doi:10.1111/evo.13

Selection on structural allelic variation biases plasticity estimates

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Received January 30, 2019
Accepted February 19, 2019

Wang and Althoff (2019) explored the capacity of *Drosophila melanogaster* to exhibit adaptive plasticity in a novel environment. In a full-sib, half-sib design, they scored the activity of the enzyme alcohol dehydrogenase (ADH) and plastic responses, measured as changes in ADH activity across ethanol concentrations in the range of 0–10% (natural variation) and 16% (the novel environment). ADH activity increased with alcohol concentration, and there was a positive association between larval viability and ADH activity in the novel environment. They also reported that families exhibiting greater plasticity had higher larval survival in the novel environment, concluding that ADH plasticity is adaptive. However, the four authors now concur that, since the study estimated plasticity from phenotypic differences across environments using full-sib families, it is not possible to disentangle the contributions of allele frequency changes at the *Adh* locus from regulatory control at loci known to influence ADH activity. Selective changes in allele frequencies may thus conflate estimates of plasticity; any type of "plasticity" (adaptive, neutral, or maladaptive) could be inferred depending on allele frequencies. The problem of scoring sib-groups after selection should be considered in any plasticity study that cannot use replicated genotypes. Researchers should monitor changes in allele frequencies as one mechanism to deal with this issue.

KEY WORDS: *Adh* **polymorphism, *Drosophila*, ethanol, phenotypic plasticity.**

Wang and Althoff (2019) recently presented a study to test whether, as mentioned in the literature (e.g., Coulautti et al. 2017), plasticity might facilitate the colonization of novel environments. They analyzed both the mean larvae survival and changes in expression of alcohol dehydrogenase (ADH) of *Drosophila melanogaster* families as a function of increased concentrations of ethanol through a nested full-sib, half-sib mating design (Lynch and Walsh 1998, pp. 570–573). The final goal was to search for an association between a plastic response, measured as changes in ADH expression across ethanol concentrations, and the concomitant adaptive value measured as larval survival at higher concentrations. Can larvae of this species adapt to a novel environment up to a concentration of 16% ethanol? This concentration is unlikely to be found in natural larval substrates, where flies feed and breed on fermenting fruits containing ethanol in concentrations as high as 6–7% (Fry 2014; Zhu and Fry 2015). The scored phenotype in Wang and Althoff (2019) was the activity of the enzyme alcohol dehydrogenase (ADH: NAD^+ oxidoreductase: EC 1.1.1.1.), which plays a key role in the ability of *D. melanogaster* to exploit alcoholic environments. They also estimated plasticity from the phenotypic difference across environments in ADH activity using the means of full-sib families in each environment.

The authors found a substantial increase in third-instar larvae ADH activity in response to increased alcohol concentrations in the food from 0% to 10%, which were taken as to be representative of the range of natural variation; and also in

a concentration of 16%, which was assumed to be the novel alcohol environment. They also found a concomitant decrease in egg to third-instar larvae viability, which dropped to 0.71 (10% alcohol) and 0.24 (16% alcohol) relative to the viability in the control (0% alcohol) environment. Increased ADH activity in the novel 16% environment was positively selected, as was ADH plasticity between 10% to 16% alcohol (with fitness measured as larval survival). Heritable variation for both ADH activity and ADH plasticity were high, with narrow-sense heritabilities $h^2 = 0.557$ and $h^2 = 0.776$, respectively. From these results, Wang and Althoff (2019) concluded that ADH plasticity is an adaptive trait, which is an important finding because adaptive trait plasticity is uncommon (Scheiner 2018).

The standard definition of phenotypic plasticity is the change in the expressed phenotype of a genotype as a function of the environment. However, Wang and Althoff (2019) estimated ADH plasticity from the phenotypic difference across environments in ADH activity using full-sib families as a surrogate of replicated genotypes and, therefore, a key question arises: what was the underlying genetic basis of the phenotypic response to increased alcohol concentrations in their experiments? This goes back to the question (Schlichting and Pigliucci 1993): what is the form of genetic control of phenotypic plasticity? In *D. melanogaster* the *Adh* locus is polymorphic for two common alleles labeled Adh^F (*Adh*-fast) and Adh^S (*Adh*-slow) on the basis of allozyme electrophoretic mobility. The Adh^F allele is generally associated with higher ADH activity than the Adh^S allele, and this activity difference is partly due to a catalytic efficiency difference and partly due to protein quantity that is not mirrored in RNA level (McDonald et al. 1980; Laurie and Stam 1988; Laurie et al. 1991). The rank order of maximum ADH activity is $\mathrm{ADH-FF} > \mathrm{ADH-FS} > \mathrm{ADH-SS}$, with fast homozygotes generally having a two- to three-fold higher activity than slow homozygotes (Middleton and Kacser 1983; Laurie et al. 1991). Therefore, although there are many *cis*- and *trans*-acting genetic factors affecting ADH activity (e.g., Laurie-Ahlberg et al. 1980; Maroni et al. 1982; Corbin and Maniatis 1990), a large part of the genetic variation in ADH activity is structural and associated with the *Adh* protein polymorphism. Most importantly, a number of laboratories have reported that Adh^F genotypes tend to survive better in alcohol stress environments than their Adh^S counterparts both at the adult (e.g., Briscoe et al. 1975; Anderson et al. 1981; Kerver and van Delden 1985) and larval stages (e.g., Morgan 1975; Kerver and van Delden 1985; Heinstra et al. 1987). This means that there is potentially – likely – a role for selective response in polymorphic populations even in a single generation of ethanol stress.

Wang and Althoff's (2019) experimental flies originated from a natural population collected in Syracuse (NY, USA) where Adh^F and Adh^S alleles had been reported to segregate at approximately intermediate frequencies (Berger 1971). However, they did not score the *Adh* enzyme polymorphism in the experimental flies and, consequently, we cannot know to what degree the increased levels of ADH activity in their 16% alcohol environment was due to allelic frequency changes (associated with larval mortality) at the target *Adh* locus and/or to regulatory control associated to genetic variability at other loci. The former will be an evolutionary response, while only the latter can be correctly a plasticity change–meaning different phenotypic values for the same genotype across environments (Ghalambor et al. 2015).

We concur with Schlichting and Pigliucci (1993) in defining plasticity genes as "regulatory loci that exert environmentally dependent control over structural gene expression and thus produce a plastic response." Along this line, selection on standing structural genetic variation at the *Adh* locus adjusting the trait means to a new optimum should be distinguished from the modification of ADH activity through gene expression change, which would be selection for plasticity per se (Schlichting and Pigliucci 1993). Signs of such plasticity are known to affect ADH. Both the second and the third chromosomes are known to contain regulatory regions that affect larval ADH activity (Maroni et al. 1982; Corbin and Maniatis 1990). Furthermore, both Adh^{SS} and Adh^{FF} homozygous larvae are plastic in their ADH activity in response to alcohol, and their plasticity can vary depending on the genetic background (Malherbe et al. 2005). Wang and Althoff's (2019) data do suggest that other loci besides *Adh* structural allelic variants are involved in regulating both ADH activity and ADH plasticity because they observed substantial ADH plasticity between the 0% to 10% alcohol environments, where larval mortality was uncorrelated to ADH activity. However, all the authors of this consensus think that the positive association of ADH plasticity with survival between the 10% to 16% alcohol environments, where higher ADH activity increased larval survival, could have been biased by frequency changes at the *Adh* locus. In what follows, we describe the sort of bias that might occur when Adh^F and Adh^S alleles are segregating in sib-groups.

Problems of Using Sib-Groups as Surrogates of Replicated Genotypes

Table 1 gives the genetic composition of the various sib-groups for the locus *Adh* (assuming random mating) when segregating for the two common alleles $Adh^F(F)$ and $Adh^S(S)$. Let us assume that we perform an experiment using two environments (treatments): environment 1 is "nonselective" and environment 2 is "selective." Nonselective here means that any mortality is random with respect to ADH activity (i.e., *Adh* genotype). This could represent the 0% or 10% alcohol environments in Wang and Althoff (2019) because they did not detect any effects of ADH activity on egg to third-instar larvae viability in the 10% alcohol environment

Table 1. Expected frequencies and distribution of genotypes in sib-groups, and recursion equations for a locus with two alleles – Adh^F (F) and Adh^S (S)– in a random mating population under selection in a stressful environment.

Mating pair	Mating pair frequency	Progeny *FF*	Progeny *FS*	Progeny *SS*	Mean ADH activity	Frequency after selection (q')	Mean ADH activity after selection
$FF \times FF$	p^4	1			y_{FF}	0	y_{FF}
$FF \times FS$	$4p^3q$	1/2	1/2		$\frac{1}{2}y_{FF}+\frac{1}{2}y_{FS}$	$\frac{\frac{1}{4}(1-hs)}{1-\frac{1}{2}hs}$	$\frac{\frac{1}{2}y_{FF}+\frac{1}{2}(1-hs)y_{FS}}{1-\frac{1}{2}hs}$
$FF \times SS$	$2p^2q^2$		1		y_{FS}	$\frac{1}{2}$	y_{FS}
$FS \times FS$	$4p^2q^2$	1/4	1/2	1/4	$\frac{1}{4}y_{FF}+\frac{1}{2}y_{FS}+\frac{1}{4}y_{SS}$	$\frac{\frac{1}{2}-\frac{1}{4}s(h+1)}{1-\frac{1}{2}s(h+\frac{1}{2})}$	$\frac{\frac{1}{4}y_{FF}+\frac{1}{2}(1-hs)y_{FS}+\frac{1}{4}(1-s)y_{SS}}{1-\frac{1}{2}s(h+\frac{1}{2})}$
$FS \times SS$	$4pq^3$		1/2	1/2	$\frac{1}{2}y_{FS}+\frac{1}{2}y_{SS}$	$\frac{\frac{3}{4}-\frac{1}{4}s(h+2)}{1-\frac{1}{2}s(h+1)}$	$\frac{\frac{1}{2}(1-hs)y_{FS}+\frac{1}{2}(1-s)y_{SS}}{1-\frac{1}{2}s(h+1)}$
$SS \times SS$	q^4			1	y_{SS}	1	y_{SS}

The phenotype is the alcohol dehydrogenase (ADH) activity (y_{FF}, y_{FS}, and y_{SS}). The relative fitness of genotypes *FF*, *FS*, and *SS* are $1, 1-hs$, and $1-s$, respectively, where s ($0 < s < 1$) is the selection differential and h ($0 \le h \le 1$) is the degree of dominance.

despite average viability dropping to 0.71 relative to the control (0% ethanol). The selective environment means that there is also mortality with respect to ADH activity (*Adh* genotype) and represents their 16% alcohol environment. Given the genotype-dependent rank order of ADH activity ($y_{FF} > y_{FS} > y_{SS}$; see above), Table 1 assumes that the relative fitness of genotypes *FF*, *FS*, and *SS* are $1, 1-hs$, and $1-s$, respectively, in the selective (high alcohol concentration) environment; where s is a positive constant ($0 < s < 1$) and h ($0 \le h \le 1$) is the degree of dominance (see, e.g., Crow and Kimura 1970, p. 183). The last column in Table 1 gives the change in ADH activity after selection in the different sib-groups.

To numerically illustrate how selection on ADH activity through changes in *Adh* allele frequencies would change the slope across environments in the sib-groups, assume we perform the following full-sib, half-sib experiment along the lines of Wang and Althoff (2019). A total of 200 sires are crossed to three dams each, and six vials for each sire × dam cross are set up with 50 eggs each (to have an accurate representation of genotypes in the offspring). Three vials are allocated to treatment 1 (nonselective environment) and the other three to treatment 2 (selective environment). In treatment 1, egg to third-instar larvae viability is, for example, 0.85 and mortality is random regarding *Adh* genotype. In treatment 2, egg to third-instar larvae viability further decreases as a function of the genotype composition in the sib-groups and viability selection for the *Adh* locus. Assuming higher or lower random mortality would obviously not change the conclusions. Simulation programs were implemented in MATLAB (version R2016b) algebra environment using tools supplied by the Statistics Toolbox (MATLAB and Statistics Toolbox Release 2016). The routine to run the analyses is provided in the Supporting Information.

Because the simulated data was a fully balanced design, variance component estimations were made by conventional least-squares (ANOVA) methods and are also restricted maximum likelihood (REML) solutions (Searle et al. 1992). The following linear model was used:

$$y_{ijkl} = \mu + T_i + S_j + D_{k(j)} + TS_{ij} + TD_{ik(j)} + \varepsilon_{ijkl},$$

where μ is the overall grand mean, y_{ijkl} is the average ADH activity in the ith ($i = 1, 2$) treatment, estimated from the surviving offspring in the lth ($l = 1, 2, 3$) vial from the kth ($k = 1, 2, 3$) dam (nested in sire) mated to sire j($j = 1, 2, \ldots, 200$), and ε_{ijkl} is the residual error. Treatment was a fixed effect; whereas sire, dam, treatment × sire, and treatment × dam(sire) were treated as random effects.

Table 2 gives the results from some simulated data. We have assumed $y_{FF} = 0.80$, $y_{FS} = 0.56$ and $y_{SS} = 0.32$; that is, a two- to three-fold higher activity in fast homozygotes in comparison with slow homozygotes (see above). The frequency of allele Adh^F was assumed to be 0.5, the selective coefficient $s = 0.5$ and the degree of dominance $h = 0.4$. This strong selective coefficient might not be unreasonable in the 16% alcohol environment used by Wang and Althoff (2019) because in this environment viability dropped to 0.34 relative to the viability in the 10% alcohol environment. [Note that for simplicity we ignore any variation in ADH activity within *Adh* structural genotypes, which is known to exist (e.g., Laurie et al. 1991).]

From Table 2, it is clear that the selective environment had a highly significant effect on average ADH activity, and that there was a highly significant treatment × dam(sire) interaction effect, which means that changes in ADH activity across environments [i.e., reaction norms between 10% to 16% alcohol concentration in Figure 2 of Wang and Althoff (2019)] in the full-sib families were not parallel. Also as expected, there was no correlation between ADH activity and viability in treatment 1 (nonselective environment), but this correlation was highly

Table 2. Analysis of variance for ADH activity in the simulated full-sib, half-sib experiment, with full-sib families raised in two treatment environments ("nonselective" and "selective").

Source of variation	d.f.	Sum of squares	Mean square	Error d.f.	Error mean square	F	P	Variance component
Treatment	1	0.3323	0.33232	199	0.000897	370.4	<0.001	
Sire	199	29.1179	0.14632	407.26	0.044459	3.29	<0.001	$\hat{\sigma}^2_S = 0.0056589989$
Dam(Sire)	400	17.6161	0.04404	400	0.000478	92.12	<0.001	$\hat{\sigma}^2_{D(S)} = 0.0072603610$
Treatment × Sire	199	0.1785	0.00090	400	0.000478	1.88	<0.001	$\hat{\sigma}^2_{T\times S} = 0.0000465699$
Treatment × Dam(Sire)	400	0.1912	0.00048	2400	0.000340	1.40	<0.001	$\hat{\sigma}^2_{T\times D(S)} = 0.0000458806$
Error	2400	0.8170	0.00034					$\hat{\sigma}^2_\varepsilon = 0.0003404307$
Total	3599	48.2532						

In the last column, caret notation denotes "an estimate of."

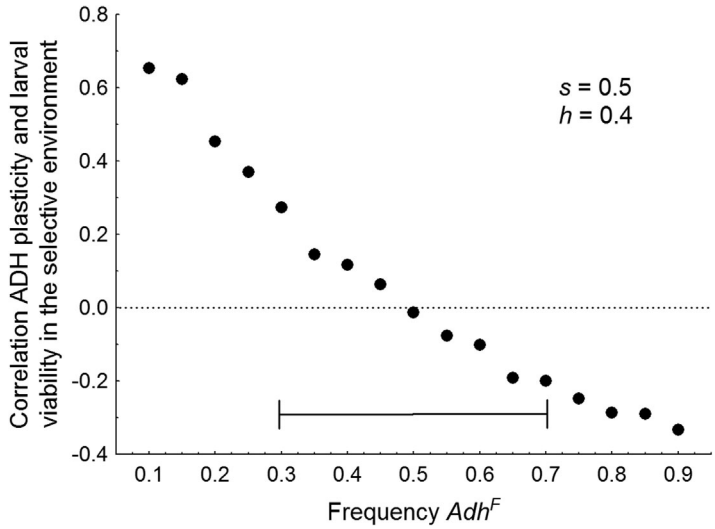

Figure 1. Correlations between ADH plasticity and larval viability in the selective environment, as a function of the frequency of allele Adh^F. The selection coefficient (s) and the degree of dominance (h) used in the simulation are given in the upper right of the graph. Each dot is the correlation estimated from simulations assuming a total of 200 sires crossed to three dams each, with three vials set up with 50 eggs each in each environment (nonselective and selective). The dotted line indicates where the correlation is zero. The horizontal bar gives the likely range of the frequency of allele Adh^F in the Syracuse (NY, USA) population sampled by Wang and Althoff (2019). See Fig. 1 A in Berger (1971).

significant in treatment 2 (selective environment; Pearson correlation $r = 0.9893$, d.f. $= 1798, P < 0.001$). The conclusions remain qualitatively the same if we assume that the frequency of Adh^F is in the range $0.3 - 0.7$ (results not shown), which is a reasonable assumption from Figure 1A in Berger (1971).

The correlation between ADH "plasticity" (i.e., the change in ADH activity of full-sib families between environments) and larval viability in the selective environment was slightly negative and nonsignificant (Pearson correlation $r = -0.0125$, d.f. $= 1798, P = 0.596$), which would suggest neutral ADH plasticity when the frequency of Adh^F equals 0.5. Importantly, from Table 1 it can be appreciated that this correlation will depend on allele frequencies (as well as on relative fitness). Figure 1 shows a plot of the correlation between ADH plasticity and larval viability in the selective environment, as a function of the frequency of allele Adh^F. If the frequency of Adh^F is in the range $0.30 - 0.45$, then this correlation is positive and can be highly significant (e.g., $r = 0.2734$, d.f. $= 1798, P < 0.001$ in a representative simulation with allele frequency 0.3), which would lead to a misleading suggestion of adaptive ADH "plasticity." On the other hand, if the frequency of Adh^F is in the range $0.55 - 0.70$, then the correlation is negative (e.g., $r = -0.1990$, d.f. $= 1798, P < 0.001$ assuming an allele frequency of 0.7) and would suggest maladaptive ADH "plasticity." The reason for this behavior is that below a frequency of 0.5 there will be a relatively high abundance of sib-groups fixed

and/or segregating for the low fitness allele Adh^S that experiences strong viability selection, and the opposite happens when the frequency of Adh^F is above 0.5. To sum up, any type of "plasticity" (adaptive, neutral, or maladaptive) could thus be obtained and, therefore, we can only speculate about the direction of the bias.

Given the above analyses, the four authors concur that changes in ADH activity between environments due to selection on the polymorphic *Adh* locus may conflate estimates of plasticity, as well as its adaptive value.

Summary

We have explained a source of bias that can arise when sib-groups are used as surrogates of replicated genotypes. Interestingly, a recent preprint by Signor and Nuzhdin (2018) has also analyzed plastic responses to high ethanol concentration in *D. melanogaster* and *D. simulans*. Their experimental flies were the F1 offspring obtained by crossing six isogenic male genotypes of each species derived from nature with females from tester stocks. This protocol allowed replicated observations of gene expression in identical twin flies. Adult flies were exposed to 15% ethanol and the results pointed to a lack of genetic variation for plasticity in *D. melanogaster*. These results suggest a different role of ADH plasticity than suggested by Wang and Althoff (2019).

However, aside from methodological differences between the two studies, there are additional differences to consider. The use of adults (Signor and Nuzhdin 2018) versus larvae (Wang and Althoff 2019) is important for interpreting the results, because the regulation of ADH expression is known to be under separate genetic control in these two life stages (Posakony et al. 1985). Additionally, Wang and Althoff (2019) quantified ADH activity rather than overall gene expression changes, and the fitness effect of the latter is much less clear than the former. Furthermore, we do not think that a lack of significant genes for interaction variance in response to alcohol in Signor and Nuzhdin (2018) is sufficient reason to suggest that there is no genetic variation in ADH plasticity.

In summary, the four authors agree that the problem of selection on structural allelic variation is sensible and should be considered in any plasticity study that cannot use replicated genotypes. For circumstances in which plasticity has a direct relationship to fitness because of differential mortality in sib-groups, shifts in allele frequencies at structural loci due to a selective environment will likely influence the phenotypic value of a trait in a novel environment and conflate estimates of plasticity. The magnitude of this effect is determined by the strength of selection and the degree of correlation between the phenotypic values in the nonselective and selective environments. If selection is strong and the correlation between trait values in the nonselective and selective environments is high, this would suggest that plasticity measures may be biased by those shifts. For example, families with the better genotypes would have higher phenotypic values both before and after selection and, thus, these values would be highly correlated. If phenotypic values were not correlated across environments, it is less likely that plasticity estimates are biased by selection.

In other cases, in which the link between plasticity and differential mortality is weak or nonexistent across sib groups, shifts in alleles at the locus under study will likely be much smaller or not occur at all. Moreover, here we highlight a case in which there is a structural locus of major effect with just two alleles. Additional analyses for more quantitative traits are needed to better understand if such a bias would occur as well. Thus, researchers need to consider not only the genetics of the trait under study, but also how selective environments may cause shifts in allele frequencies that contribute to changes in phenotypic values. Ideally, studies of plasticity would monitor changes in allele frequencies and know the effects of these alleles on both overall trait values and trait plasticity. Accomplishing this will be a difficult and laborious task, even in model organisms, but one that will lead to better understanding of the role of plasticity in novel environments. When this is not possible, researchers need to acknowledge that plasticity estimates may be biased when there is differential mortality among environments.

AUTHOR CONTRIBUTIONS

M.S. conceived the idea and performed simulations; M.S and M.M. presented the idea; M.S., M.M., S.P.W. and D.M.A. contributed to the writing of this manuscript.

ACKNOWLEDGMENTS

We thank Prof. Mohamed Noor for encouraging us to find a general agreement on the subject. The manuscript benefited from comments and suggestions from M. Noor and R. Fuller. M.S. is funded by grant CGL2017-89160-P from the Ministerio de Economía, Industria y Competitividad (Spain), and Grant 2017 SGR 01379 from Generalitat de Catalunya. M.M. is funded by cE3c Unit FCT UID/BIA/00329/2013. S.W. is funded by Sigma Xi. D.M.A is funded by grants NSF 1556568 and 1655544.

DATA ARCHIVING

The doi for our data is https://doi.org/10.5061/dryad.2mj7019.

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Associate Editor: R. C. Fuller
Handling Editor: Mohamed A. F. Noor

Supporting Information

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Supporting Information