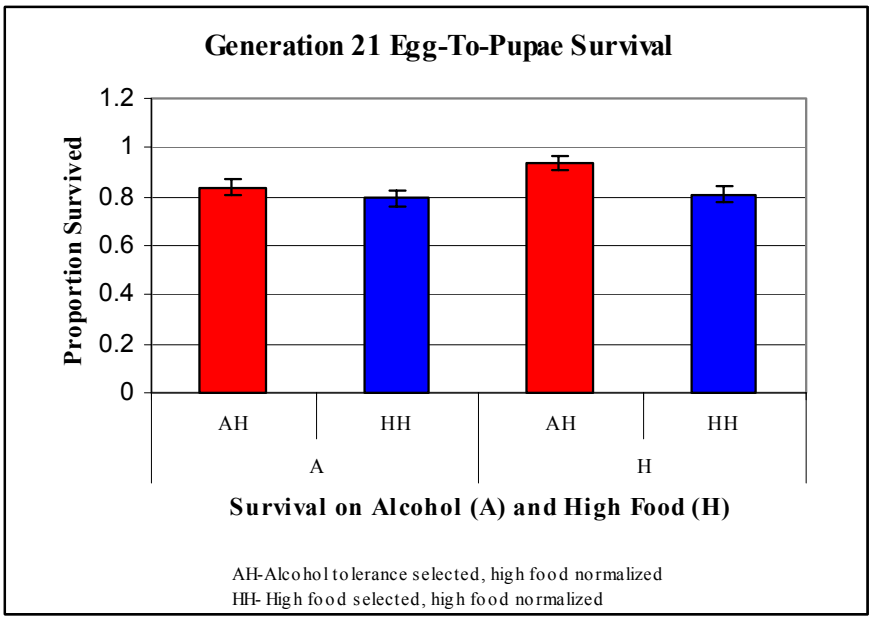


Figure 3 Generation 9: Egg to pupae survival - After eight generations of selection, alcohol treated lines have increased fitness in inducing environment relative to controls. More significantly they remained less likely to survive in the environment of selection (p=0.0213).

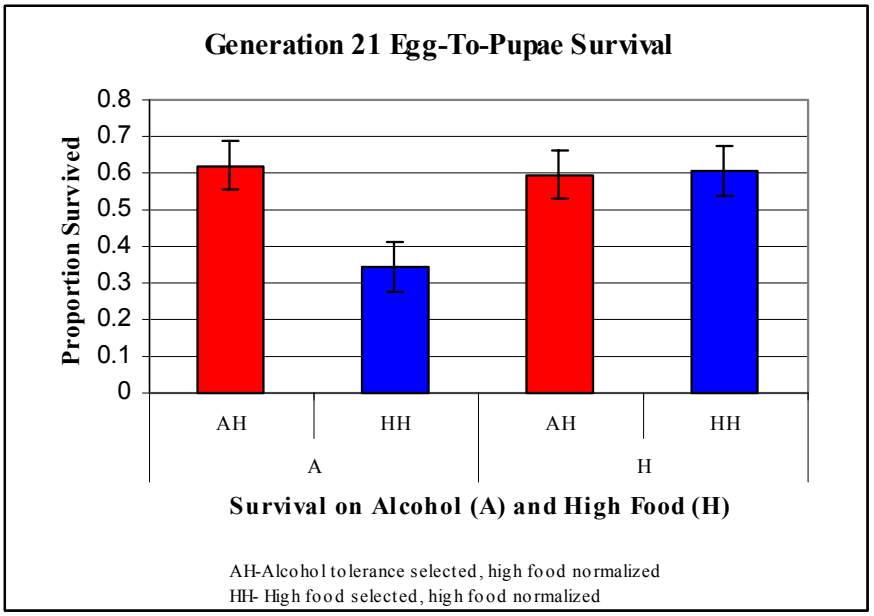


Figure 4 Generation 21: Egg to pupae survival - After 21 generations of selection, alcohol treated lines have increased fitness in inducing environment relative to controls and decreased the difference in fitness between them to a point of statistical insignificance (p=0.288).

| Source | F | *p* |
|---|---|---|
| **Maternal** | 28.37 | <0.0001 |
| **Selection** | 17.19 | <0.0001 |
| **Current** | 25.70 | <0.0001 |

Table 1 Egg-to-pupae survival: High v. Alcohol- Across generations, egg-to-pupae survivorship is predicted by maternal condition, current environment, and membership in a selection regime.

Egg to Pupae Survival after Selection in Low Food

Low food treated lines, by contrast, exhibited a different trend. Also represented in Figures 5-7, it is obvious that selection via a reduction in nutrition did not translate into either increased egg-to-pupae survival relative to controls or themselves after 21 generations of selection. Over all generations, the only consistent predictor of low food survivorship is maternal condition (Table 2).

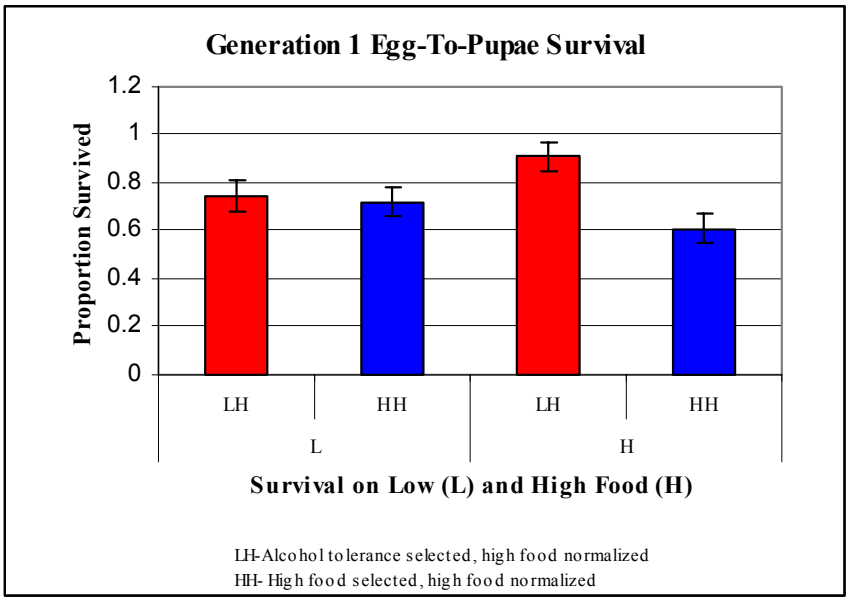


Figure 5 Generation 1 Egg to pupae Survival (Low food)

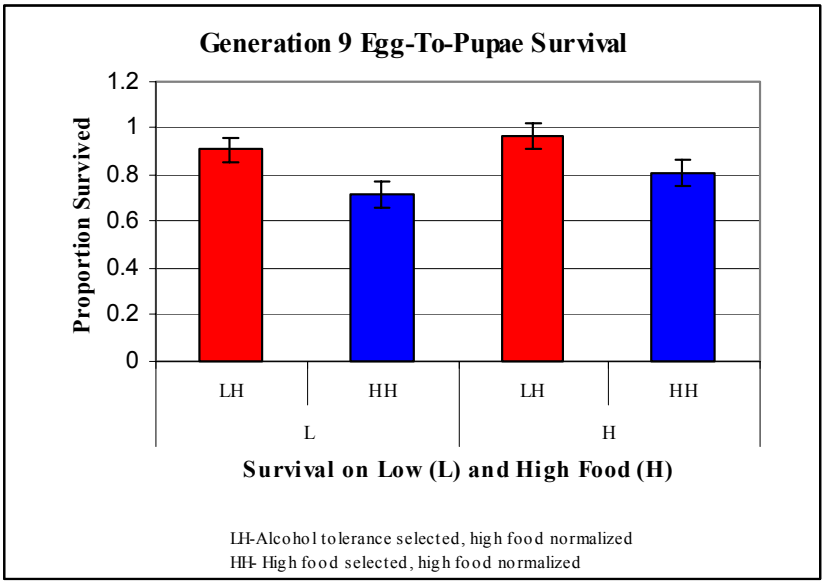


Figure 6 Generation 9 Egg to pupae Survival (Low food)

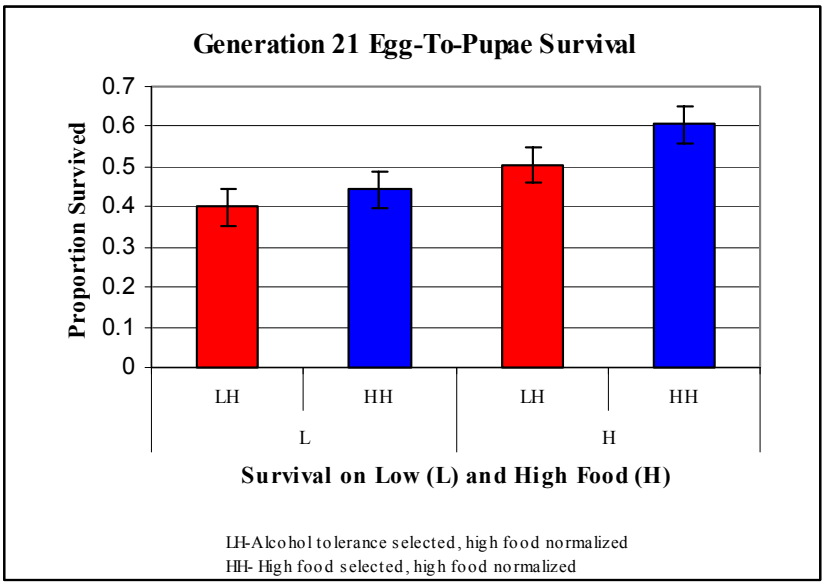


Figure 7 Generation 21 Egg to pupae Survival (Low food) – No effect of selection is observed

| Source | F | *p* |
|---|---|---|
| **Maternal** | 19.25 | <0.0001 |
| **Selection** | 0.21 | 0.650 |
| **Current** | 0.08 | 0.781 |

Table 2 Egg-to-pupae survival: High v. Low - Across generations, egg-to-pupae survivorship is predicted by maternal condition, only.

Acute Survival in High Alcohol

The results for acute survival under high alcohol conditions after 17 generations of selection, relative to sex and by replicate, are summarized in Fig. 8(a)&(b). Fitting nonparametric survival using Weibull and lognormal distributions finds that only females differ significantly (Table 3). Though a similar analysis of this comparison in generation one yielded the same results, here maternal effect can not explain the difference as the selected lines in this representation were normalized on high food medium two generations prior to selection.

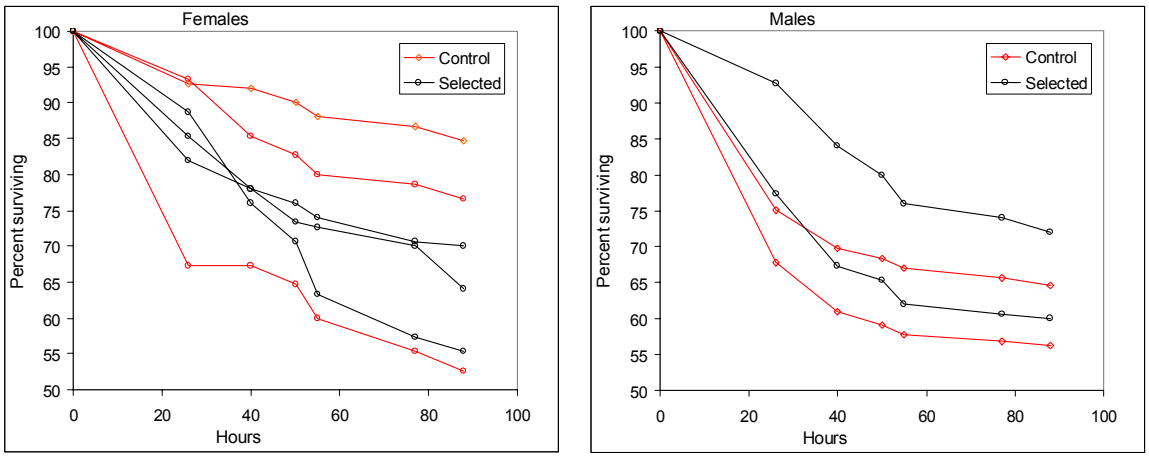


Figure 8 Survivorship by sex: High v. Alcohol - Selected females are significantly different between controls and selected lines. On the other hand males appear not to have increased persistence under high alcohol conditions in response to selection.

| **Females** | **$\chi 2$** | ***p*** | **Males** | **$\chi 2$** | ***p*** |
|---|---|---|---|---|---|
| Weibull: | 4.15 | <0.05 | Weibull: | 0.36 | >0.54 |
| Lognormal: | 1.1 | <0.003 | Lognormal: | 13.29 | >0.30 |

Table 3 Survivorship by sex: High v. Alcohol - Females are significantly between controls and selected replicates after 17 generations of selection. Males remain undifferentiated.

Acute Survival in Low Food

Results of acute survival in empty agar yield a significant response in both males and females after 17 generations of selection (Fig. 9 (a) & (b)). Weibull distributions yield an insignificant relationship among females but the lognormal distribution is significant (Table 4). Males, on the other hand, are significantly different between

controls and selected replicates no matter which distribution is applied in the nonparametric survival fit.

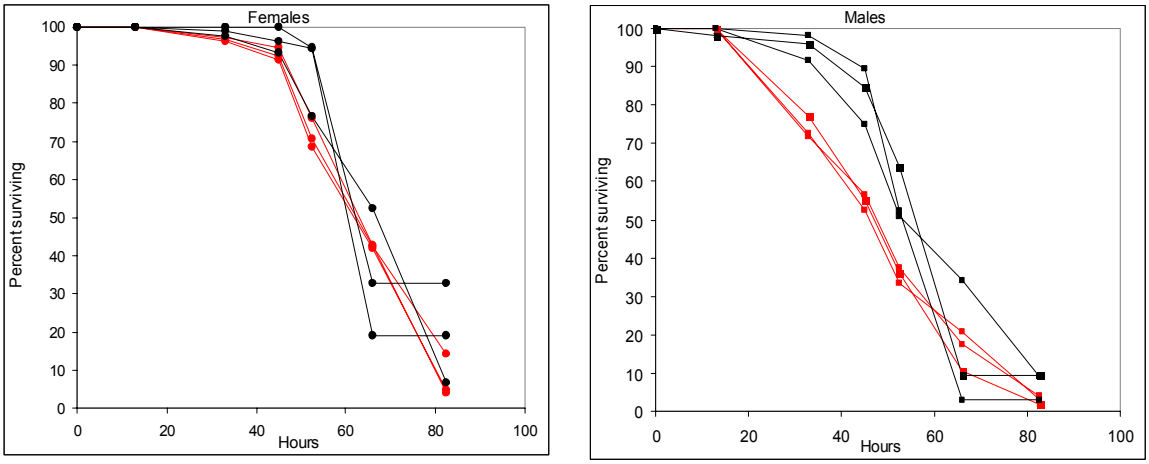


Figure 9 Selected males and females are significantly different between controls and selected lines.

| **Females** | **$\chi 2$** | ***p*** | **Males** | **$\chi 2$** | ***p*** |
|---|---|---|---|---|---|
| Weibull: | 0.44 | >0.5 | Weibull: | 4.85 | <0.03 |
| Lognormal: | 11.04 | <0.001 | Lognormal: | 38.6 | P<0.0001 |

Table 4 Females and males are significantly between controls and selected replicates after 17 generations of selection. Selected lines appear to be persisting longer under acute conditions than controls.

Summary

In summary, both selection regimes responded significantly in at least one phenotype variable measured. Lines selected for tolerance in high alcohol conditions both increased egg-to-pupae survival and adult persistence under acute conditions, even if among females only. By contrast, low food selected lines demonstrated no increased survival from egg-to-pupae in low food conditions, but adults did increase persistence significantly under acute conditions in both sexes.

The genuine measure of an increase in adaptive plasticity in this case is whether or not those increases in survival matched that already attained in the environment of noninduction but exceeded controls within the environment of induction. The increase of survival of high alcohol selected lines in the egg-to-pupae trial clearly demonstrates this. Among low food selected lines, the broad increase in persistence under acute conditions demonstrates adaptive evolution but could only be qualified as the acquisition of adaptive

plasticity contingent on the assumption that persistence under non-acute conditions would exceed or match that of controls.

## Gene Expression

### Selection in High Alcohol

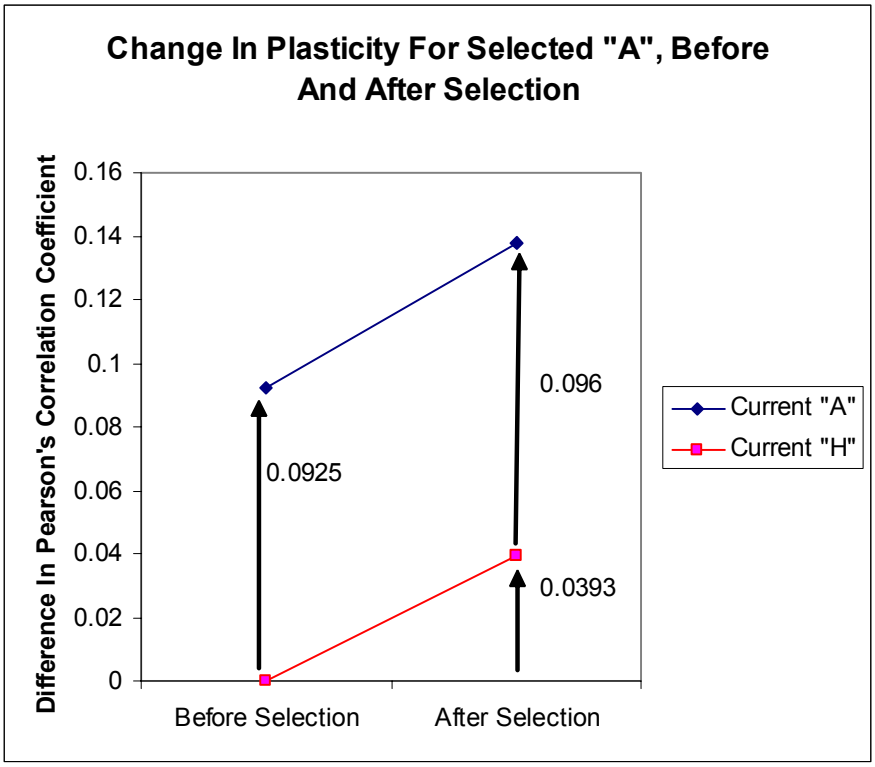


Figure 10 Change in plasticity for selected "A", before and after selection - There is less similarity between noninduced and induced in high alcohol after selection than before selection. The dissimilarity within the noninduced before/after selection comparison is much smaller than the plastic response.

Differences in Pearson's Correlation Coefficient between induction in high alcohol and noninduction, before and after selection, demonstrate the change in plastic response is consistent with the Baldwin Effect. There is less similarity between induction in high alcohol and non-induction after selection than before (Fig. 10). The difference attributable to selection alone is comparatively less, indicating new regulation of the existing plastic response to induction has possibly emerged.

In terms of the effect of this regulation on overall plasticity, an overview of response to induction between alcohol selected flies induced on a high food medium

(AH), control lines induced on an alcohol treated medium (HA), and alcohol selected lines being induced on an alcohol treated medium (AA) relative to controls (HH) (Fig. 11) was performed via comparison on a fitted line plot. Broadly one observes that under conditions of induction, overall gene expression appears to decrease, but there is apparent up and down regulation of genes well outside of confidence interval for the fit.

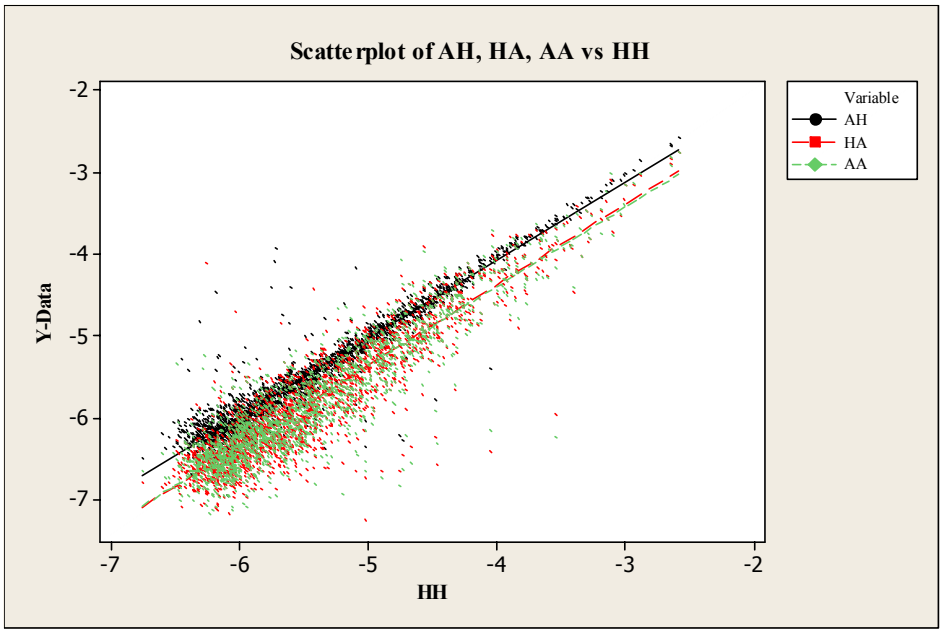


Figure 11 Scatterplot of AH, HA, & AA vs. HH - A broad signature of down-regulation in response to induction with high alcohol in controls and selected lines is matched with strongest changes in gene expression both above and below the fit.

A two-way ANOVA of the impact of selection and current conditions on overall gene expression finds that current is the strongest predictor of changes in gene expression, but that selection comes relatively close to significance (Table 5). However, the interaction of selection and current environment is insignificant.

| Source | SS | F | *p* |
|---|---|---|---|
| Selection | 1.1 | 2.18 | 0.14 |
| Current | 1685.7 | 3237.74 | <0.0001 |
| Selection*Current | 0.0 | 0.07 | 0.786 |

Table 5 Overall gene expression: before v. after selection in high alcohol - Current environment has a significant impact on overall gene expression. Selection approaches significance, but the interaction of current environment and selection is insignificant.

Whether or not this change in overall gene expression corresponds to overall response to induction, a measure of difference between induced and noninduced gene expression, before and after selection, was taken and compared by ANOVA and represented as an interval plot (Fig. 12).

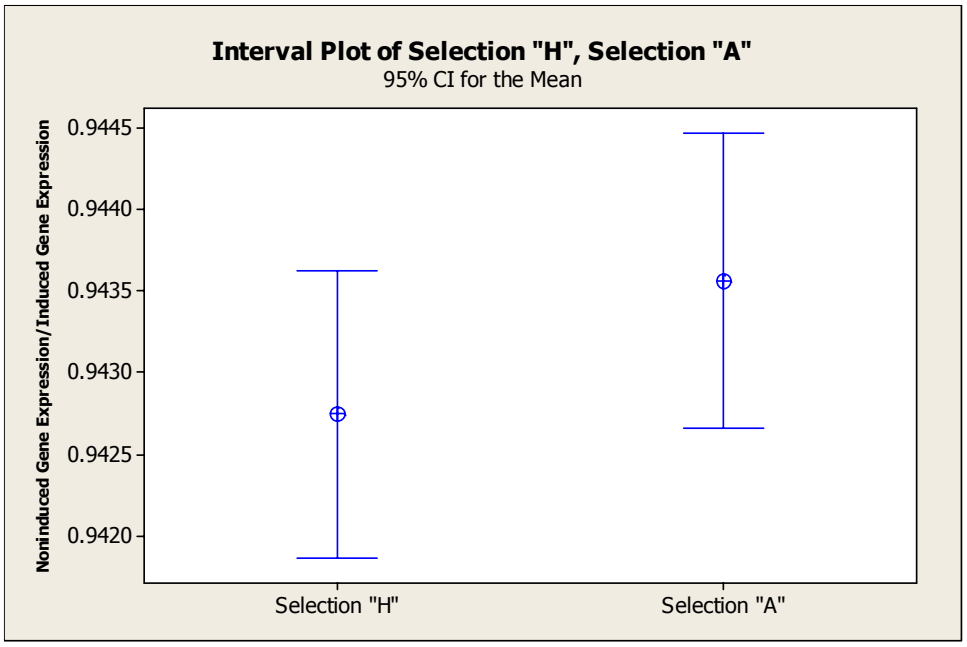


Figure 12 Interval plot of selection H, selection A- Plotting the overall response of gene expression to induction in high alcohol reveals less change in overall gene expression among alcohol selected lines from Induction to noninduction.

The results of a one-way ANOVA demonstrate that the response of net gene expression to induction in high alcohol approaches significance ($F=1.61$, $p = 0.204$).

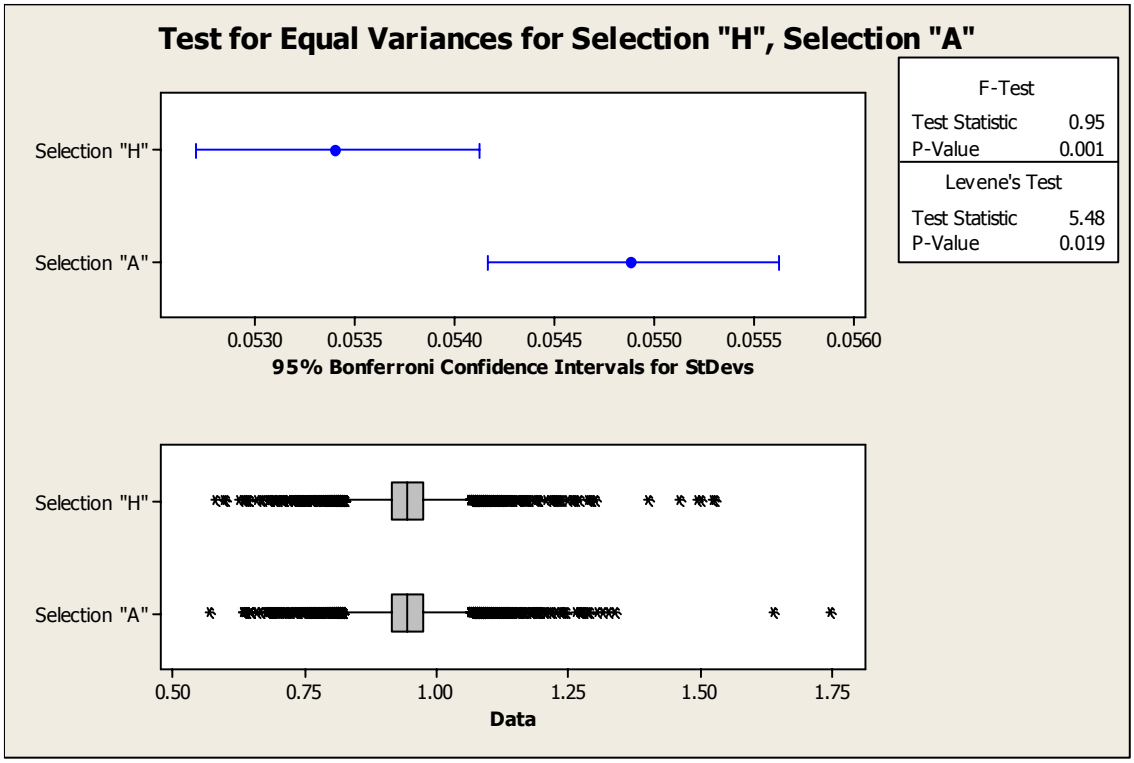


Figure 13 Test for Equal Variances for selection H, selection A - The effect of induction before (H) and after (A) selection is unequal; selected lines demonstrate significantly greater variance.

A test of equal variances demonstrates this may be influenced by unequal variances between the responses of controls ("H") relative to flies selected for tolerance in high alcohol conditions (Fig. 13). However, a Mann-Whitney test of the medians only confirms the ANOVA results, and no significant difference exists in the overall response to induction before and after selection ($p = 0.31$) though the variance is significantly different ($p = 0.001$).

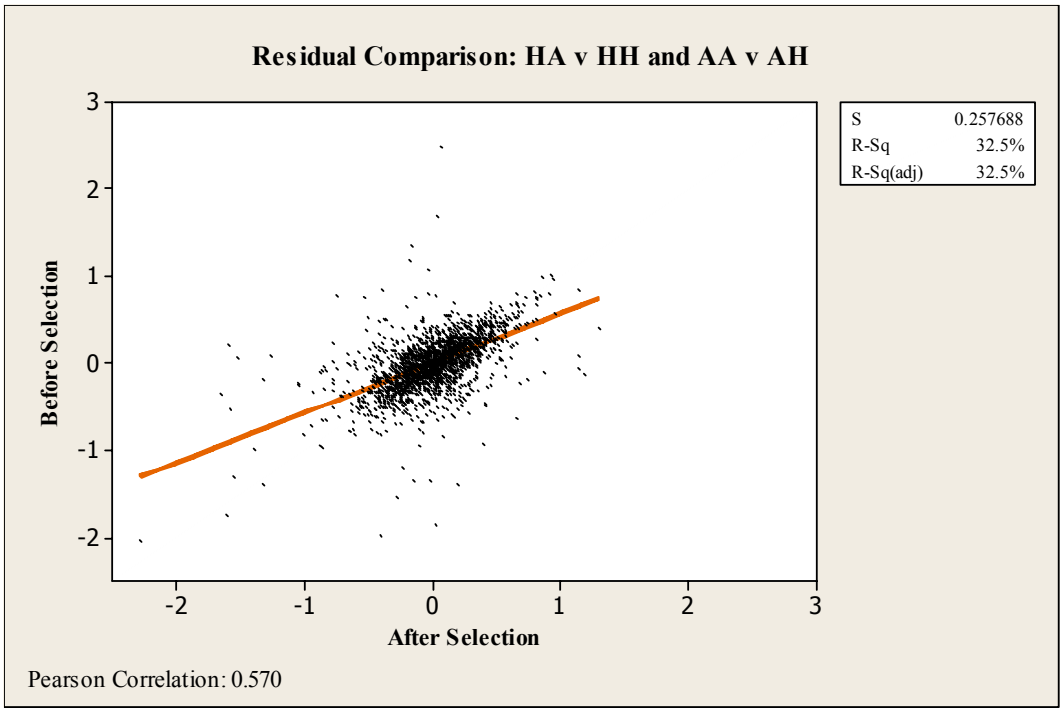


Figure 14 Residual comparison HH v HH and AA v AH - There is a low correlation between genes responding to induction before and after selection for tolerance to high alcohol conditions.

An analysis of the residuals between induction and non-induction, before and after selection, reveals that there is relatively little similarity in the behavior of genes responding to induction in high alcohol (Fig. 14). Regression of HA versus AA reveals that while otherwise similar, there are individual genes that vary in response to induction in high alcohol (Fig. 15).

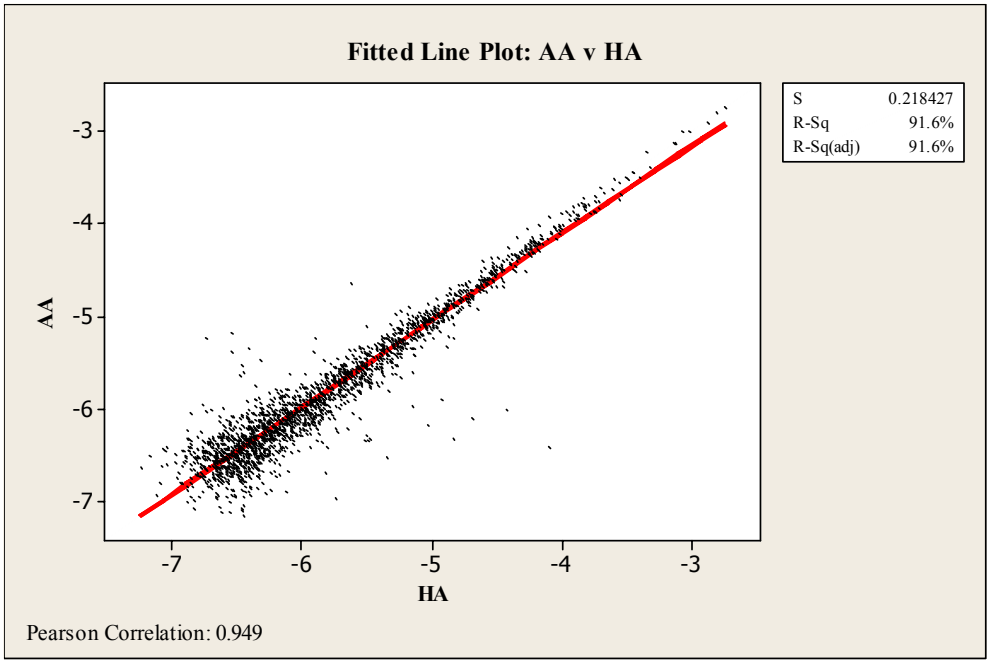


Figure 15 Fitted line regression: AA v HA - While over-all gene expression in response to induction is strongly correlated before and after selection, individual differences in gene expression are significant.

A detailed survey of individual genes that changed expression (at a significance level of $p < 0.05$) after selection demonstrates that many of the previous results can be attributed to a transition in the assemblage of genes responding to selection (Fig. 16). The initial impression one obtains from this analysis is that change in expression among regulatory genes are affecting changes in expression among structural genes.

The impression is more than hypothetical, however, when the genes identified as having changed regulation are put through a Flybase search for molecular function. While relatively few genes are identified as having explicit regulatory roles in comparison with genes that ultimately changed regulation, the proportion of those genes that changed expression and are regulatory is strongly associated with the nature of that change.

The total number of genes identified as significantly up regulated before selection but down regulated after equals 527. The number of genes identified as downregulated before selection but up regulated after equals 446. The number of genes identified as not significantly expressed before but up regulated after equals 282. The number of genes not significantly expressed before but down regulated after equals 211. Finally, genes identified as up regulated before but not after equal 384 and genes identified as down

regulated before but not after equal 171. The total number of genes that changed regulation in some fashion is 2021.

In contrast, the number of regulatory genes identified as significantly up regulated before selection but down regulated after selection is 17. The number of genes down regulated before selection but up regulated after equals 12. The numbers of genes not significantly expressed before but up regulated after equals 8 and the number of genes not expressed before but down regulated after equals 9. Finally, the number of genes that were up regulated before selection but not after equals 17 and the number of genes down regulated before but not after equals 2. The total number of genes explicitly identified as having functions of regulation from Flybase that changed expression in response to selection is 65.

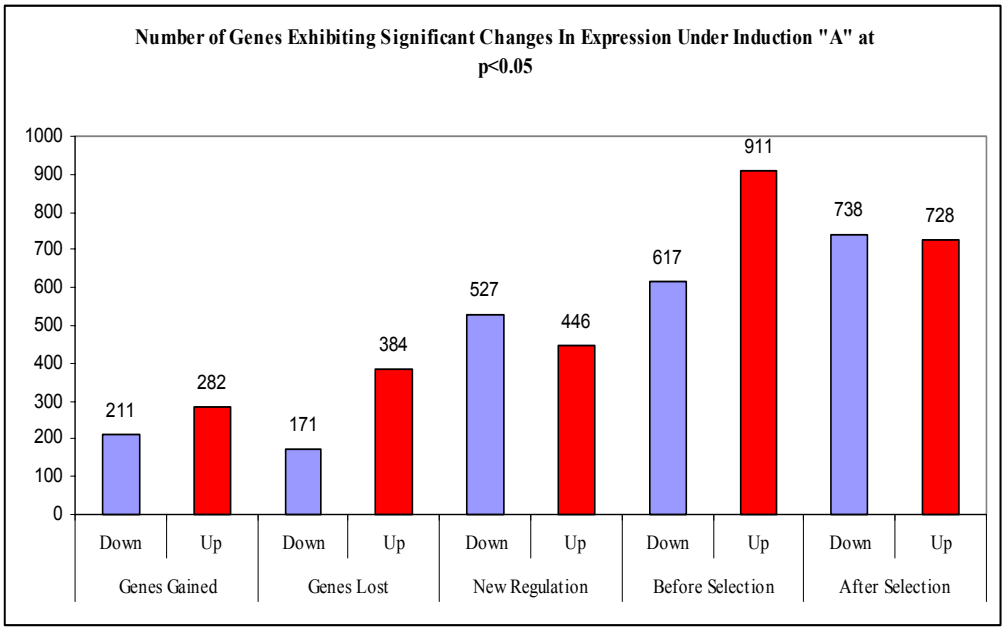


Figure 16 Genes that changed expression to induction before/after selection "A"- Change in gene assemblage constituting the plastic response to induction under high alcohol conditions before/after selection at $p<0.05$.

When the number of regulatory genes of each category (Up regulated before but not significantly expressed after selection, down regulated before but not significantly expressed after selection, not significantly expressed before selection but up after, not significantly expressed before but down after, up regulated before but down after, and down regulated before but up after) are divided by the total number of genes of each

group (all genes that changed) one is able to represent the proportion of regulatory genes that changed expression relative to all genes that changed expression. The relationship between the proportions of genes identified as having regulatory functions relative to the share of all genes of each category that similarly changed expression is explored; the correlation is significant (Fig. 17).

The only exception appears to occur among regulatory genes significantly up regulated before selection but not after. When contrasted with Fig. 16, it is logical to infer that, for the most part, regulatory genes represented most of the genes that changed expression within this category and, likely, many of these positively control expression of the share of all genes that similarly changed expression after selection.

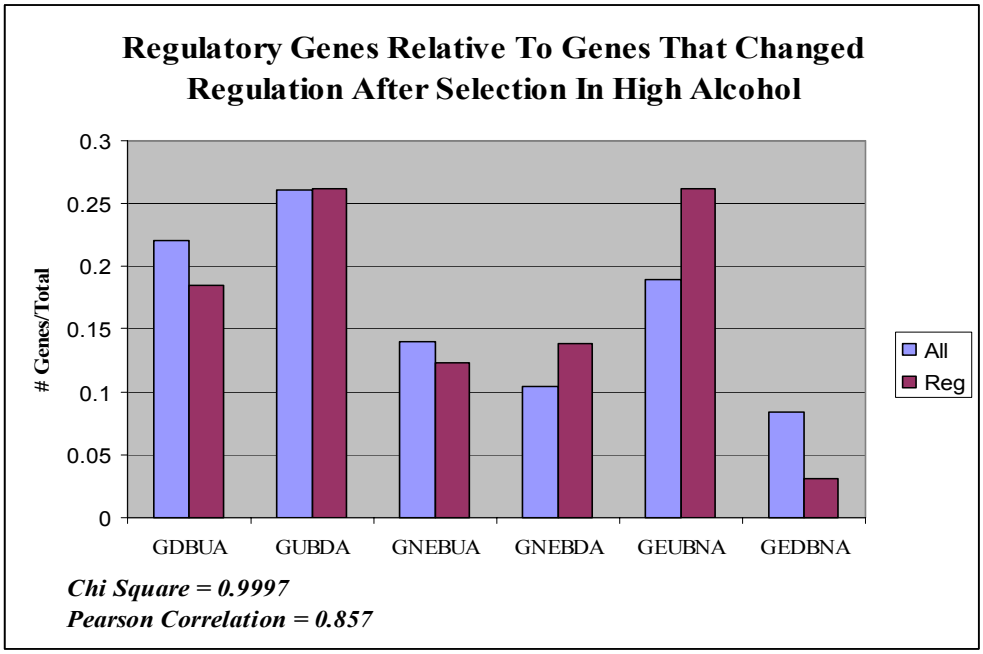


Figure 17 Regulatory genes/share of total genes that changed expression after selection - GDBUA: "Genes Down Regulated Before/Up After"; GUBDA: "Genes Up Regulated Before/Down After", GNEBUA: "Genes Not Significantly Expressed Before Selection/Up After", GNEBDA: "Genes Not Expressed Significantly Before/Down After", GEUBNA: "Genes Significantly Expressed Up Before/Not Significantly Expressed After", GEDNA: "Genes Expressed Down Before/Not Significantly Expressed After Selection".

Finally, a One-Way ANOVA of the residuals of genes significantly up or down regulated fails to distinguish between before and after selection ($p = 0.58$). However, the pattern of gene expression for genes that were significantly expressed before and after selection (Fig.18) matches the trend observed in overall gene expression (Fig.19).

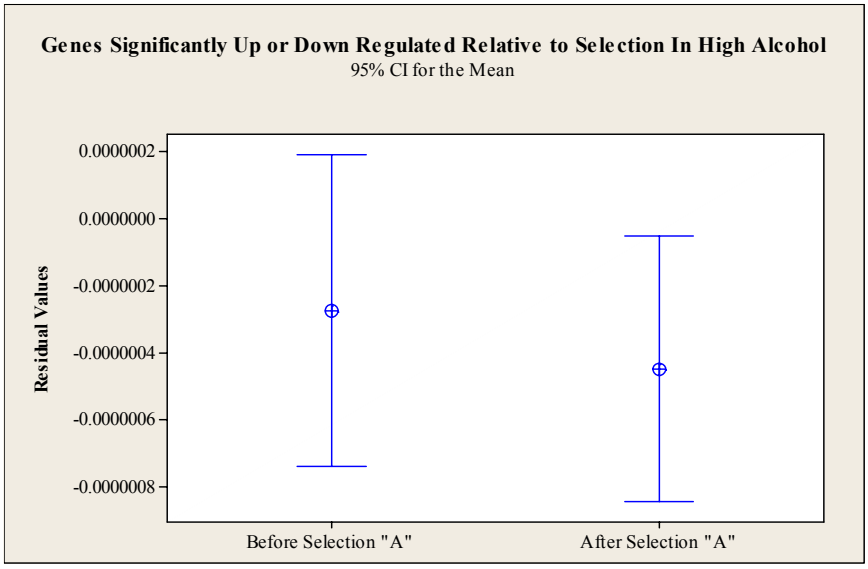


Figure 18 Residuals of genes significantly up or down regulated relative to selection A - An analysis of the residuals of genes that changed regulation in response to selection finds the effect negligible ($p = 0.58$). However, the pattern of gene expression for genes that significantly changed regulation after selection matches that of observed in overall gene expression (Fig. 19).

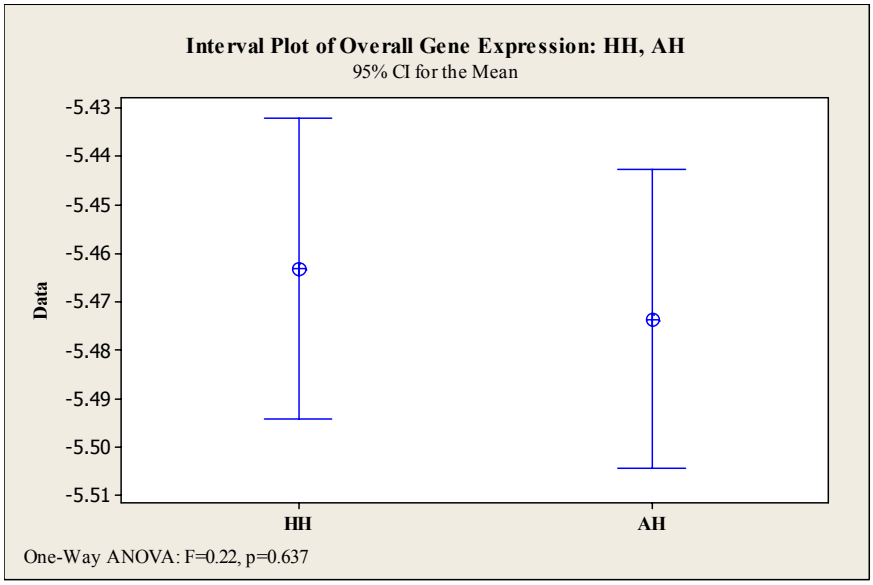


Figure 19 Interval plot of overall gene expression: HH, AH - The pattern of overall gene expression observed after selection matches that of genes that significantly changed regulation in response to selection. This supports the observation that patterns of over-all gene expression can be observed for the 'fingerprint' of new adaptive plasticity via the acquisition of new regulation.

Selection in Low Food

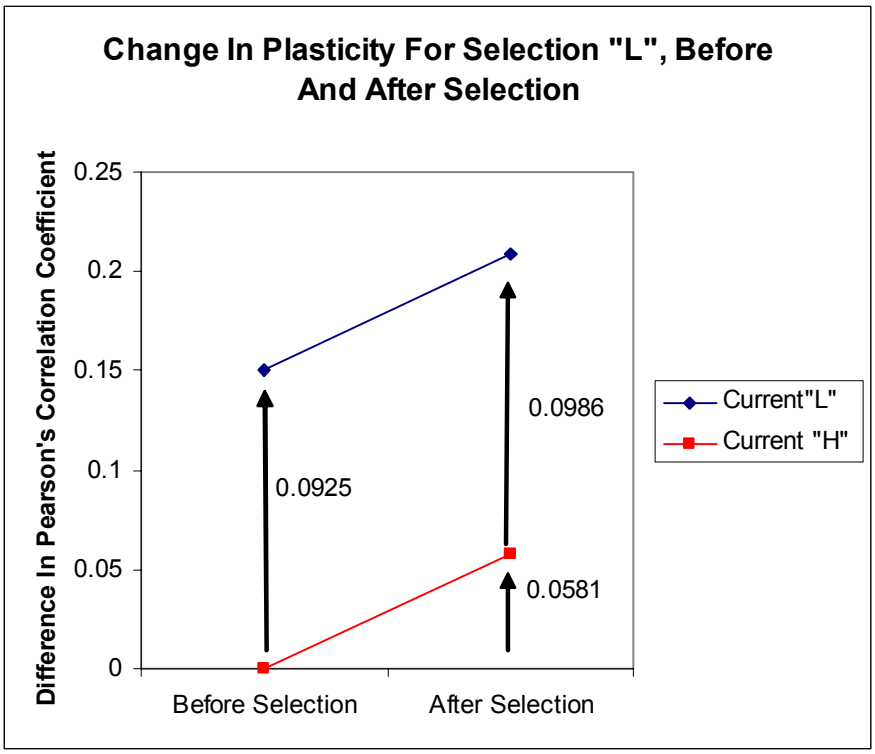


Figure 20 Change in plasticity for selection L, before and after selection - There is less similarity between noninduced and induced in larvae selected for tolerance to low food conditions after selection than before selection. The dissimilarity within the noninduced before/after selection comparison is much smaller than the plastic response

Just as the difference was greater between induction and noninduction after selection in high alcohol, the same is true for selection for tolerance to low food conditions (Fig. 20). However, the difference in correlation attributable to selection was much greater (a difference of 0.0581in Pearson's Correlation Index).

In terms of the effect of this regulation on overall plasticity, an overview of response to induction between low-food selected flies induced on a high food medium (LH), control lines induced on an low food medium (HL), and low food selected larvae induced on an the selective medium (LL) relative to controls (HH) was expressed as a fitted-line plot. Broadly, one observes that under conditions of induction, overall gene expression appears to favor the down regulation of less commonly expressed genes in exchange for enhanced expression among more commonly expressed genes (Fig. 21).

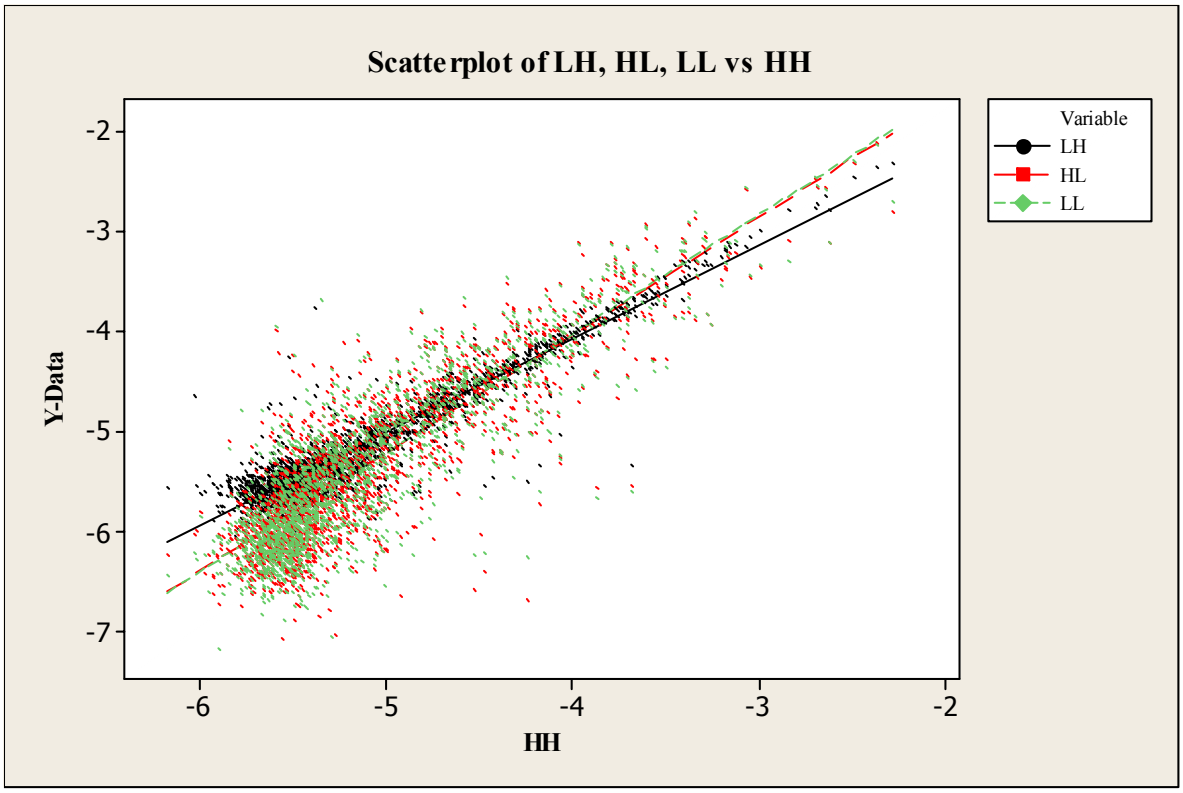


Figure 21 Scatterplot of LH, HL, & LL v. HH - A broad signature of down-regulation of less commonly expressed genes in favor of up regulation for more commonly expressed genes under conditions of induction.

A review of the normal distribution of overall expression reveals that induction has a significant effect (Fig. 22). A further analysis of overall gene expression before and after selection demonstrates a similar pattern as observed after selection in high alcohol (Fig. 23). However, no such pattern exists under conditions of induction before or after selection (Fig. 24).

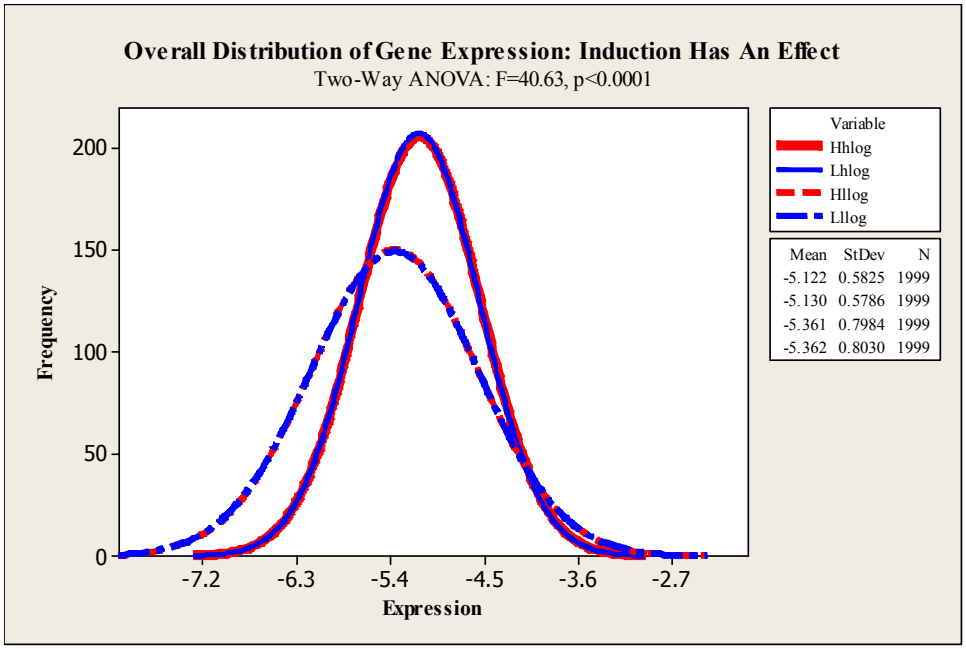


Figure 22 Overall distribution of gene expression: induction has an effect.

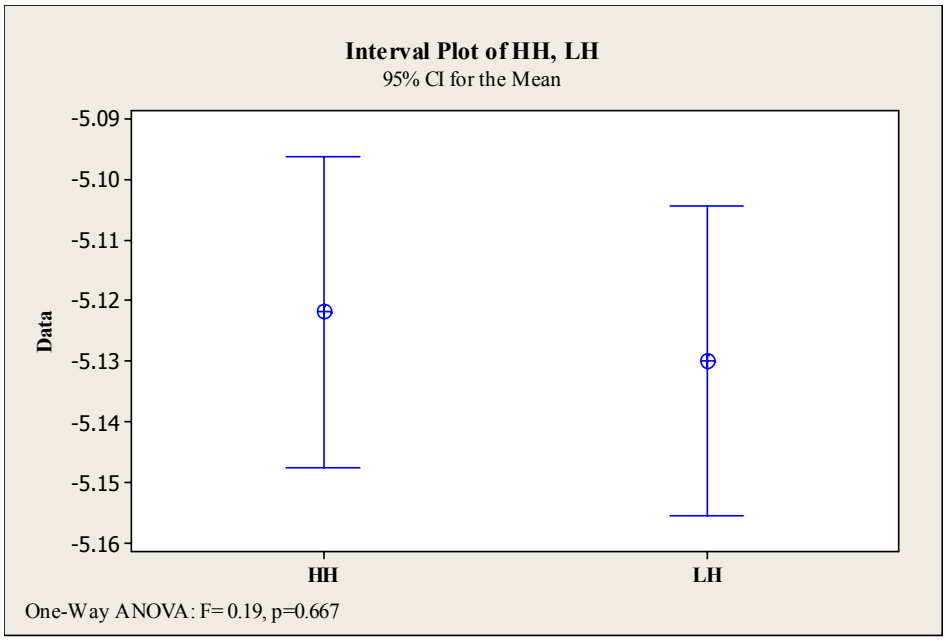


Figure 23 Interval plot of HH, LH - Overall gene expression, while somewhat down regulated in "L" selected lines, is not significantly different from controls.

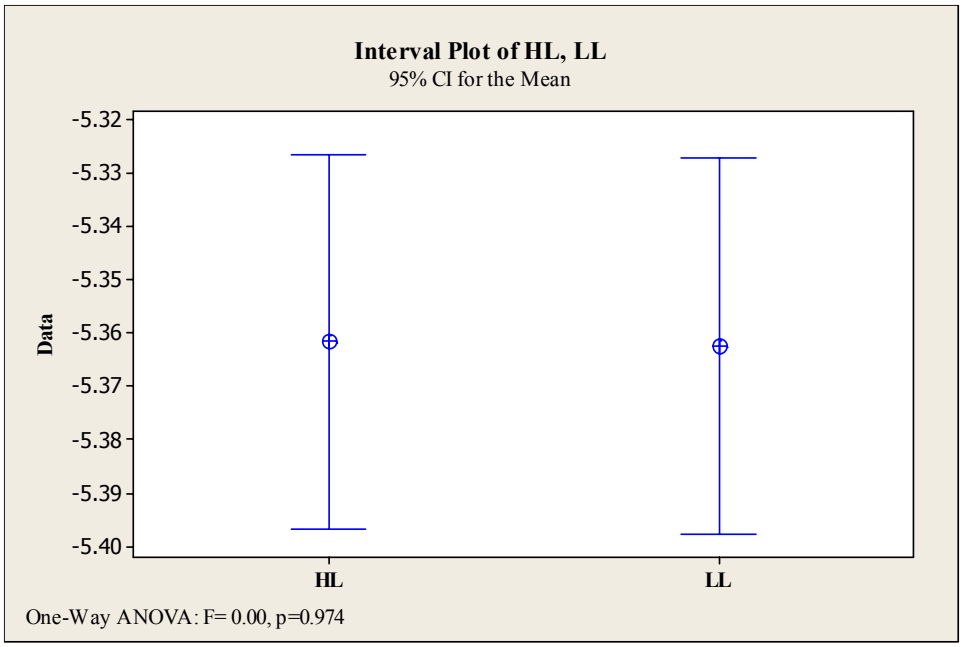


Figure 24 Interval plot of HL, LL - Overall gene expression under induction: Selected and nonselected lines are undifferentiable.

Overall gene expression before and after selection, before and after induction in low food, is best predicted by current conditions. Unlike selection in high alcohol conditions, though, selection does not approach significance though the interaction of selection and current does (Table 6).

| **Source** | **SS** | **F** | ***p*** |
|---|---|---|---|
| Selection | 0.0 | 0.03 | 0.54 |
| Current | 921.6 | 1964.12 | <0.0001 |
| Selection*Current | 0.4 | 0.89 | 0.345 |

Table 6 Overall gene expression: before v. after selection in low food - Current environment has a significant impact on overall gene expression. Selection approaches significance, but the interaction of current environment and selection is highly insignificant.

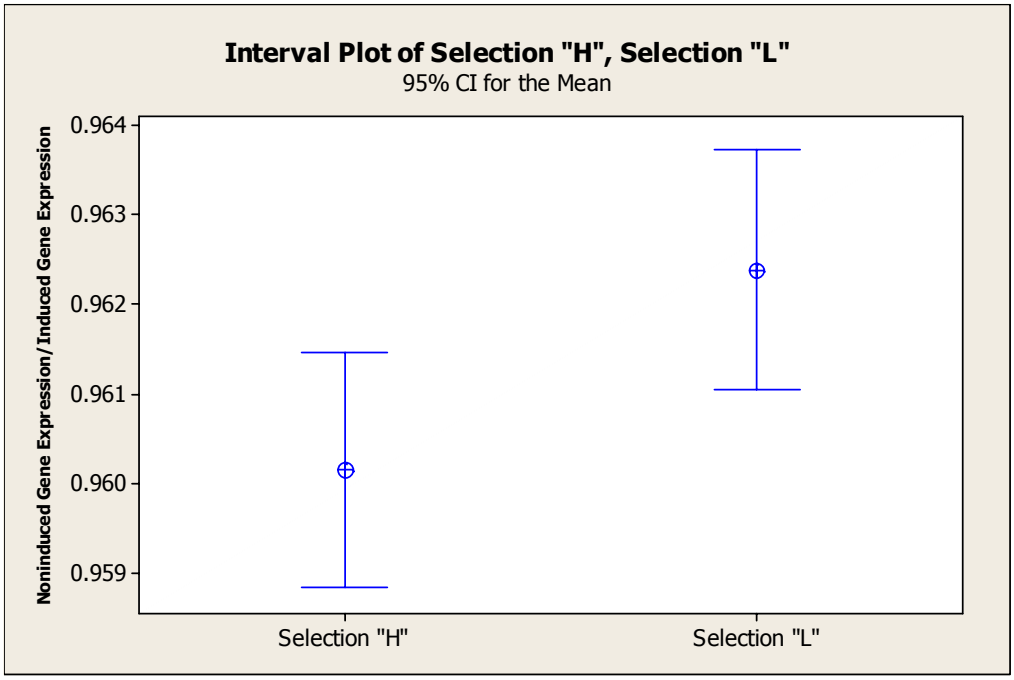


Figure 25 Interval plot of selection H, selection L - The overall response to induction, in terms of changes in overall gene expression, is significant less after selection (One-Way ANOVA: $F = 5.42$, $p = 0.02$).

On the other hand, the overall response of gene expression to induction is significant (Fig. 25). When noninduced gene expression is divided by induced gene expression, before and after selection, the magnitude of the response among low food selected lines is much less than that of controls. When contrasted with the difference observed in overall gene expression among noninduced selected lines and controls (and by comparison the substantial lack of difference in overall gene expression after induction) this result is completely logical. Lines selected for tolerance to low food conditions, already somewhat down-regulated, have less distance to travel under conditions of induction. This suggests the manifestation of the new plastic response may be in the form of a more 'thrifty' over-all gene expression among low food selected lines.

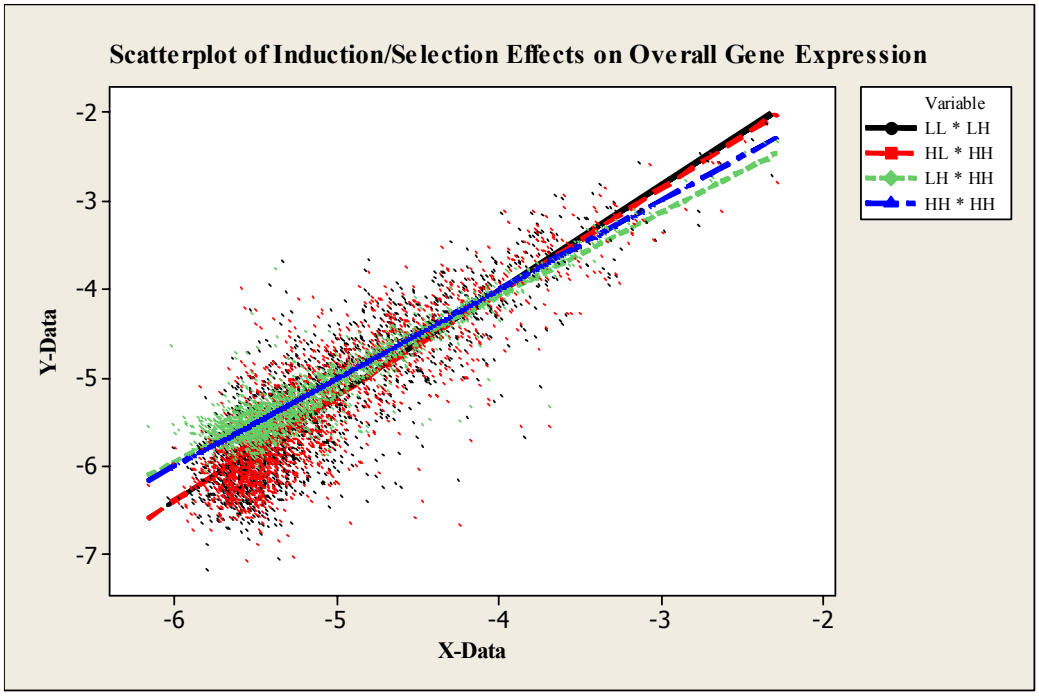


Figure 26 Scatterplot of induction/selection effects on overall gene expression - Thriftier expression among more commonly transcribed genes, combined with more efficient function of less commonly transcribed genes, leads to less distance to travel under induction after selection.

One way to visualize this effect is to revisit comparative overall gene expression (Fig. 26). When LL is compared to LH, the effect is synonymous with what is observed between HL and HH. However, when a 1-to-1 fit is established by using HH as both the predictor and the response, we see that LH demonstrates a great deal of reduced expression among more commonly expressed genes. At this juncture it is reasonable to apply a little common sense to the observation and deduce that this effect is consistent with probability theory. Under this scenario it is possible the response to induction is mediated by competition. This is a likely explanation, as more commonly transcribed genes will likely be more represented in sites of translation. Under conditions of nutritional stress, this would mean that these more commonly transcribed genes 'rob' the raw materials of translation from less commonly transcribed genes by weight of representation, alone.

On the other hand, when these observations are paired with the relationship of regulatory genes that changed expression (relative to all genes that significantly changed expression) an interesting pattern is observed, though (Fig. 27).

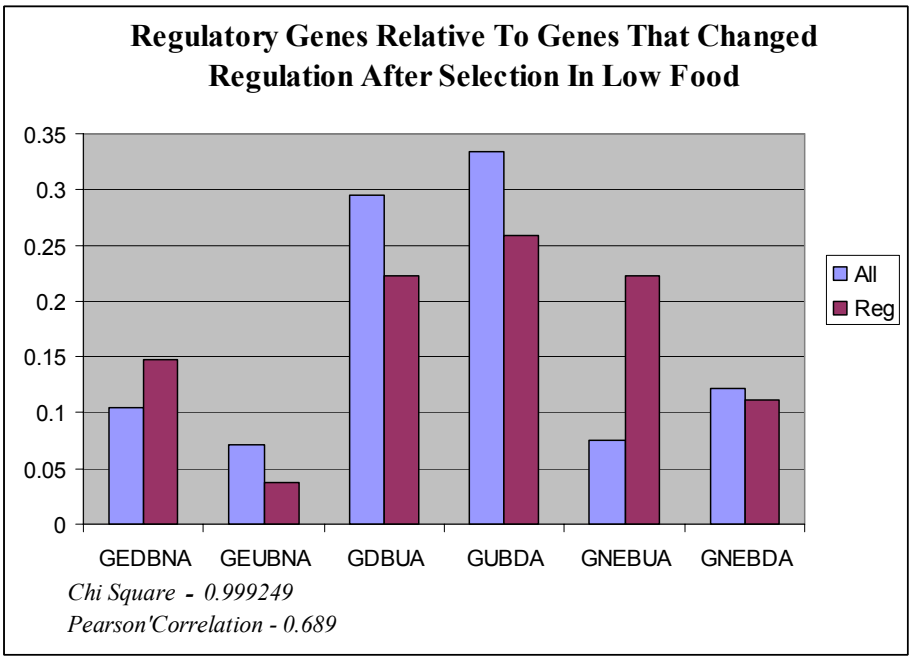


Figure 27 Regulatory genes relative to genes that changed regulation after selection in L - GEDBNA: "Genes Expressed Down Before/Not Significantly Expressed After Selection", GEUBNA: "Genes Significantly Expressed Up Before/Not Significantly Expressed After" GDBUA: "Genes Down Regulated Before/Up After"; GUBDA: "Genes Up Regulated Before/Down After", GNEBUA: "Genes Not Significantly Expressed Before Selection/Up After", GNEBDA: "Genes Not Expressed Significantly Before/Down After". The share of regulatory genes identified as not expressed before selection but up regulated after represent a disproportionate share of genes that generally changed expression, similarly.

While the proportion of regulatory genes to genes that generally changed regulation in response to selection is comparable, one notable exception is the proportion of regulatory genes that were not significantly expressed before but were up-regulated after selection. Here, one observes that of genes of that category regulatory genes are significantly more represented in this class of altered regulation. While overall numbers of regulatory genes that changed regulation in response to selection were relatively small (27 of the 975 genes that demonstrated new regulation after selection); that such a disproportionate share of regulatory genes would be upregulated in a genome that generally exhibited decreased over all expression is significant.

The breakdown of all genes that responded to selection with new regulation demonstrates an interesting effect (Fig. 28). Before selection, equitable numbers of genes were up-regulated as down in the environment of induction. However, after selection substantially more genes are down-regulated as up in the environment of induction.

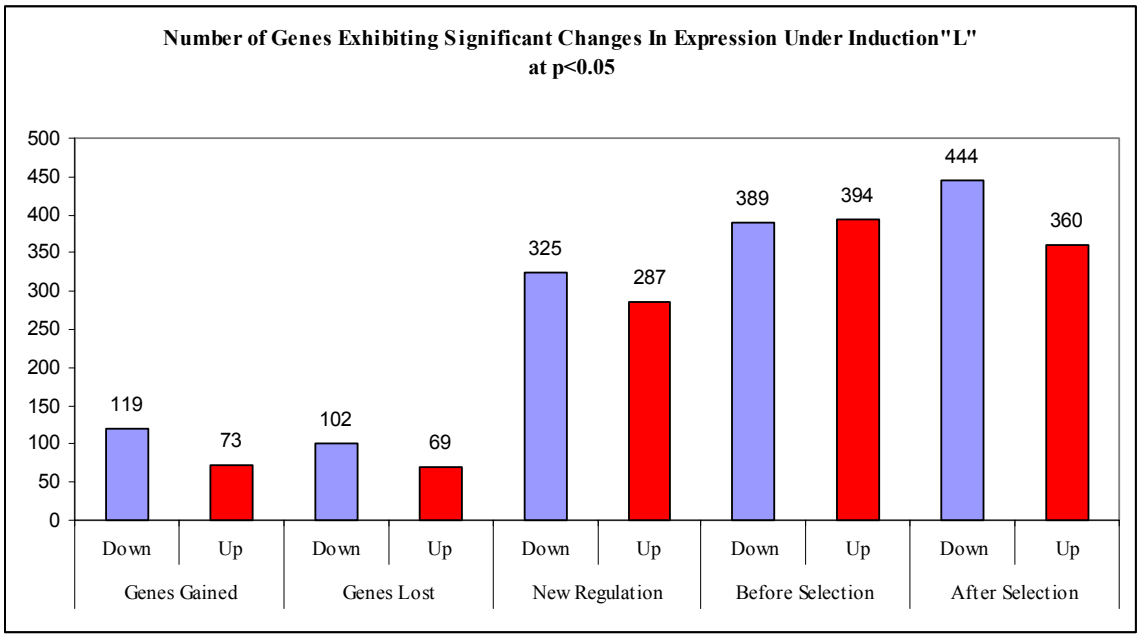


Figure 28 Number of genes exhibiting significant changes in expression under induction L at $p < 0.05$ - More genes are significantly down regulated after selection than up regulated. Before selection, the relationship of genes down regulated to up regulated was equitable. This suggests that regulatory genes not expressed significantly before selection, but significantly up regulated after, have downregulated many less commonly expressed genes that formally responded to induction with up regulation.

The impression one gains from this and the former observation is that the down-regulation of many genes in response to induction is to some degree deliberate. The simplest explanation for the up-regulation of more commonly expressed genes in response to induction at the expense of genes less commonly expressed (where competition for resources in the sites of translation dictated the difference) begins to fail at this juncture. What emerges in its place is the possibility that new regulation is mediating the transition of transcriptional emphasis from less commonly expressed genes to be weighted in favor of those more commonly expressed (and presumably more essential to life-functions such as metabolism).

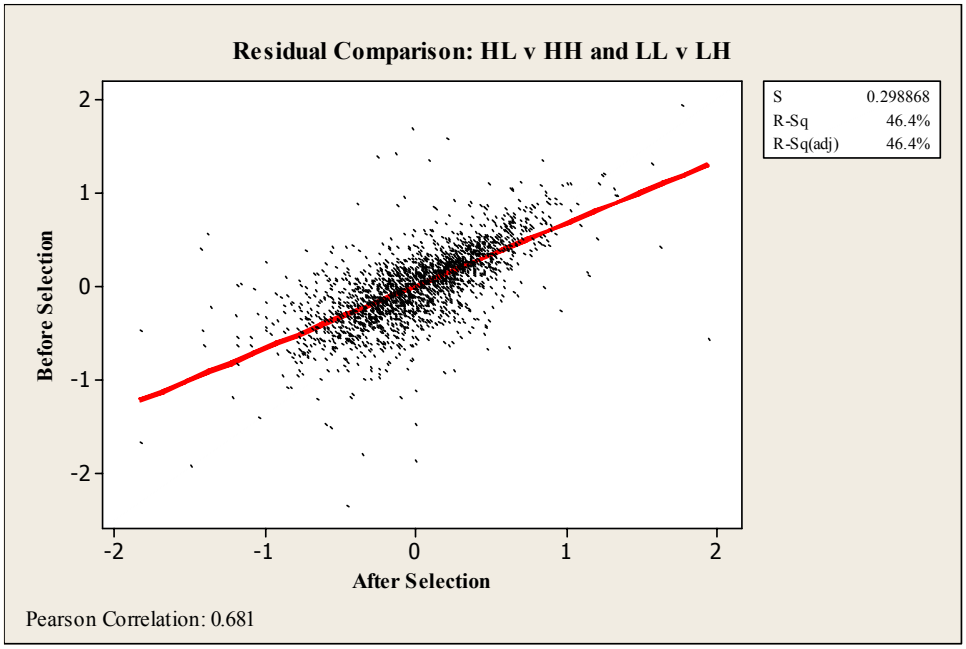


Figure 29 Residual comparison: HL v. HH & LL v. LH - Genes responding to induction appear to be largely the same before and after selection, but is likely being variably regulated.

When the residuals of induced genes (before and after selection) are compared, what is apparent is that under conditions of induction a greater share of genes seem to be of the same plastic response in both regimes than what was observed after selection in high alcohol (Fig. 29 & 30). What is suggested, when taken in the context of the previous observations, is that the plastic response to induction under conditions of low food is essentially the same after selection as before. However, it is likely the difference can be accounted for by the assumption of a new plastic response by genes that have a stronger effect of regulation. Furthermore, this new regulation appears to 'shorten the distance' between induction and noninduction in selected lines.

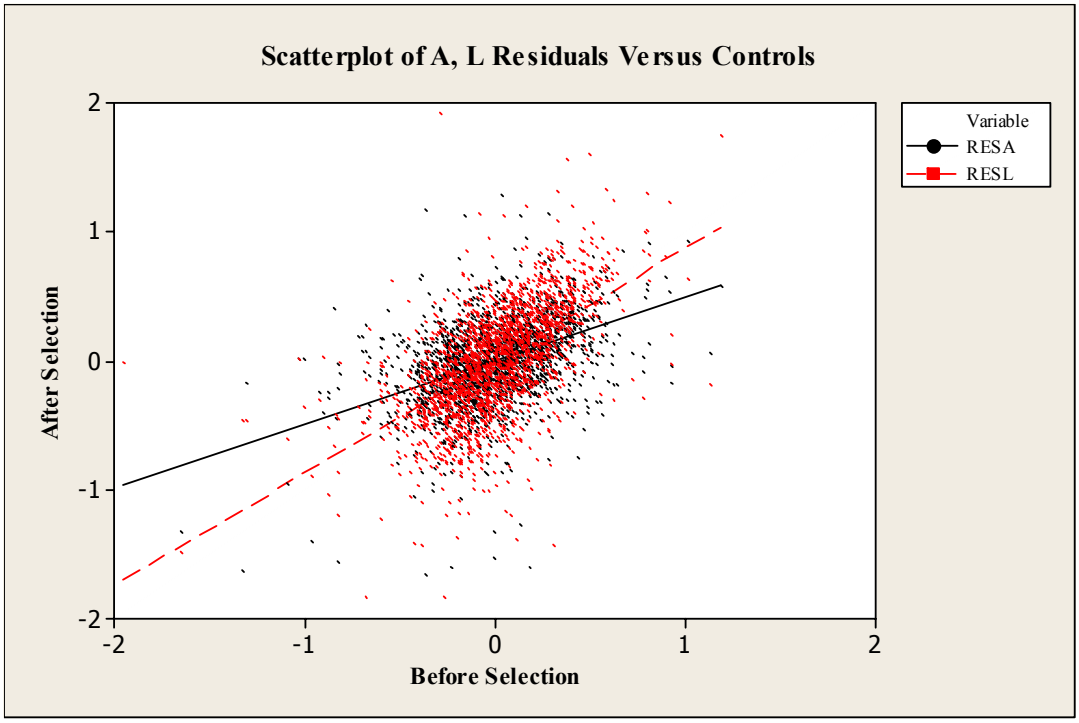


Figure 30 Scatterplot of A, L residuals versus controls - Here is a comparison of residuals after selection for tolerance to low food and high alcohol. The fingerprint of the residuals, in each, suggests different paths to the assumption of new adaptive plastic responses. The less significant slope of residuals induced under high alcohol suggests a new plastic response has partially displaced the former; while the more significant slope of residuals induced under low food conditions, combined with the observation of low correlation from noninduction to induction, implies the existing plastic response is being differentially regulated.

Summary

In summary, it is observed that both selected lines expanded or enhanced adaptive plasticity. Lines selected for tolerance to high alcohol conditions appear to have assumed a new plastic response to induction that is difficult to discriminate against the background of overall gene expression or response to induction. This response is only visible when residuals are compared and a detailed inventory of genes changing regulation in response to selection is conducted. While the proportions of genes that changed regulation is highly correlated with the proportions of regulatory genes that changed regulation (implying chance sampling), the fact that egg-to-pupae survivorship in high alcohol improved can not be ignored.

Furthermore, the detailed inventory of genes responding to selection demonstrates relatively large assemblage turnover in the plastic response to induction, after selection. This observation is supported by the significantly different variances assumed in response to induction between selected lines and controls. Thus, it is reasonable to conclude that

while the new adaptive response to induction by alcohol does not exhibit a significant fingerprint on overall gene expression, it is visible in the response to induction. Lastly, the disparity of correlation between induction and noninduction after selection links the former and latter observations as logically consistent with the substitution of the former plastic response with a new.

Low food selected lines accomplished the acquisition of new adaptive plasticity through different means and, possibly, enhanced net plasticity as a consequence. This appears to have come about on the part of low food selected lines via the assumption of a 'thriftier' basal gene expression profile that enhances the response to induction in low food. The maintenance of this profile, absent induction, appears in part to have come about by the acquisition of regulation and this could be implicit in the disparity of correlation between selected lines and controls. However, further testing would be required to elevate this possibility beyond the realm of informed speculation taken from existing data.

Likewise, under induction the simplest explanation for the disparity of expression between more commonly expressed genes and less commonly expressed genes is one of competition at sites of translation. However, evidence of positive regulation post-induction, after selection, more strongly implies this response is mediated.

# CHAPTER 4

# DISCUSSION

It is possible that the initial concerns regarding the cost associated with maintaining phenotypic plasticity are overstated, pending a broader acceptance of the mechanistic definition of plasticity as the default setting of unregulated gene expression. While this is not conceptually novel, this author is unaware of it being explicitly stated as such. Generally, what manifestations of the definition that are rendered regarding phenotypic plasticity acknowledge the variable expression of a genotype, but by the same token scant attention is attributed to exactly how it is the variable expression of the genotype itself is accomplished.

Additionally, it is observed that little distinction is generally made between net plasticity and adaptive plasticity. As such, the findings of C.H. Waddington and J. Mark Baldwin are often needlessly pitted in the literature as dichotomous alternatives in the discussion surrounding the evolution of phenotypic plasticity. On the other hand, most empirical evidence (including that which has been obtained in this study) and recent theoretical work suggests they are, in fact, complimentary (32).

For example, it would be convenient to characterize the basal gene expression of noninduced low food selected lines as ‘canalized’. After all, it remains in the absence of induction. On the other hand, this profile exhibits properties of being a newly acquired plastic response of the phenotype to induction in low food conditions. Furthermore, there is no reason to assume the ability to mobilize the profile associated with induction is confined to development or is selectively insignificant. Indeed, it would not be surprising to find that the rapid mobilization of this induced gene expression profile accounts for the increased persistence of adults under acute conditions of nutritional stress relative to controls.

The conceptual significance of this is that this ‘hidden’ variation, though canalized as a plastic response to induction, manifests the phenotype in accordance with Baldwin’s original predictions. Baldwin speculated that much plasticity is present early in the phylogenetic history of an organism and that ‘organic selection’ only later emerges to

enhance its manifestation as an adaptive phenotype. Subsequent authors have extrapolated on this observation to suggest an expansion of net plasticity is the outcome of the Baldwin Effect when in fact this is but one of two possibilities originally stated.

The other possibility is that new adaptive plasticity is acquired (implicit but largely unstated explicitly in subsequent literature) via the assumption of a novel plastic response that wholly or partially displaces the former. In this scenario, net plasticity is neither increased nor decreased but is, rather, subjected to new regulation of the adaptive response of the phenotype.

Such an example can be found within the data surrounding selection for tolerance to high alcohol. Here, it is observed that the existing plastic response was partially supplanted by a novel one. The evidence of this redistribution of existing adaptive plasticity is found in the gain, loss, and novel regulation of genes after selection that is not reflective of the plastic response to induction observed before selection. It is further strongly supported as adaptive in so much as it coincides with an increase in egg-to-pupae survival under conditions of high alcohol stress while retaining fitness comparable to controls under conditions of noninduction. While perhaps empirically novel here, conceptually this is consistent with original predictions of the Baldwin Effect and such a result has already been established as possible in previous theoretical work (33, 34).

With these observations in mind, one is tempted to indulge a broader view of the potential impact of the Baldwin Effect on evolution. From the synthesis of these observations it could be deduced that selection's most significant impact on plasticity is likely biased to neither increases nor decreases plasticity, *per se*. Rather, the effect of selection on plasticity is likely merely the redistribution of the assemblage of genotypes driving the plastic response. This is of substantial evolutionary consequence as the new pleiotropic limitations imposed by induction would have the effect of resetting the start point for subsequent evolution of the phenotype.

In such a way it appears selection has, via assimilation or canalization of a new 'hidden' variation, incorporated to limited degree a former phenotypic extreme as a new genotypic norm within low food selected lines. Much of this hidden variation is likely maintained as undetected regulatory differences maintaining a thriftier basal expression profile. It is also likely this regulatory effect can be detected in existing data or further

experimentation. Whichever is the case, though, the effect likely translated into a cascade of new phenotypes simply not assayed as a part of this study but for which ample anecdotal evidence exists as offhand observations in the laboratory.

Likewise, a similar effect is observed in high alcohol selected lines but with different mechanism and likely impacts on subsequent evolution. Here, it is observed that selection has perhaps more perfectly 'hidden' the plastic response to induction amidst the background of noninduced gene expression. Under conditions of high alcohol stress this different plastic response is visible, however, in the form of a novel assemblage and regulation of genes responding to induction. The likely effect of this on evolution is not so much the often speculated buffering effect predicted as to be a function of plasticity, but a reduced likelihood the change, absent induction, will be manifested in a cascade of novel, pleiotropically linked, phenotypes that result in substantially divergent character of the organism.

Both the former and the latter observations imply a more conservative approach is warranted where the Baldwin Effect or canalization is invoked as either more, less, or equally important predictors of evolutionary trajectory. As it is broadly observed within these data and found in theoretical studies, the true impact of selection on novel adaptive plasticity is truly contingent upon how close the organism is to reaching the fixation of a similar adaptation through mutation. As such, prior exposure to the inducing environment (often unquantifiable), stated previously as the 'phenotypic memory' to induction, could be equally as important in determining whether or not adaptive plasticity itself is enhanced or canalized.

The consequences of such an observation lend themselves to determining the future course of investigation. As it was observed previously, many adaptive surfaces in nature go unmeasured. Furthermore, it is difficult to determine to what degree a population may have been exposed to a particular inducing environment prior to experimental selection for adaptive plasticity relative to it. Resolving the latter is likely key to understanding whether subsequent evolution of the plastic response will result in canalization, a partial or whole redistribution of genotypes participating in the plastic response, or the enhancement of regulation for the existing plastic response to induction.

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