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Membrane lipid physiology and toxin catabolism underlie ethanol and acetic acid tolerance in *Drosophila melanogaster*

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Summary

Drosophila melanogaster **has evolved the ability to tolerate and utilize high levels of ethanol and acetic acid encountered in its rotting-fruit niche. Investigation of this phenomenon has focused on ethanol catabolism, particularly by the enzyme alcohol dehydrogenase. Here we report that survival under ethanol and acetic acid stress in *D. melanogaster* from high- and low-latitude populations is an integrated consequence of toxin catabolism and alteration of physical properties of cellular membranes by ethanol. Metabolic detoxification contributed to differences in ethanol tolerance between populations and acclimation temperatures *via* changes in both alcohol dehydrogenase and acetyl-CoA synthetase mRNA expression and enzyme activity. Independent of changes in ethanol catabolism, rapid thermal shifts that change membrane fluidity had dramatic effects on ethanol tolerance. Cold temperature treatments upregulated phospholipid metabolism genes and enhanced acetic acid tolerance, consistent with the predicted effects of restoring membrane fluidity. *Phospholipase D* was expressed at high levels in all treatments that conferred enhanced ethanol tolerance, suggesting that this lipid-mediated signaling enzyme may enhance tolerance by sequestering ethanol in membranes as phophatidylethanol. These results reveal new candidate genes underlying toxin tolerance and membrane adaptation to temperature in *Drosophila* and provide insight into how interactions between these phenotypes may underlie the maintenance of latitudinal clines in ethanol tolerance.**

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Key words: acetic acid tolerance, acetyl-CoA synthetase, alcohol dehydrogenase, *Drosophila*, SREBP, ethanol tolerance, lipid-mediated signaling, membrane fluidity, phospholipase D.

Introduction

Ethanol and acetic acid are environmental stressors for *Drosophila* species that inhabit rotting fruit, and adaptations for enhanced utilization of these substances may have allowed for niche expansion in *D. melanogaster* (McKenzie and McKechnie, 1979; McKenzie and Parsons, 1972). Ethanol tolerance in *D. melanogaster* covaries positively with latitude across continents (Cohan and Graf, 1985; David et al., 1986), as does the frequency of the high-activity allele of *alcohol dehydrogenase* (*Adh*) (Berry and Kreitman, 1993; David et al., 1986; Oakeshott et al., 1982), the gene encoding the first enzymatic step in ethanol catabolism. Whereas these latitudinal clines are clearly maintained by natural selection (Berry and Kreitman, 1993), the ecological, physiological and genetic mechanisms underlying this maintenance remain elusive.

The molecular, biochemical and thermostability differences between *Adh* variants have been well characterized (Anderson and McDonald, 1983; Chambers et al., 1984; Geer et al., 1993; Laurie et al., 1990; Milkman, 1976). The *Adh-F* allele confers greater ADH activity, but the *Adh-S* allele can maintain activity at higher temperatures (Anderson and McDonald, 1983; Milkman, 1976). This ecologically relevant trade-off between enzyme thermostability and catalytic activity may be a driving force maintaining observed latitudinal clines in *Adh* allele frequencies. However, it is not clear that adaptive change in ethanol tolerance is always manifested as genotypic change at the *Adh* locus. Laboratory selection for enhanced tolerance has been shown to increase the high-activity ADH-F allozyme frequency (Chakir et al., 1996; van Delden et al., 1975), but in other cases, adaptation to different ethanol environments did not have a consistent effect on *Adh* allele frequencies (Cohan and Graf, 1985; Gibson et al., 1979; Gibson and Wilks, 1988). The lack of a consistent response to selection suggests that genetic background can alter the relationship between *Adh* and ethanol tolerance, and two-locus analyses indicate that this is the case (Bokor and Pecsenye, 1997; Pecsenye et al., 1997; Pecsenye et al., 1994). Furthermore, the strength of the relationship between *Adh* genotype, ADH activity and ethanol

tolerance is population dependent (e.g. Chakir et al., 1993; Merçot et al., 1994). Whereas ADH clearly contributes to ethanol catabolism, other genes and cellular processes must play a significant role in determining ethanol tolerance.

ADH does not function in isolation, but is embedded in a pathway that catabolizes both ethanol and acetic acid to acetyl-CoA (Fig. 1). Acetaldehyde dehydrogenase (ALDH) dehydrogenates the acetaldehyde produced by ADH into acetate, and *Drosophila Aldh* mutants lacking ALDH activity have compromised ethanol tolerance (Fry and Saweikis, 2006). *Aldh* may contribute to the evolution of tolerance in natural populations, as female ALDH activity increased when laboratory *D. melanogaster* populations were evolved on a high-ethanol diet (Fry et al., 2004). Acetyl-CoA synthetase (AcCoAS) ligates both ingested acetate and that produced by ALDH to coenzyme A (CoA) to form acetyl-CoA. Ethanol and acetic acid tolerances are strongly positively correlated across *Drosophila* species and populations of *D. melanogaster* (Chakir et al., 1993; Chakir et al., 1996). The two traits share a common genetic basis that may be due, in part, to AcCoAS, the shared enzyme in the catabolism of both ethanol and acetic acid (Chakir et al., 1996; Chakir et al., 1993). Flux through biochemical pathways depends upon the entire complement of enzymatic steps (Kacser and Burns, 1981), demanding a pathway approach to understanding the contribution of all three enzymes to toxin tolerance.

Survival under toxin stress is a complex physiological process, of which toxin metabolism is only one component. Cell membrane fluidity and phospholipid composition potentially mediate tolerance to both ethanol and acetic acid. Ethanol inserts into the lipid bilayer of cell membranes, increasing fluidity (i.e. decreasing the order of lipids in the bilayer) and disrupting the function of proteins embedded in membranes (Baker and Kramer, 1999; Geer et al., 1993; Rubin and Rottenberg, 1982; Sun and Sun, 1985; Taraschi and Rubin, 1985). Ethanol also modifies membrane lipid composition in mammals and flies through interactions with the lipid-derived signaling enzymes, phospholipases C and D (PLC and PLD) (Baker and Kramer, 1999; Gustavsson, 1995; Hoek and Rubin, 1990; Miller et al., 1993c; Shukla et al., 2001). In the presence of ethanol, PLD converts the membrane phospholipid, phosphatidylcholine (PC), to the abnormal phospholipid, phosphatidylethanol (PEth), disrupting the normal PLD-mediated signaling cascade (Fig. 1). Adaptive changes that increase membrane order or mediate the interaction with lipid-derived signaling may be another mechanism for countering the toxic effects of ethanol.

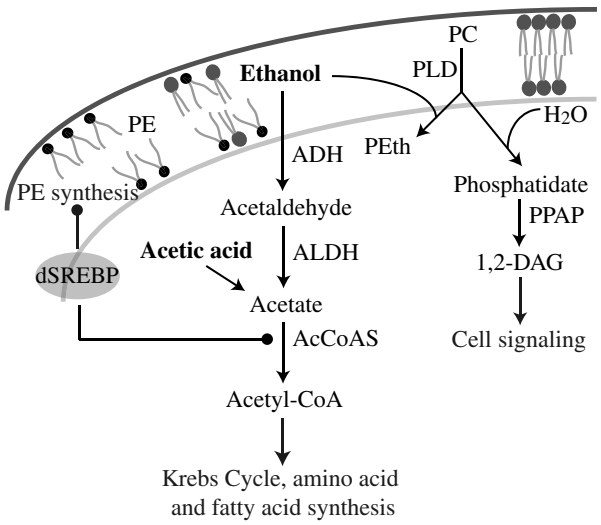

Fig. 1. Systems of genes/enzymes underlying ethanol metabolism and lipid-derived signaling pathways, along with the regulatory effects of the sterol regulatory element binding protein (dSREBP). The two parallel curved lines represent the plasma membrane. AcCoAS, acetyl-CoA synthetase; ADH, alcohol dehydrogenase; ALDH, acetaldehyde dehydrogenase; DAG, diacylglycerol; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PEth, phosphatidylethanol; PLD, phospholipase D; PPAP, phosphatidate phosphatase.

Ectotherms regulate membrane fluidity in response to environmental temperature change, and this homeoviscous or homeophasic adaptation is commonly achieved through altered membrane lipid composition (Cossins and Prosser, 1978; Hazel, 1995; Hazel and Williams, 1990; Hochachka and Somero, 2002; McElhaney, 1984; Sinensky, 1974). *Drosophila* membranes are composed primarily of PC and phosphatidylethanolamine (PE) (Jones et al., 1992), with the latter destabilizing membranes. When PE levels are low, the *Drosophila* sterol regulatory element binding protein (dSREBP) upregulates transcription of genes involved in fatty acid and PE biosynthesis, including *AcCoAS* (Dobrosotskaya et al., 2002; Rawson, 2003). Presumably this regulatory control of *AcCoAS* results from an essential role in fatty acid synthesis. However, this regulation may feed back on ethanol and acetic acid tolerance as a result of the dual role that AcCoAS has in the catabolism of these toxins. Tolerance, particularly of acetic acid, may then be influenced by pathways responding to the state of the lipid membrane. The thermal dependence of membrane fluidity in ectotherms makes these pathways tantalizing candidates underlying the maintenance of latitudinal clines in ethanol tolerance.

Here we report the effect of temperature treatments designed to modify cell membrane fluidity on ethanol and acetic acid tolerance in genetic lines of *D. melanogaster* derived from high- and low-latitude Australian populations. We also quantified the corresponding biochemical and transcriptional responses in systems of genes and enzymes underlying membrane phospholipid regulation and metabolism, as well as the complete ethanol and acetic acid catabolic pathway. Tolerance of ethanol and acetic acid was the integrated result of multiple metabolic and cellular processes that depended on the state of both the detoxification pathway and the cell membrane. These data describe a temperature-dependent relationship between toxin metabolism, cell membrane physiology and survival in the presence of ethanol and acetic acid with implications for the evolution of toxin tolerance in natural populations of *Drosophila*.

Materials and methods

Population samples, experimental design and candidate pathways

The goal of this experiment was to predictably modify membrane fluidity and physiology by modulating temperature and to test for effects on ethanol and acetic acid tolerance. We measured 3–5-day old adult males from ten *Drosophila melanogaster* isofemale lines from each of two Eastern Australia populations [Innisfail (I), 17°S, 146°E and South Tasmania (RK), 42°S, 147°E] that were provided by Dr Ary Hoffmann (University of Melbourne). In an additional experiment, we surveyed ethanol and acetic acid tolerance of *D. melanogaster* isofemale lines from Austria, Pennsylvania and Zimbabwe, as well as the closely related species, *D. mauritiana*, *D. simulans* and *D. yakuba*. Isofemale lines are generated through sib-mating the progeny of single wild-caught females. The intent was to capture a natural genome that will experience little laboratory adaptation, given that there is minimal genetic variation among a single female's progeny. When the experimental environment is controlled, phenotypic differences between lines and populations are due to genetic variation among them. All lines were acclimated to laboratory conditions for over 2 years prior to the experiment.

Flies were reared from eggs in multiple bottles of cornmeal–agar–yeast *Drosophila* medium at controlled densities. Austrian, Pennsylvanian and Zimbabwe *D. melanogaster*, *D. mauritiana*, *D. simulans* and *D. yakuba* males were reared and assayed for ethanol and acetic acid tolerance only at 24°C. Australian populations were reared from eggs at either 15°C or 26°C. Males were assayed at their rearing temperature to quantify the effects of temperature acclimation. Siblings of these males were assayed during a thermal shift from a 15°C rearing temperature to 26°C or from a 26°C rearing temperature to 15°C. A rapid downward temperature shift is expected to reduce membrane fluidity, whereas an upward thermal shift should increase membrane fluidity (Cossins et al., 1981; Hazel, 1995; Hazel and Williams, 1990; Hochachka and Somero, 2002). Flies from all four temperature treatments (two acclimated and two shifted) were assayed for ethanol or acetic acid tolerance.

Across the same temperature treatments, Australian flies were collected for enzyme activity and relative mRNA transcript abundance assays. We measured both activity and mRNA abundance for the three enzymes catalyzing ethanol and acetic acid catabolism, ADH, ALDH and AcCoAS, as well as for the phospholipid signaling enzyme, PLD. PLD and the downstream phosphatidate phosphatase (PPAP), encoded by the gene *wunen*, deplete PC from cell membranes (Fig. 1). We measured relative mRNA abundance of *wunen*, *desat1* [coding for a Δ9-desaturase (Labeur et al., 2002) that potentially increases fatty acid unsaturation in the phospholipid pool] the PE-biosynthesis transcription factor *dSrebp*, and three PE biosynthesis genes, *CDP-ethanolamine diglyceride transferase* (*Cdpet*), *phosphoethanolamine cytidylyltransferase* (*Pect*) and *sphinganine-1-phosphate lyase* (*Sply*) (Dobrosotskaya et al., 2002).

Tolerance assays

We quantified tolerance as the percentage of toxin causing 50% mortality after 48 h (LD_{50}). A tolerance assay consisted of five Parafilm-sealed vials each containing 20 male flies exposed to a gradient of toxin concentrations. Each vial contained a Whatman disk soaked in 1 ml of a 3% sucrose solution supplemented with either 4, 8, 11, 14 or 20% ethanol or 3, 7, 9, 11 or 15% acetic acid. Vials were kept at either 15°C or 26°C, and 48 h later we scored the number of live and dead flies. LD_{50} values were estimated from at least two replicate assays per line within each of the temperature treatments. For each line in each temperature treatment (N=80) we fitted probit regressions, relating mortality to toxin concentration using the SAS PROBIT procedure. From these fitted curves we obtained estimates of the toxin LD_{50} with 95% confidence limits (i.e. the fiducial or inverse confidence limits). Non-overlapping 95% confidence intervals obtained from independent probit regressions are highly conservative tests for differences in LD_{50} (Payton et al., 2003).

Enzyme activities in candidate pathways

We exposed two replicate groups of 20 males (3–5-day old) from each Australian *D. melanogaster* line in each temperature treatment to 1 ml of a 5% ethanol, 3% sucrose solution in a sealed vial. This allows for any ethanol-dependent induction in transcription or translation and attempts to better match the experimental conditions used for expression and activity measures with those used in the tolerance assays. After 24 h we lightly ether-anesthetized, weighed and homogenized the 20 flies in 1 ml of cold homogenization buffer (0.02 mol l^{-1} Tris-HCl, pH 7.5). We added an additional membrane-disrupting buffer to a portion of this homogenate (final concentrations: 0.01 mol l^{-1} Tris-HCl, 0.2 mol l^{-1} sucrose, 1 mmol l^{-1} EDTA, 1 mmol l^{-1} dithiothreitol, 1% Triton X-100, 2 mg ml^{-1} deoxycholic acid). Detection of maximal ALDH activity in *Drosophila* requires this membrane disruption (Anderson and Barnett, 1991; Heinstra et al., 1989; Lietaert et al., 1985). All homogenates were centrifuged at 400 ***g*** for 4 min at 4°C. Portions of homogenates were placed into 96-well UV-transparent plates that were stored at –80°C. Replicate homogenates were assayed twice both within and across plates. Plates were brought to room temperature before kinetic assays were performed in a 96-well plate spectrophotometer (Molecular Devices, Sunnyvale, CA, USA). Each maximal enzyme activity assay was performed at a single controlled temperature across all samples, regardless of the experimental temperature treatment.

ADH (EC 1.1.1.1) oxidizes ethanol to acetaldehyde, and we detected the resulting NADH as an increase in absorbance at 340 nm over 10 min at 24°C. The final concentrations for the 250 μl assay were 2.5 mol l^{-1} reagent alcohol, 5 mmol l^{-1} βNAD, 0.1 mol l^{-1} Tris-HCl, pH 7.5, 1 mmol l^{-1} EDTA and 1.2 flies ml^{-1}. ALDH (EC 1.2.1.3) oxidizes acetaldehyde to acetate, producing NADH. We monitored this reaction at

340 nm for 10 min at 27°C (final concentrations: 1.4 mmol l^{-1} acetaldehyde, 2 mmol l^{-1} βNAD, 2 mmol l^{-1} pyrazole, 25 mmol l^{-1} $Na_4P_2O_7$ pH 10 and 3.75 flies ml^{-1}). ADH can also convert acetaldehyde to acetate, as well as back to ethanol. Pyrazole is a potent inhibitor of ADH activity (Anderson and Barnett, 1991; Heinstra et al., 1989), ensuring that we assayed primarily ALDH activity at this enzymatic step. We measured AcCoAS (EC 6.2.1.1) activity using an enzyme-coupled assay that monitors the pyrophosphate released during the ATP-dependent ligation of acetate and CoA (Upson et al., 1996). To eliminate free phosphate in the fly homogenate, we incubated the reaction with the enzymes for 10 min before adding sodium acetate, CoA and ATP and monitored absorbance at 360 nm at 27°C for 10 min. The final concentrations for this 250 μl assay were 1 mmol l^{-1} sodium acetate, 1 mmol l^{-1} ATP, 1 mmol l^{-1} CoA, 0.2 mmol l^{-1} 2-amino-6-mercapto-7-methylpurine ribonucleoside, 1 U ml^{-1} purine nucleoside phosphorylase, 0.03 U ml^{-1} inorganic pyrophosphatase, 20 mmol l^{-1} Tris-HCl, pH 7.5, 1 mmol l^{-1} $MgCl_2$, 0.1 mmol l^{-1} sodium azide and 0.4 flies ml^{-1}.

PLD (EC 3.1.4.4) cleaves PC to produce phosphatidic acid and choline. We monitored choline production for 10 min at 37°C using an enzyme-coupled fluorescent kinetic assay, with excitation at 530 nm and detection of emission at 590 nm. Addition of choline oxidase oxidizes the resulting choline to betaine and produces H_2O_2. In the presence of a peroxidase and 10-acetyl-3,7-dihydrophenoxazine (Amplex Red), the H_2O_2 is converted to the fluorescent molecule resorufin. The final concentrations for this 200 μl assay were 50 μmol l^{-1} Amplex Red, 1 U ml^{-1} horseradish peroxidase, 0.1 U ml^{-1} choline oxidase, 0.25 mmol l^{-1} PC and 2.5 flies ml^{-1}. Enzymes and reagents for PLD and AcCoAS assays were obtained from Molecular Probes (Eugene, OR, USA).

The activity assays were optimized to have a linear increase in absorbance over the measurement time and saturating substrate levels. Maximal enzyme activities were calculated as the rate of change in absorbance over time for each of the 640 assays per enzyme. Enzyme V_{max} values estimated from these protocols capture variation both in the abundance of enzyme present in the whole fly, as well as any kinetic differences in the enzymes. To control for overall differences in protein abundance between samples we quantified the total protein content for each homogenate using a modified Lowry protocol (Clark and Keith, 1989).

Relative mRNA abundance in candidate pathways

We measured relative mRNA transcript abundances for genes involved in membrane lipid and ethanol metabolic processes (Table S1 in supplementary material) using quantitative real-time PCR amplification of cDNA on an ABI Prism 7000 (Applied Biosystems, Foster City, CA, USA) [for review of qRT-PCR, see Ginzinger (Ginzinger, 2002)]. Searching the annotated *D. melanogaster* genome (FlyBase Consortium, 2003) by the EC enzyme nomenclature for each enzyme, revealed that the majority of our candidates were encoded by a single locus. When multiple possible loci existed, we chose the locus for which there was the most functional information. This does not exclude the possibility that other loci contributed to the enzymatic function we were investigating. For *AcCoAS* we additionally probed two putative genes encoding acetyl-CoA synthetases that have not been functionally characterized (*CG6432* and *CG8732*).

We extracted mRNA using a standard Trizol extraction from each of two replicate groups of 15 3–5-day-old males from each line in each temperature treatment after 24 h exposure to 1 ml of a 5% ethanol, 3% sucrose solution. cDNA was made from each extraction by reverse transcription from the 3′ poly(A)mRNA tail. We probed each gene using minor-groove binding fluorescent probes that spanned exon junctions when possible. We diluted cDNA samples 16-fold and used 10 μl of cDNA in a 50 μl reaction with final concentrations of 200 μmol l^{-1} dATP, dCTP and dGTP, 400 μmol l^{-1} dUTP, 900 nmol l^{-1} each primer, 250 nmol l^{-1} probe and 0.025 U μl^{-1} AmpliTaq Gold. The cycling parameters were a 10 min hold at 95°C followed by at least 30 cycles of 15 s at 95°C and 60 s at 60°C. All reagents were obtained from Applied Biosystems (Foster City, CA, USA).

Relative gene expression was assayed twice per sample and analyzed as the inverse of the relative cycle number at which the amplification curves crossed a set threshold (1/Ct) for each of the 320 mRNA expression assays per gene. We amplified the ribosomal gene *RpL32* in all samples to control for experimental variability in both mRNA extraction and cDNA synthesis. The expression assays were optimized for repeatability and for their efficacy to discriminate levels of transcript (Ståhlberg et al., 2003) by running preliminary assays on serial dilutions of a standard cDNA pool. Primer and probe sequences, as well as optimized $MgCl_2$ concentrations are provided in Table S1 in supplementary material. All supplementary materials are also available from the authors on request.

Adh genotypes

To determine the effect of genetic variants at the *Adh* locus on ethanol tolerance we genotyped the *Adh*-F/S amino acid-altering nucleotide polymorphism and the ∇1 intronic insertion-deletion polymorphism (Kreitman, 1983). The ∇1 polymorphism also shows a latitudinal cline in allele frequency and affects ADH protein levels (Laurie and Stam, 1994). Genotyping was performed on genomic DNA extracted from pools of 20 flies, allowing us to assess the probability that the pools of flies used for phenotyping contained a single allele or both alleles at each of the polymorphic sites. We used a restriction enzyme, *Hpy*CH4IV, to distinguish the *Adh*-F and *Adh*-S alleles, and allele-specific PCR to type the *Adh*-∇1 polymorphism. The resulting products from both assays were visualized using gel electrophoresis. PCR primers, detailed protocols and resulting genotypes for each line are given in Table S2 in supplementary material.

Statistical analyses

For all traits we tested the fixed effects of population, rearing temperature (T_{Rear}), exposure temperature (T_{Expose}) nested in

T_{Rear} and the interactions between population and the two temperature effects. The effect of rearing temperature was used to assess acclimation effects, whereas the effect of exposure temperature nested in rearing temperature tested for effects of the thermal shift. Analyses of variance results are presented in Table 1. Ethanol and acetic acid LD_{50} measures were analyzed using a general linear model. Mixed analysis of variance models of enzyme activity and gene expression data were fitted using maximum likelihood estimation. Mixed models included the random effects of genetic line nested in population and replicate pools of flies. Weight, protein and a plate standard rate were included as covariates of enzyme activity. *RpL32* measures were used as a covariate of mRNA expression. We also tested for the fixed effect of *Adh* genotype on *Adh* expression, ADH activity and ethanol tolerance using a general linear model. All statistical models were fit in SAS (SAS Institute, Cary, NC, USA).

Results

The correlation between ethanol and acetic acid tolerances

Ethanol and acetic acid tolerances were tightly correlated across *Drosophila* species (Pearson correlation coefficient, r=0.925, Fig. 2) and across Austrian, Pennsylvania and Zimbabwe populations of *D. melanogaster* (r=0.904, Fig. 2) reared and assayed at 24°C. The strongest correlation between ethanol and acetic acid tolerances within the Australian populations was observed when flies were acclimated to and assayed at 15°C (r=0.512, Fig. 2). These correlations support the strong positive relationship previously observed for these phenotypes across *Drosophila* species (Chakir et al., 1993; Chakir et al., 1996).

Effects of Adh genotype

Five of the high-latitude Tasmania lines were fixed for the *Adh*-F allele, and five of the low-latitude Innisfail lines were fixed for the *Adh*-S variant (Table S2 in supplementary material). Five lines from each population were segregating both *Adh* genotypes. Consistent with previous observations (Laurie and Stam, 1994), *Adh*-F and *Adh*-S were always associated with the *Adh*-∇1F and *Adh*-∇1S intronic variants, respectively (Table S2 in supplementary material). *Adh* genotype explained significant amounts of the variation in *Adh* expression (F=5.65, P=0.005, R^2=0.128) and ADH activity (F=33.17, P<0.0001, R^2=0.463), but little of the variation in ethanol tolerance (F=3.73, P=0.031, R^2=0.088) observed across treatments (Fig. 3). *Adh* genotype remained a strong predictor of *Adh* expression (F=13.8, P=0.001, R^2=0.27) and ADH activity (F=109.2, P<0.0001, R^2=0.74) and a poor predictor of tolerance (F=3.92, P=0.06, R^2=0.094) when lines still segregating variation at *Adh* (N=10 lines) were removed from the analysis. Although genetic variation at *Adh* underlies much of the variation in the biochemical function of ADH, other loci and cellular processes must contribute to the variation we observed in ethanol tolerance across temperature treatments.

Table 1. *Population and temperature effects on ethanol and acetic acid tolerances, enzyme activities and gene expression*

	Fixed effect									
	Population		T_{Rear}		T_{Expose} (T_{Rear})		Pop×T_{Rear}		Pop×T_{Expose} (T_{Rear})	
Trait	F	P	F	P	F	P	F	P	F	P
Acetic acid tolerance	0.07		23.16	– – – –	2.19		0.40		0.44	
Ethanol tolerance	12.35	+++	45.26	++++	41.96	****	0.37		0.77	
AcCoAS activity (EC 6.2.1.1)	3.71		28.99	++++	3.38	*	1.23		1.86	
AcCoAS expression (CG9390)	5.73	+	9.52	++	3.25	*	3.26		0.71	
CG6432 (putative AcCoAS)	21.59	+++	10.53	++	55.62	****	2.60		0.14	
CG8732 (putative AcCoAS)	2.72		37.76	++++	1.43		0.31		0.89	
ADH activity (EC 1.1.1.1)	51.81	++++	291.2	++++	9.88	****	7.77	**	1.60	
Adh expression (CG3481)	11.58	++	0.17		5.38	**	0.33		1.67	
ALDH activity (EC 1.2.1.3)	0.56		3.07		6.27	**	17.63	****	0.32	
Aldh expression (CG3752)	0.50		90.14	– – – –	1.09		0.63		0.48	
Cdpet expression (CG6016)	0.03		24.12	++++	4.85	**	5.07	*	0.60	
desat1 expression (CG5887)	0.57		18.17	++++	1.09		1.66		1.16	
Pect expression (CG5547)	1.82		8.24	++	0.24		0.60		0.62	
PLD activity (EC 3.1.4.4)	0.05		48.02	++++	4.76	**	5.09	*	0.91	
Pld expression (CG12110)	1.89		4.62	+	6.95	**	2.40		0.6	
Sply expression (CG8946)	5.65	+	4.82	+	18.98	****	3.25		0.24	
Srebp expression (CG8522)	1.92		6.50	–	1.44		0.94		0.57	
wunen expression (CG8804)	0.00		5.99	–	7.63	***	1.68		0.02	

The number of symbols indicates the level of significance from mixed model ANOVAs; +, increased values in Tasmania or 26°C reared flies, –, increased values in Innisfail or 15°C reared flies;* significant at *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.
CG numbers are gene identifiers (FlyBase Consortium, 2003).

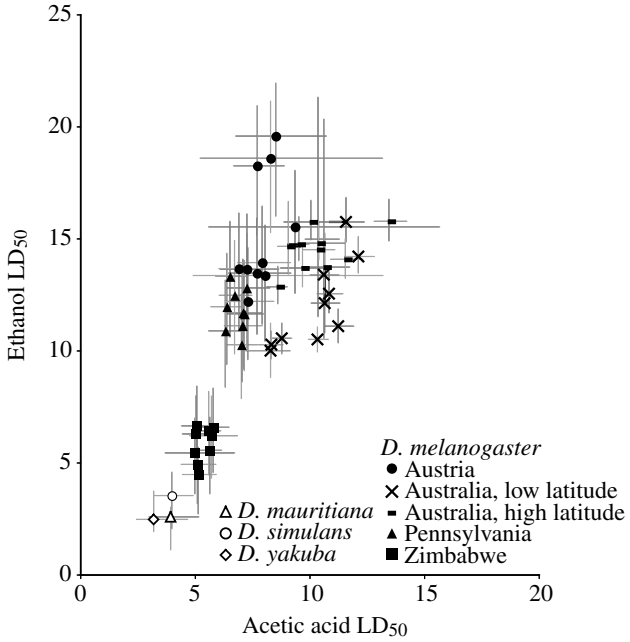

Fig. 2. Correlation between ethanol and acetic acid tolerances across species of *Drosophila* and populations of *D. melanogaster*. Points represent the lethal dose of toxin causing a 50% male mortality (LD_{50}) in each genetic line. Error bars represent 95% confidence intervals for LD_{50} values. Australian population data are from flies acclimated to and tested at 15°C. All others are from a survey of tolerances in flies reared and assayed at 24°C.

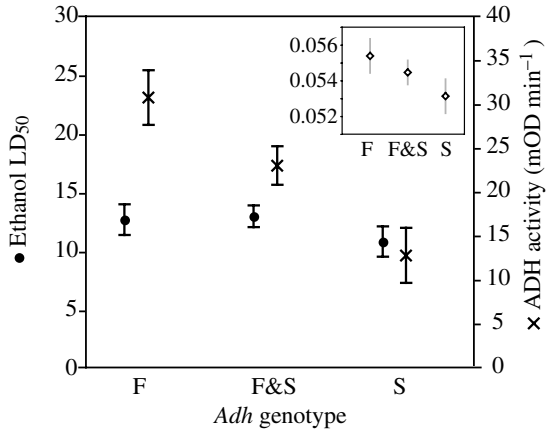

Fig. 3. ADH activities and ethanol tolerances for each *Adh* genotypic class. Lines are categorized as having only the *Adh*-F allele (F, *N*=5 lines), only the *Adh*-S allele (S, *N*=5 lines) or having both alleles (F&S, *N*=10 lines). Data are least square means ± 1 standard error. The inset contains *Adh* expression data (1/Ct) for the three genotype classes.

Differential ethanol tolerance and catabolism between populations and rearing temperatures

Differences in ethanol tolerance were large between extreme lines from the two Australian populations (Fig. 4). However, there was substantial variation in ethanol tolerance within each population, and the range of ethanol tolerances across the two latitudinally extreme Australian populations was well within that of *D. melanogaster* sampled from Africa, N. America and Europe (Fig. 2). The high-latitude Australian population (Tasmania) had significantly greater ethanol tolerance than did the low-latitude Australian population (Innisfail) across acclimation temperatures (Fig. 5A), consistent with previously described latitudinal clines for ethanol tolerance in *D. melanogaster* (Cohan and Graf, 1985; David et al., 1986). The high-latitude population also had significantly greater ADH activity, *Adh* expression and *AcCoAS* expression across rearing temperatures (Fig. 5B,C), suggesting that differences in ethanol catabolism contribute to populational differences in ethanol tolerance.

Acclimation to 26°C enhanced ethanol tolerance relative to 15°C acclimation in both populations (Fig. 5A). Flies reared at 26°C also had greater ADH and AcCoAS activities, as well as increased *AcCoAS* expression, relative to siblings reared at 15°C (Fig. 5B,C). There was a significant interaction effect between population and rearing temperature on ADH activity (Table 1), manifested as a greater response to temperature in the high-latitude Tasmania population. Expression differences between populations and acclimation temperatures for the two putative AcCoAS encoding loci, *CG6432* and *CG8732*, were similar to those of the *AcCoAS* locus (Fig. 5D). Enhanced expression and/or activity of two of the three steps in the ethanol catabolic pathway at 26°C coincided with the enhanced ethanol tolerance observed under high-temperature acclimation. A notable deviation was a much lower level of *Aldh* expression in the more ethanol-tolerant, high-temperature acclimated flies relative to low-temperature acclimated flies (Table 1). However, no change in ALDH activity accompanied this change in gene expression (Table 1).

Induction of membrane lipid biosynthesis and signaling pathways

In addition to changes in ethanol catabolism, the suite of membrane phospholipid biosynthesis genes examined were also differentially expressed across acclimation temperatures. Three genes underlying the final steps of PE synthesis (Fig. 6A) were expressed at higher levels in flies exposed to ethanol after 26°C acclimation relative to 15°C acclimation. Expression of *Sply* and *Cdpet* was population dependent, with the high-latitude Tasmania population having overall higher levels of *Sply* expression and a greater temperature response in *Cdpet* expression (Fig. 6B). *desat1*, a Δ9-desaturase that potentially mediates levels of unsaturated fatty acids, was also more highly expressed at 26°C (Fig. 6B).

The lipid-mediated signaling enzyme, PLD, had higher activity in flies reared at 26°C relative to flies reared at 15°C (Fig. 7A). Changes in gene expression at the *Pld* locus were consistent with this response (Fig. 7B). There was a significant interaction effect with population at the level of enzyme activity, with PLD activity responding more strongly to temperature in the high-latitude Tasmania population (Fig. 7A). The PLD activity assay had high background levels

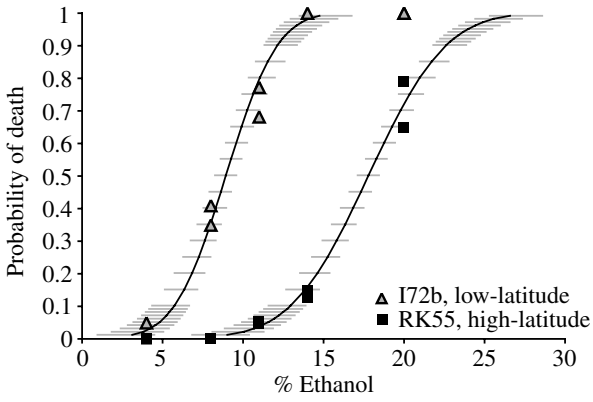

Fig. 4. Mortality curves for extreme high and low ethanol tolerant lines acclimated to and assayed at 26°C. Symbols represent the two replicate observations for each line at each of five ethanol concentrations. Lines with 95% confidence intervals (horizontal bars) are fits from probit regressions from which we obtained estimates of LD_{50}.

of activity, presumably caused by free choline in the fly homogenates. The observed increases in PLD activity may then reflect both increases in enzyme abundance and choline levels.

The temperature-dependent changes in lipid biosynthesis and signaling pathways in the presence of low levels of ethanol may reflect an enhanced physiological response at warmer temperatures to both membrane ethanol and the acetate derived from ethanol catabolism. The greater response at 26°C corresponds with enhanced ethanol tolerance at 26°C. Although it is not clear whether this response in lipid pathways is an adaptive or a physiological/biochemical response, it supports the hypothesis that these pathways affect ethanol tolerance by altering membrane lipid physiology and/or increasing flux through the ethanol catabolic pathway by drawing on the pool of acetyl-CoA.

Rapid thermal shifts, ethanol tolerance and the induction of gene expression

When warm-acclimated ectotherms are placed at 15°C, their cell membranes should become more ordered and less fluid (Hochachka and Somero, 2002; Los and Murata, 2004; Sinensky, 1974). We predicted that this temperature shift would confer enhanced tolerance, because ethanol disrupts membrane function by making membranes more fluid and less ordered. The shift from 26°C to 15°C dramatically enhanced tolerance in both populations, with the mean LD_{50} increasing from 12.3% to 15.3% ethanol (Fig. 8). Conversely, flies acclimated to 15°C and shifted to 26°C should have had more fluid membranes that were more ethanol sensitive. Flies shifted from 15°C to 26°C had a large decrease in LD_{50} of more than 4% ethanol (Fig. 8). The overall effect of these thermal shifts was highly significant and strikingly consistent across lines

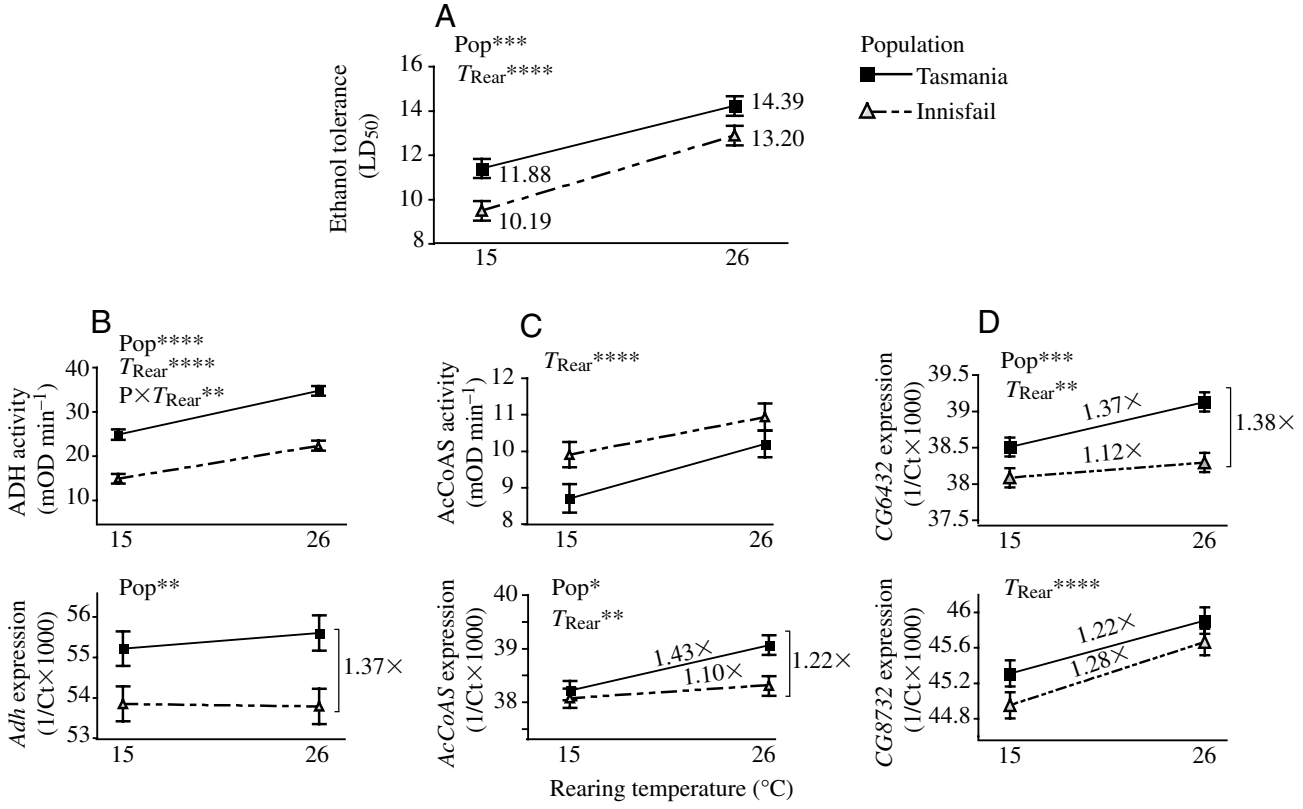

Fig. 5. Population and temperature acclimation effects on ethanol tolerance (A), the ethanol catabolic enzymes, ADH (B), AcCoAS (C) and two putative AcCoAS-encoding loci (D). Shown are least square means ± 1 standard error and significant fixed effects from mixed-model ANOVAs. Fold expression changes were calculated from serial dilution standard curves. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

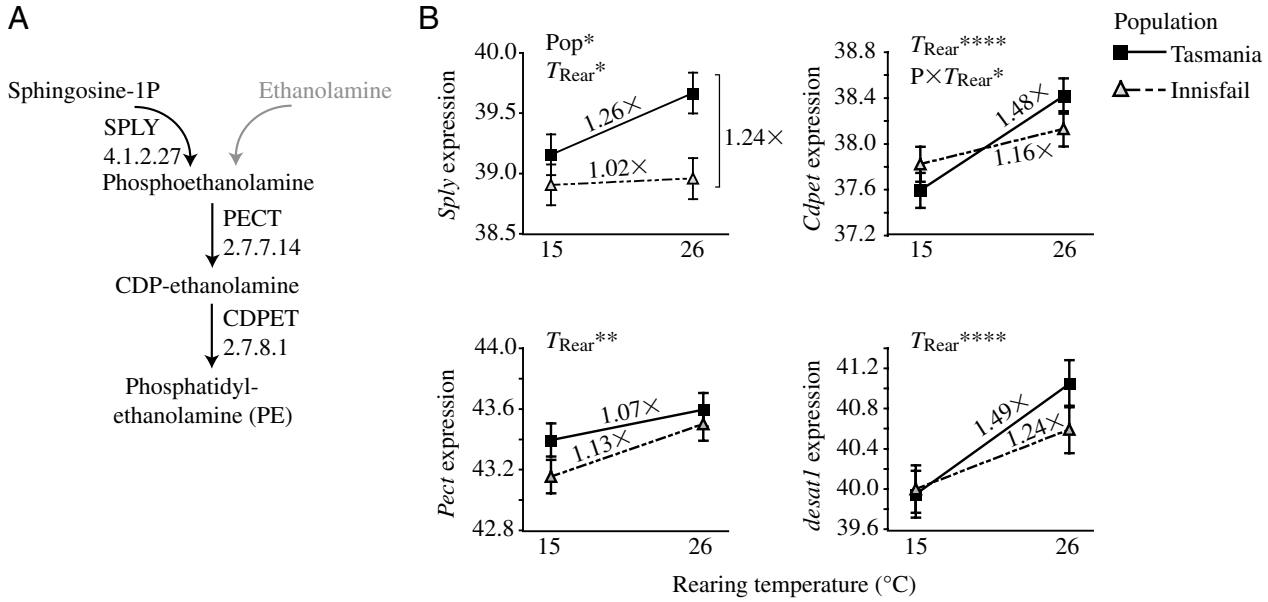

Fig. 6. Phosphatidylethanolamine (PE) biosynthesis pathway (A) and the population and temperature acclimation effects on expression of genes encoding enzymes in this pathway, as well as a fatty acid desaturase (B). Shown are least square means ± 1 standard error and significant fixed effects from mixed-model ANOVAs. Units of expression are (1/Ct)×1000. Fold expression changes were calculated from serial dilution standard curves. CDPET, CDP-ethanolamine diglyceride transferase; PECT, phosphoethanolamine cytidylyltransferase; SPLY, sphinganine-1-phosphate lyase. *P<0.05, **P<0.01, ****P<0.0001.

within both populations (Table 1; Fig. S1 in supplementary material). The magnitude of the thermal shift effects on ethanol tolerance was greater than the observed differences in tolerance between populations and acclimation temperatures. Changes in membrane fluidity and order clearly have the potential to play a critical role in ethanol tolerance.

The effects of rapid thermal shifts on ethanol tolerance were independent of changes in ethanol catabolism. We did not observe corresponding changes in Adh, Aldh or AcCoAS expression or enzyme activity as a result of the temperature shifts. Although there were significant effects of T_{Expose} (T_{Rear}) on Adh and AcCoAS activity and expression (Table 1), activity or expression was lower in the thermal-shift treatments that conferred greater ethanol tolerance. The one exception to this pattern was the putative AcCoAS-encoding locus, *CG6432*, for which significant changes in gene expression levels did correspond to the patterns of ethanol tolerance for the downward thermal shift (discussed below). The lack of an overall correspondence between changes in the ethanol catabolic pathway and changes in ethanol tolerance across thermal shifts, suggests that the temperature effects on ethanol tolerance were independent of changes in toxin metabolism and were presumably the result of changes in membrane fluidity.

In addition, the downward thermal shift induced expression of genes that would counter an increase in membrane order, providing evidence that the thermal shifts did alter cell membrane fluidity and/or order. The PE biosynthesis genes, *Sply* and *Cdpet*, as well as the PC-depleting genes, *Pld* and *wunen*, were expressed at higher levels in flies shifted from 26°C to 15°C (Fig. 9A,B). The protein products of these genes are predicted to decrease the PC/PE ratio, a response that restores membrane order during the initial phase of cold temperature acclimation (Hazel and Landrey, 1988; Pruitt, 1988).

Low temperature acclimation and acetic acid tolerance

We predicted that low-temperature acclimation would have an impact on acetic acid tolerance through pleiotropic effects of dSREBP. AcCoAS detoxifies acetic acid, and expression of *AcCoAS* is regulated by dSREBP in *D. melanogaster* (Dobrosotskaya et al., 2002; Seegmiller et al., 2002). SREBP regulates membrane lipid homeostasis, and there is evidence that this is a direct response to membrane fluidity (Thewke et al., 2000). During cold acclimation when membranes are too ordered, we predicted that dSREBP would be activated to restore membrane order by increasing PE levels and that its targets, including *AcCoAS*, would be transcriptionally upregulated. In this case we would expect greater tolerance, particularly of acetic acid, in flies reared at 15°C. We did not see an increase in ethanol tolerance in cold-acclimated flies (Fig. 5A). However, acetic acid tolerance was significantly enhanced in cold-acclimated relative to warm-acclimated flies, as was *dSrebp* expression (Fig. 10). We did not detect corresponding increases in expression of dSREBP targets, including *AcCoAS*, in cold-acclimated flies. SREBP is post-translationally activated (Rawson, 2003), and the observed increase in *dSrebp* expression probably represents the replenishing of dSREBP protein during acclimation to 15°C. The transcriptional upregulation of dSREBP targets may have occurred during a transient timeframe that we captured for

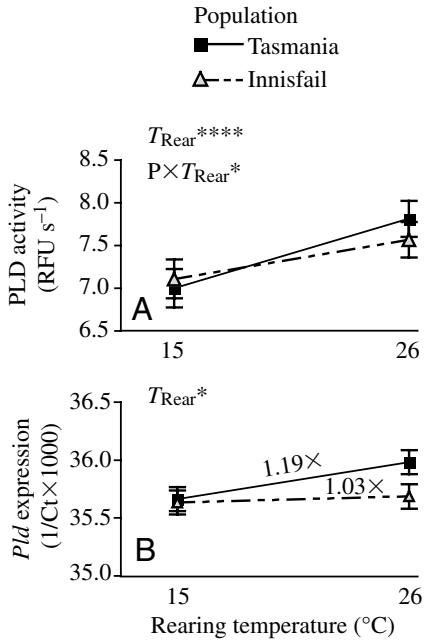

Fig. 7. Population and temperature acclimation effects on activity (A) and expression (B) of the lipid-mediated signaling enzyme, phospholipase D (PLD). Shown are least square means ± 1 standard error and significant fixed effects from mixed-model ANOVAs. Fold expression changes were calculated from serial dilution standard curves. *P<0.05, ****P<0.0001.

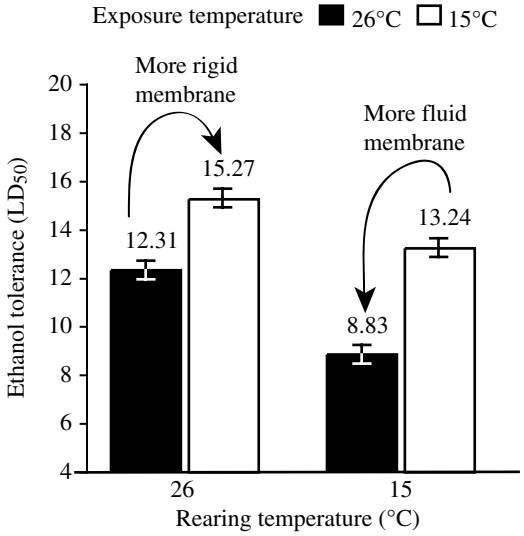

Fig. 8. The effect of rapid thermal shifts on ethanol tolerance. Flies reared at 26°C and shifted to 15°C (left) had increased ethanol tolerance, whereas flies reared at 15°C and shifted to 26°C (right) had decreased tolerance. Data are least square means ± 1 standard error.

several targets during the 24 h rapid cold shift (Fig. 9A), including a putative AcCoAS-encoding locus (Fig. 9C). The flies used for gene expression quantification were exposed to low levels of ethanol rather than acetic acid, which may also explain why we did not observe consistent induction of expression across all putative AcCoAS-encoding loci. Although the observed increase in acetic acid tolerance at low rearing temperatures is consistent with predictions based on membrane acclimation, experiments to dissect the response of AcCoAS to temperature under low acetic acid exposure are needed to understand whether dSREBP may feedback on acetic acid tolerance *via* its regulation of *AcCoAS* expression.

Discussion

The catabolic detoxification and utilization of ethanol

Ethanol catabolism to acetyl-CoA presents a biochemical and physiological challenge, as both of the metabolic intermediates are toxic. The reward of efficient flux through this pathway is an energetically valuable pool of acetyl-CoA that can be converted into fatty acids and amino acids, as well as shunted into the Krebs Cycle. For species that develop, feed and oviposit on rotting fruit, adaptations that increase ethanol catabolism may open up nutrient-rich niches. We observed that increased expression and activity of two of the three ethanol catabolism genes/enzymes, ADH and AcCoAS, accompanied enhanced ethanol tolerance in the Tasmania population and in warm-acclimated flies. Much attention has focused on the evolutionary response of Adh, while AcCoAS has been understudied in this response (Chakir et al., 1993; Chakir et al., 1996). Selection to enhance ethanol tolerance is not always linked to evolutionary change at *Adh* (Cohan and Graf, 1985; Gibson et al., 1979), and the effect of background loci, such as *AcCoAS*, may be critical in elucidating the evolution of tolerance in natural *Drosophila* populations. The toxic nature of the intermediates in this pathway gives some insight into why this may be the case. The control of flux is shared by the full complement of enzymes in a pathway (Kacser and Burns, 1973; Kacser and Burns, 1981). Selection to increase flux through a pathway could result in increased enzymatic activity at all steps. However, allelic combinations at *Adh*, *Aldh* and *AcCoAS* that result in the accumulation of toxic intermediates will decrease survival under ethanol stress. A high activity *Adh* variant may enhance survival when paired with a high activity *AcCoAS* variant, but decrease survival when paired with a low activity *AcCoAS* allele that causes accumulation of acetaldehyde or acetate. Stabilizing selection on metabolic intermediates can lead to correlated activities of the flanking enzymes (Clark, 1991). This is consistent with the correlated changes we observed in Adh and AcCoAS that were predictive of survival under ethanol stress.

We did not observe correlated changes in Aldh. Warm-acclimated, high-ethanol tolerant flies actually had less *Aldh* transcript and similar ALDH activity relative to cold-acclimated flies. Complete loss of function at *Aldh* compromises ethanol tolerance in *D. melanogaster* (Fry and Saweikis, 2006), and laboratory selection for enhanced ethanol tolerance increased ALDH activity (Fry et al., 2004) and acetaldehyde tolerance (Cohan and Hoffmann, 1986).

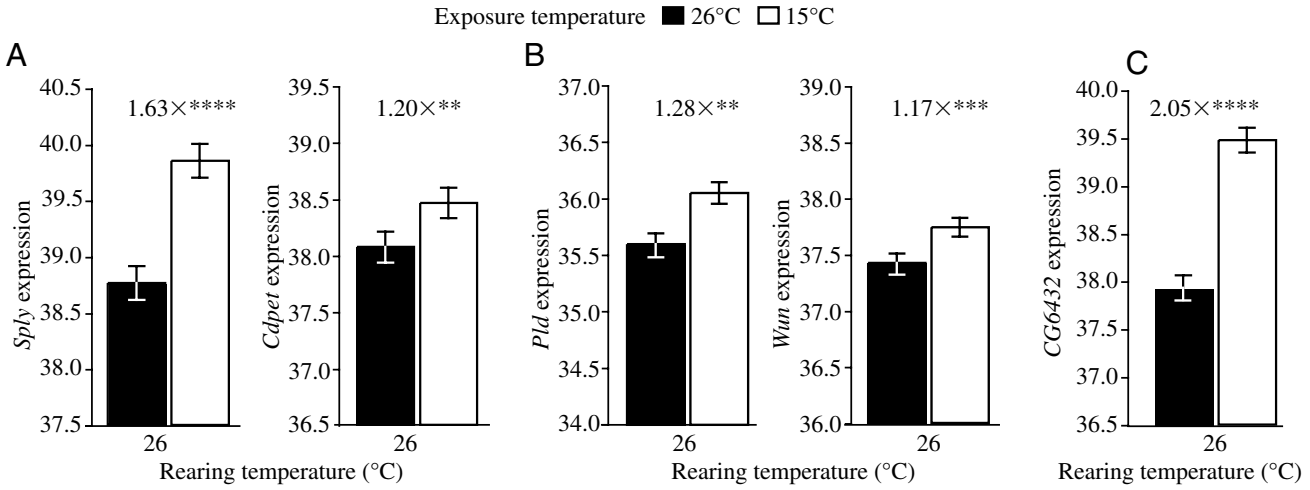

Fig. 9. The effects of a rapid thermal shift from 26°C to 15°C, which should increase membrane rigidity, on the expression of genes involved in phosphatidylethanolamine (PE) biosynthesis (A; *Sply*, *Cdpet*), membrane lipid signaling (B; *Pld*, *Wun*) and encoding a putative AcCoAS (C; *CG6432*). Shown are least square means ± 1 standard error and significant fixed effects of $T_{Expose}(T_{Rear})$ from mixed-model ANOVAs. Units of expression are (1/Ct)×1000. Fold expression changes were calculated from serial dilution standard curves. ***P*<0.01, ****P*<0.001, *****P*<0.0001.

However, the substantial variation we observed in *Aldh* expression across temperature treatments and in ALDH activity between genetic lines was not predictive of ethanol tolerance. ALDH catabolizes the highly toxic intermediate, acetaldehyde, and we might expect strong selection for reduced acetaldehyde accumulation to increase ALDH activity. It is possible that selection may have increased ALDH activity in these populations to the plateau of the concave relationship between enzyme activity and pathway flux, where variation in enzyme activity has little effect on phenotypes related to flux (Hartl et al., 1985; Kacser and Burns, 1981).

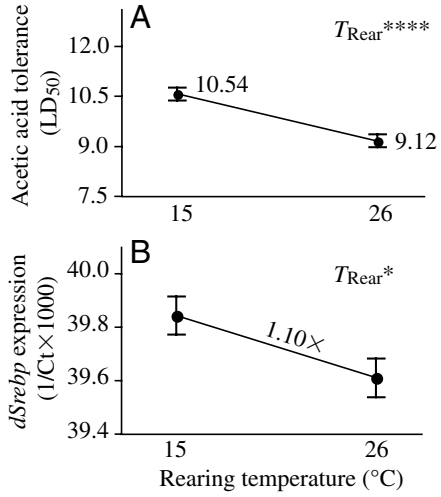

Fig. 10. The response of acetic acid tolerance (A) and *dSrebp* expression (B) to temperature acclimation. Shown are least square means ± 1 standard error and significant fixed effects. **P*<0.05, *****P*<0.0001.

Acclimation to 26°C increased components of ethanol and lipid metabolism relative to 15°C acclimation. Although all flies in our study were exposed to a low dose of ethanol, making it impossible to assess basal *versus* induced levels of mRNA and enzyme activity, this result may still reflect a stronger response of ethanol-inducible pathways at higher temperatures. In *D. melanogaster* there is greater induction of ADH activity at high temperature (Pecsenye et al., 1996). The observed acclimation response is opposite to population differentiation along the latitudinal cline where high-latitude, cold-adapted populations have increased catabolic potential and enhanced tolerance. When the high- and low-latitude populations differed in their response to temperature, it was typically a greater response in the high-latitude Tasmanian population. The high-latitude population appears to have acquired enhanced tolerance through modification of overall levels of ethanol catabolism, as well as an increased ability to induce biochemical detoxification at high temperatures.

Membrane physiology in response to both environmental ethanol and temperature

Catabolism of environmental ethanol may be energetically beneficial, but the presence of ethanol is a toxic challenge to cellular membranes (Baker and Kramer, 1999; Rubin and Rottenberg, 1982; Sun and Sun, 1985; Taraschi and Rubin, 1985). In *Drosophila*, dietary ethanol increases fluidity in deep regions of the lipid bilayer (Miller et al., 1993a). Alteration of membrane fluidity, order and/or content by ethanol disrupts the function of proteins imbedded in membranes, and this may underlie detrimental effects of ethanol on mitochondrial health (Chi and Arneborg, 1999; Rubin and Rottenberg, 1982; Taraschi and Rubin, 1985). Manipulating the phospholipid pool affects ethanol tolerance in yeast (Swan and Watson, 1999; You et al.,

2003) and *Drosophila* (McKechnie and Geer, 1993), presumably by changing physical properties of membranes. Within the range of allowable fluidity, adaptive modification of membranes to resist toxic effects of ethanol may make ethanol-rich foods an accessible habitat for *Drosophila*.

We tested whether membrane fluidity affects ethanol tolerance in *D. melanogaster* without dietary manipulation of the phospholipid pool. Rather, we took advantage of the fact that ectotherm membrane fluidity changes in response to environmental temperature (Cossins and Prosser, 1978; Hazel, 1995; Hazel and Williams, 1990; Hochachka and Somero, 2002; McElhaney, 1984; Sinensky, 1974). Alteration of ectotherm membrane fluidity by modulating environmental temperature is well established (Cossins et al., 1981; Cossins and Prosser, 1978; Los and Murata, 2004; Sinensky, 1974), and this approach has been used to investigate membrane acclimation and adaptation in *E. coli* (Sinensky, 1974), cyanobacteria (Horvath et al., 1998; Los et al., 1993; Los and Murata, 2004; Vigh et al., 1998), crayfish (Pruitt, 1988), crabs (Cuculescu et al., 1999) and fishes (Cossins and Prosser, 1978; Hazel and Landrey, 1988; Hazel et al., 1998; Tiku et al., 1996; Zehmer and Hazel, 2003). The dramatic increase in tolerance that accompanied the inferred increase in membrane order suggests that modification of membrane physiology to counter the membrane-disrupting effects of ethanol could be as important as toxin metabolism in determining ethanol tolerance in *Drosophila*. Although changes in fluidity are a probable mechanism underlying the observed differences in ethanol tolerance, we cannot exclude the role of additional biological changes that may have accompanied the thermal shifts.

The rapid thermal shifts not only indicate that physical properties of membranes impact ethanol tolerance in *Drosophila*, they also reveal ecologically relevant effects of temperature on ethanol tolerance. *D. melanogaster* living in eastern Australia can experience temperature fluctuations similar to those used in this experiment during a 24 h period (Fig. S2 in supplementary material). Our findings indicate that survival of *Drosophila* under ethanol stress in nature should be sensitive to temperature fluctuations and the resulting physiological status of cellular membranes. The effect of membrane fluidity on ethanol tolerance during thermal shifts was similar across populations. However, measures of lipid composition and phase properties of lipid bilayers in these populations will indicate whether adaptive membrane changes contribute to the overall enhanced ethanol tolerance of the high-latitude population.

The ratio of unsaturated to saturated fatty acids in membranes plays a large role in the cellular response to temperature and ethanol (Kajiwara et al., 1996; Los et al., 1993; Swan and Watson, 1999; Tiku et al., 1996; You et al., 2003), making the desaturase gene, *desat1*, a key candidate underlying temperature-dependent changes in ethanol tolerance in *Drosophila*. However, patterns of *desat1* expression were not consistent with predicted responses to temperature or ethanol. We observed no induction of *desat1* 24 h after the downward thermal shift and less *desat1* transcript in cold-acclimated relative to warm-acclimated flies. *desat1* functions in pheromone biosynthesis in *D. melanogaster* (Labeur et al., 2002) and thus may have little influence on membrane lipid desaturation. The *D. melanogaster* genome contains six additional desaturases (Roelofs and Rooney, 2003). Investigation of these desaturases in laboratory-selected and natural populations that differ greatly in ethanol or thermal tolerance will elucidate to what extent alteration of lipid unsaturation contributes to the evolution of these tolerances in *Drosophila*.

The thermal shifts provide insight on the initial phases of homeoviscous or homeophasic adaptation in *D. melanogaster*, a physiological and evolutionary response that has been relatively understudied in *Drosophila*. Cold hardening increases levels of unsaturated to saturated fatty acids in *Drosophila* lipids (Overgaard et al., 2005). A comparative study of Japanese *Drosophila* species supports the role of desaturation in cold-acclimating membranes, but found no evidence of adaptive change in the percentage of unsaturated fatty acids between species (Ohtsu et al., 1998). Consistent with this observation, the high- and low-latitude Australian populations had similar levels of *desat1* expression, although it remains to be determined if *desat1* has a primary role in desaturating fatty acids in *Drosophila*. Changes in the PC/PE ratio also contribute to homeophasic adaptation. PE destabilizes the lipid bilayer, and increased levels of PE relative to PC are thought to maintain membranes in the fluid phase rather than shifting to the gel phase at cold temperatures (Hazel, 1995; McElhaney, 1984). Cold-acclimated ectotherms have lower PC/PE ratios than do warm-acclimated ectotherms (Hazel and Williams, 1990). Increases in PE and decreases in PC accompany the early phase of cold-acclimation in trout (Hazel and Landrey, 1988), and winter-active species of crayfish decrease the PC/PE ratio in response to cold (Pruitt, 1988). We observed an increase in the expression of *Pld* and *wunen* in flies that were rapidly shifted from 26°C to 15°C. These gene products deplete PC from membranes, which should result in a decreased PC/PE ratio. In *Drosophila*, the transcription factor dSREBP responds to physical properties of membranes, inducing expression of genes involved in PE biosynthesis (Dobrosotskaya et al., 2002; Rawson, 2003). Cold-shifted flies had increased expression of two dSREBP targets, *Cdpet* and *Sply*, which should increase synthesis of PE and decrease the PC/PE ratio. These results indicate that modification of the PC/PE ratio through PE biosynthesis and PLD-mediated depletion of PC plays a role in membrane acclimation to temperature in *Drosophila*.

The pleiotropic roles of acetyl-CoA synthetase and phospholipase D

The induction of dSREBP targets in response to downward thermal shifts, coupled with increased *dSrebp* expression in cold-acclimated flies suggests that the dSREBP regulatory cascade responds to the effects of cold temperature on membranes. Because active dSREBP enhances *AcCoAS* expression (Dobrosotskaya et al., 2002; Seegmiller et al., 2002), we predicted that cold-acclimating flies would have

increased *AcCoAS* expression. In this way membrane acclimation to temperature might feed back onto ethanol and acetic acid tolerance *via* increased flux through the catabolic pathway. We observed significantly enhanced acetic acid tolerance in cold-acclimated flies, but no similar increase in ethanol tolerance. This is potentially due to lower levels of Adh expression and activity in cold-acclimated flies, which would impact ethanol but not acetic acid tolerance. What remains to be determined is whether increased levels of AcCoAS activity underlie this enhanced acetic acid tolerance at low temperature. A putative AcCoAS encoding locus was induced in flies shifted to cold temperature, suggesting that AcCoAS activity could be enhanced in cold-acclimating flies, but this was not the locus previously shown to be under transcriptional control of dSREBP (Dobrosotskaya et al., 2002; Seegmiller et al., 2002). In addition, flies used for expression and enzyme activity measurements in this experiment were induced with ethanol, not acetic acid, making it impossible to infer the response to acetic acid induction. Although whole-organism survival under acetic acid stress was consistent with predictions based on the regulation of membrane physiology, the role of AcCoAS in this response needs further investigation.

AcCoAS has dual biochemical roles, responding to both an increased need for fatty acid synthesis and the presence of ethanol and acetic acid. At times these may be complementary roles, as increased availability of environmental ethanol and acetic acid will increase availability of acetate for flux into lipids. However, this pleiotropy may also constrain the role of AcCoAS in metabolizing acetic acid and ethanol. In the absence of active dSREBP, levels of *AcCoAS* were 20% of normal levels (Dobrosotskaya et al., 2002), suggesting that the availability of AcCoAS for acetic acid detoxification may be dependent upon the status of phospholipid levels in cellular membranes. The *D. melanogaster* genome has three putative AcCoAS-encoding loci, and experiments to detect differential expression of these loci in response to cold-temperature and acetic acid stress will be informative in understanding whether the roles of AcCoAS may be decoupled across loci.

Dietary ethanol has an impact on membrane lipid-mediated signal transduction in mammals (Gustavsson, 1995; Hoek and Rubin, 1990; Shukla et al., 2001) and *Drosophila* (Miller et al., 1993b; Miller et al., 1993c). It is unclear whether this is a physiological response or an adaptation to dietary ethanol. Ethanol effectively competes with water as a substrate for PLD, resulting in the accumulation of phosphatidylethanol (PEth) at the expense of the normal signaling molecule, 1,2-DAG (Fig. 1) (Gustavsson, 1995). PEth increases membrane fluidity, but the presence of PEth in membrane lipid bilayers may also confer some tolerance to ethanol-induced membrane disruption (Omodeo-Sale et al., 1991). In our experiments, *Pld* expression and PLD activity were increased in the more ethanol tolerant warm-acclimated and thermally down-shifted flies. The induction of *Pld* may have been a response to temperature (see above), but it coincided with and may have contributed to the enhanced ethanol tolerance observed in these *D. melanogaster* populations. If PEth is less toxic to membranes than is free ethanol, then PLD might confer enhanced tolerance by sequestering ethanol in membranes as PEth.

PLD and AcCoAS have highly conserved signaling and biochemical function. Our results suggest that both enzymes also have the capacity to confer enhanced tolerance of environmental toxins in *D. melanogaster*. The pleiotropic roles of both enzymes make them interesting, physiologically, as they respond to the simultaneous environmental inputs of temperature, ethanol and acetic acid, but also evolutionarily, as they evolve under a mixture of evolutionary forces. The loci encoding PLD and AcCoAS probably experience strong stabilizing selection to maintain their critical functions in cellular signaling and lipid homeostasis. Yet, given their potential to mediate ethanol tolerance, it is intriguing to understand how these genes have evolved along the *D. melanogaster* lineage as this species has diverged to tolerate higher levels of ethanol and acetic acid.

Implications for the maintenance of latitudinal clines in ethanol tolerance

Temperature is a natural candidate for the ecological pressure maintaining worldwide latitudinal clines in *D. melanogaster* ethanol tolerance and *Adh* allele frequencies. *Adh*-S is at higher frequencies at warmer latitudes, and it encodes the more thermotolerant but lower activity ADH-S protein variant. *Adh-S* is also associated with the *In(2L)t* inversion, which confers a fitness advantage at high temperatures (van Delden and Kamping, 1997). Our results support the importance of maintaining efficient biochemical flux through the ethanol catabolic pathway but also suggest that the response of cellular membranes to temperature impacts tolerance. Flies from high latitudes must acclimate and adapt to lower temperatures. If this involves regulation of membrane lipids *via* dSREBP activation, then high-latitude flies should have higher baseline or inducible levels of AcCoAS activity. Provided that flux is not limited by the upstream steps, an increase in AcCoAS should increase flux of both ethanol and acetic acid through the detoxification pathway, contributing to enhanced tolerance of both toxins. Thus, temperature potentially mediates the maintenance of latitudinal clines in tolerance both through selection for thermotolerant catabolic alleles at warm latitudes and through membrane homeostatic responses to temperature gradients across latitudes.

To the extent that temperature and toxin stress together impact *Drosophila* fitness, selection should shape genetic variation within the toxin metabolic pathway, in membrane lipid composition and regulation, and in the interactions between these processes. Investigation of toxin tolerance in temperature-selected populations, as well as membrane adaptations in populations selected for enhanced toxin tolerance, will be invaluable for understanding how multiple selection pressures drive the evolution of this physiological performance phenotype in natural populations.

Conclusions

The ability of *D. melanogaster* to utilize the high levels of ethanol and acetic acid found in their ecological niche requires

a dynamic and temperature-dependent suite of genetic, biochemical and physiological responses. Our findings suggest that environmental temperature mediates the ability of *Drosophila* to tolerate ethanol in their habitat through alterations of both membrane physiology and biochemical flux through the ethanol catabolic pathway. Temperature may also mediate tolerance to environmental acetic acid *via* feedback from the dSREBP regulatory cascade on *acetyl-CoA synthetase*. These observations move us away from a single-gene understanding of ethanol tolerance towards an understanding of how several systems of genes controlling multiple physiological responses interact to determine survival under a mixture of environmental pressures.

List of abbreviations

AcCoAS	acetyl-CoA synthetase
ADH	alcohol dehydrogenase
ALDH	acetaldehyde dehydrogenase
Cdpet	*CDP-ethanolamine diglyceride transferase*
CoA	coenzyme A
DAG	diacylglycerol
dSREBP	*Drosophila* sterol regulatory element binding protein
PC	phosphatidylcholine
PE	phosphatidylethanolamine
Pect	*phosphoethanolamine cytidylyltransferase*
PEth	phosphatidylethanol
PLD	phospholipase D
PPAP	phosphatidate phosphatase
qRT-PCR	quantitative real-time PCR
Sply	*sphinganine-1-phosphate lyase*

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References

Anderson, S. M. and Barnett, S. E. (1991). The involvement of alcohol dehydrogenase and aldehyde dehydrogenase in alcohol/aldehyde metabolism in *Drosophila melanogaster*. *Genetica* **83**, 99-106.

Anderson, S. M. and McDonald, J. F. (1983). Biochemical and molecular analysis of naturally occurring *Adh* variants in *Drosophila melanogaster*. *Proc. Natl. Acad. Sci. USA* **80**, 4798-4802.

Baker, R. C. and Kramer, R. E. (1999). Cytotoxicity of short-chain alcohols. *Annu. Rev. Pharmacol. Toxicol.* **39**, 127-150.

Berry, A. and Kreitman, M. (1993). Molecular analysis of an allozyme cline: alcohol dehydrogenase in *Drosophila melanogaster* on the east coast of North America. *Genetics* **134**, 869-893.

Bokor, K. and Pecsenye, K. (1997). Strains of *Drosophila melanogaster* differ in alcohol tolerance. *Hereditas* **126**, 103-113.

Chakir, M., Peridy, O., Capy, P., Pla, E. and David, J. R. (1993). Adaptation to alcoholic fermentation in *Drosophila*: a parallel selection imposed by environmental ethanol and acetic acid. *Proc. Natl. Acad. Sci. USA* **90**, 3621-3625.

Chakir, M., Capy, P., Genermont, J., Pla, E. and David, J. R. (1996). Adaptation to fermenting resources in *Drosophila melanogaster*: ethanol and acetic acid tolerances share a common genetic basis. *Evolution* **50**, 767-776.

Chambers, G. K., Wilks, A. V. and Gibson, J. B. (1984). Variation in the biochemical properties of the *Drosophila* alcohol dehydrogenase allozymes. *Biochem. Genet.* **22**, 153-168.

Chi, Z. and Arneborg, N. (1999). Relationship between lipid composition, frequency of ethanol-induced respiratory deficient mutants, and ethanol tolerance in *Saccharomyces cerevisiae*. *J. Appl. Microbiol.* **86**, 1047-1052.

Clark, A. G. (1991). Mutation-selection balance and metabolic control theory. *Genetics* **129**, 909-923.

Clark, A. G. and Keith, L. E. (1989). Rapid enzyme kinetic assays of individual *Drosophila* and comparisons of field-caught *D. melanogaster* and *D. simulans*. *Biochem. Genet.* **27**, 263-277.

Cohan, F. M. and Graf, J. (1985). Latitudinal cline in *Drosophila melanogaster* for knockdown resistance to ethanol fumes and for rates of response to selection for further resistance. *Evolution* **39**, 278-293.

Cohan, F. M. and Hoffmann, A. A. (1986). Genetic divergence under uniform selection. II. Different responses to selection for knockdown resistance to ethanol among *Drosophila melanogaster* populations and their replicate lines. *Genetics* **114**, 145-164.

Cossins, A. R. and Prosser, C. L. (1978). Evolutionary adaptation of membranes to temperature. *Proc. Natl. Acad. Sci. USA* **75**, 2040-2043.

Cossins, A. R., Bowler, K. and Prosser, C. L. (1981). Homeoviscous adaptation and its effect upon membrane-bound proteins. *J. Therm. Biol.* **6**, 183-187.

Cuculescu, M., Pearson, T., Hyde, D. and Bowler, K. (1999). Heterothermal acclimation: an experimental paradigm for studying the control of thermal acclimation in crabs. *Proc. Natl. Acad. Sci. USA* **96**, 6501-6505.

David, J. R., Merçot, H., Capy, P., McEvey, S. F. and Van Herrewege, J. (1986). Alcohol tolerance and *Adh* gene frequencies in European and African populations of *Drosophila melanogaster*. *Genet. Sel. Evol.* **18**, 405-416.

Dobrosotskaya, I. Y., Seegmiller, A. C., Brown, M. S., Goldstein, J. L. and Rawson, R. B. (2002). Regulation of SREBP processing and membrane lipid production by phospholipids in *Drosophila*. *Science* **296**, 879-883.

FlyBase Consortium (2003). The FlyBase database of the Drosophila genome projects and community literature [http://flybase.org/]. *Nucleic Acids Res.* **31**, 172-175.

Fry, J. D. and Saweikis, M. (2006). *Aldehyde dehydrogenase* is essential for both adult and larval ethanol resistance in *Drosophila melanogaster*. *Genet. Res.* **87**, 87-92.

Fry, J. D., Bahnck, C. M., Mikucki, M., Phadnis, N. and Slattery, W. C. (2004). Dietary ethanol mediates selection on aldehyde dehydrogenase activity in *Drosophila melanogaster*. *Integr. Comp. Biol.* **44**, 275-283.

Geer, B. W., Heinstra, P. W. and McKechnie, S. W. (1993). The biological basis of ethanol tolerance in *Drosophila*. *Comp. Biochem. Physiol.* **105B**, 203-229.

Gibson, J. B. and Wilks, A. V. (1988). The alcohol dehydrogenase polymorphism of *Drosophila melanogaster* in relation to environmental ethanol, ethanol tolerance and alcohol dehydrogenase activity. *Heredity* **60**, 403-414.

Gibson, J. B., Lewis, N., Adena, M. A. and Wilson, S. R. (1979). Selection for ethanol tolerance in two populations of *Drosophila melanogaster* segregating alcohol dehydrogenase allozymes. *Aust. J. Biol. Sci.* **32**, 387-398.

Ginzinger, D. G. (2002). Gene quantification using real-time quantitative PCR: an emerging technology hits the mainstream. *Exp. Hematol.* **30**, 503-512.

Gustavsson, L. (1995). ESBRA 1994 award lecture. Phosphatidylethanol formation: specific effects of ethanol mediated via phospholipase D. *Alcohol Alcohol.* **30**, 391-406.

Hartl, D. L., Dykhuizen, D. E. and Dean, A. M. (1985). Limits of adaptation: the evolution of selective neutrality. *Genetics* **111**, 655-674.

Hazel, J. R. (1995). Thermal adaptation in biological membranes: is homeoviscous adaptation the explanation? *Annu. Rev. Physiol.* **57**, 19-42.

Hazel, J. R. and Landrey, S. R. (1988). Time course of thermal adaptation in plasma membranes of trout kidney. II. Molecular species composition. *Am. J. Physiol.* **255**, R628-R634.

Hazel, J. R. and Williams, E. E. (1990). The role of alterations in membrane lipid composition in enabling physiological adaptation of organisms to their physical environment. *Prog. Lipid Res.* **29**, 167-227.

Hazel, J. R., McKinley, S. J. and Gerrits, M. F. (1998). Thermal acclimation of phase behavior in plasma membrane lipids of rainbow trout hepatocytes. *Am. J. Physiol.* **275**, R861-R869.

Heinstra, P. W., Geer, B. W., Seykens, D. and Langevin, M. (1989). The metabolism of ethanol-derived acetaldehyde by alcohol dehydrogenase (EC 1.1.1.1) and aldehyde dehydrogenase (EC 1.2.1.3) in *Drosophila melanogaster* larvae. *Biochem. J.* **259**, 791-797.

Hochachka, P. W. and Somero, G. N. (2002). *Biochemical Adaptation:*

Mechanism and Process in Physiological Evolution. Oxford: Oxford University Press.

Hoek, J. B. and Rubin, E. (1990). Alcohol and membrane-associated signal transduction. *Alcohol Alcohol.* **25**, 143-156.

Horvath, I., Glatz, A., Varvasovszki, V., Torok, Z., Pali, T., Balogh, G., Kovacs, E., Nadasdi, L., Benko, S., Joo, F. et al. (1998). Membrane physical state controls the signaling mechanism of the heat shock response in *Synechocystis* PCC **6803**, identification of *hsp17* as a 'fluidity gene'. *Proc. Natl. Acad. Sci. USA* **95**, 3513-3518.

Jones, H. E., Harwood, J. L., Bowen, I. D. and Griffiths, G. (1992). Lipid composition of subcellular membranes from larvae and prepupae of *Drosophila melanogaster*. *Lipids* **27**, 984-987.

Kacser, H. and Burns, J. A. (1973). The control of flux. *Symp. Soc. Exp. Biol.* **27**, 65-104.

Kacser, H. and Burns, J. A. (1981). The molecular basis of dominance. *Genetics* **97**, 639-666.

Kajiwara, S., Shirai, A., Fujii, T., Toguri, T., Nakamura, K. and Ohtaguchi, K. (1996). Polyunsaturated fatty acid biosynthesis in *Saccharomyces cerevisiae*: expression of ethanol tolerance and the *FAD2* gene from *Arabidopsis thaliana*. *Appl. Environ. Microbiol.* **62**, 4309-4313.

Kreitman, M. (1983). Nucleotide polymorphism at the *alcohol dehydrogenase* locus of *Drosophila melanogaster*. *Nature* **304**, 412-417.

Labeur, C., Dallerac, R. and Wicker-Thomas, C. (2002). Involvement of *desat1* gene in the control of *Drosophila melanogaster* pheromone biosynthesis. *Genetica* **114**, 269-274.

Laurie, C. C. and Stam, L. F. (1994). The effect of an intronic polymorphism on *alcohol dehydrogenase* expression in *Drosophila melanogaster*. *Genetics* **138**, 379-385.

Laurie, C. C., Heath, E. M., Jacobson, J. W. and Thomson, M. S. (1990). Genetic basis of the difference in *alcohol dehydrogenase* expression between *Drosophila melanogaster* and *Drosophila simulans*. *Proc. Natl. Acad. Sci. USA* **87**, 9674-9678.

Lietaert, M. C., Libion-Mannaert, M., Wattiaux-De Coninck, S. and Elens, A. (1985). *Drosophila melanogaster* aldehyde dehydrogenase. *Experientia* **41**, 57-58.

Los, D. A. and Murata, N. (2004). Membrane fluidity and its roles in the perception of environmental signals. *Biochim. Biophys. Acta* **1666**, 142-157.

Los, D., Horvath, I., Vigh, L. and Murata, N. (1993). The temperature-dependent expression of the desaturase gene *desA* in *Synechocystis* PCC6803. *FEBS Lett.* **318**, 57-60.

McElhaney, R. N. (1984). The relationship between membrane lipid fluidity and phase state and the ability of bacteria and mycoplasmas to grow and survive at various temperatures. *Biomembranes* **12**, 249-278.

McKechnie, S. W. and Geer, B. W. (1993). Long-chain dietary fatty acids affect the capacity of *Drosophila melanogaster* to tolerate ethanol. *J. Nutr.* **123**, 106-116.

McKenzie, J. A. and McKechnie, S. W. (1979). A comparative study of resource utilization in natural populations of *Drosophila melanogaster* and *D. simulans*. *Oecologia* **40**, 299-309.

McKenzie, J. A. and Parsons, P. A. (1972). Alcohol tolerance: an ecological parameter in the relative success of *Drosophila melanogaster* and *Drosophila simulans. Oecologia* **10**, 373-388.

Merçot, H., Defaye, D., Capy, P., Pla, E. and David, J. R. (1994). Alcohol tolerance, ADH activity, and ecological niche of *Drosophila* species. *Evolution* **48**, 749-757.

Milkman, R. (1976). Further evidence of thermostability variation within electrophoretic mobility classes of enzymes. *Biochem. Genet.* **14**, 383-387.

Miller, R. R., Jr, Dare, A. O., Moore, M. L., Kooser, R. G. and Geer, B. W. (1993a). Long-chain fatty acids and ethanol affect the properties of membranes in *Drosophila melanogaster* larvae. *Biochem. Genet.* **31**, 113-131.

Miller, R. R., Jr, Yates, J. W. and Geer, B. W. (1993b). Dietary ethanol reduces phosphatidylcholine levels and inhibits the uptake of dietary choline in *Drosophila melanogaster* larvae. *Comp. Biochem. Physiol. Comp. Physiol.* **104**, 837-844.

Miller, R. R., Jr, Yates, J. W. and Geer, B. W. (1993c). Dietary ethanol stimulates the activity of phosphatidylcholine-specific phospholipase D and the formation of phosphatidylethanol in *Drosophila melanogaster* larvae. *Insect Biochem. Mol. Biol.* **23**, 749-755.

Oakeshott, J. G., Gibson, J. B., Anderson, P. R., Knibb, W. R., Anderson, D. G. and Chambers, G. K. (1982). *Alcohol dehydrogenase* and *glycerol-3-phosphate dehydrogenase* clines in *Drosophila melanogaster* on different continents. *Evolution* **36**, 86-96.

Ohtsu, T., Kimura, M. T. and Katagiri, C. (1998). How *Drosophila* species acquire cold tolerance – qualitative changes of phospholipids. *Eur. J. Biochem.* **252**, 608-611.

Omodeo-Sale, F., Lindi, C., Palestini, P. and Masserini, M. (1991). Role of phosphatidylethanol in membranes. Effects on membrane fluidity, tolerance to ethanol, and activity of membrane-bound enzymes. *Biochemistry* **30**, 2477-2482.

Overgaard, J., Sorensen, J. G., Petersen, S. O., Loeschcke, V. and Holmstrup, M. (2005). Changes in membrane lipid composition following rapid cold hardening in *Drosophila melanogaster*. *J. Insect Physiol.* **51**, 1173-1182.

Payton, M. E., Greenstone, M. H. and Schenker, N. (2003). Overlapping confidence intervals or standard error intervals: what do they mean in terms of statistical significance? *J. Insect Sci.* **3**, 34.

Pecsenye, K., Lefkovitch, L. P., Giles, B. E. and Saura, A. (1994). The influence of the *Odh-Aldox* region of the third chromosome on the response of *Drosophila melanogaster* to environmental alcohol. *Hereditas* **121**, 237-248.

Pecsenye, K., Lefkovitch, L. P., Giles, B. E. and Saura, A. (1996). Differences in environmental temperature, ethanol and sucrose associated with enzyme activity and weight changes in *Drosophila melanogaster*. *Insect Biochem. Mol. Biol.* **26**, 135-145.

Pecsenye, K., Bokor, K., Lefkovitch, L. P., Giles, B. E. and Saura, A. (1997). Enzymatic responses of *Drosophila melanogaster* to long- and short-term exposures to ethanol. *Mol. Gen. Genet.* **255**, 258-268.

Pruitt, N. L. (1988). Membrane lipid composition and overwintering strategy in thermally acclimated crayfish. *Am. J. Physiol.* **254**, R870-R876.

Rawson, R. B. (2003). The SREBP pathway – insights from Insigs and insects. *Nat. Rev. Mol. Cell Biol.* **4**, 631-640.

Roelofs, W. L. and Rooney, A. P. (2003). Molecular genetics and evolution of pheromone biosynthesis in Lepidoptera. *Proc. Natl. Acad. Sci. USA* **100**, 9179-9184.

Rubin, E. and Rottenberg, H. (1982). Ethanol-induced injury and adaptation in biological membranes. *Fed. Proc.* **41**, 2465-2471.

Seegmiller, A. C., Dobrosotskaya, I., Goldstein, J. L., Ho, Y. K., Brown, M. S. and Rawson, R. B. (2002). The SREBP pathway in *Drosophila*: regulation by palmitate, not sterols. *Dev. Cell* **2**, 229-238.

Shukla, S. D., Sun, G. Y., Gibson Wood, W., Savolainen, M. J., Alling, C. and Hoek, J. B. (2001). Ethanol and lipid metabolic signaling. *Alcohol. Clin. Exp. Res.* **25**, 33S-39S.

Sinensky, M. (1974). Homeoviscous adaptation – a homeostatic process that regulates the viscosity of membrane lipids in *Escherichia coli*. *Proc. Natl. Acad. Sci. USA* **71**, 522-525.

Ståhlberg, A., Åman, P., Ridell, B., Mostad, P. and Kubista, M. (2003). Quantitative real-time PCR method for detection of B-lymphocyte monoclonality by comparison of kappa and lambda immunoglobulin light chain expression. *Clin. Chem.* **49**, 51-59.

Sun, G. Y. and Sun, A. Y. (1985). Ethanol and membrane lipids. *Alcohol. Clin. Exp. Res.* **9**, 164-180.

Swan, T. M. and Watson, K. (1999). Stress tolerance in a yeast lipid mutant: membrane lipids influence tolerance to heat and ethanol independently of heat shock proteins and trehalose. *Can. J. Microbiol.* **45**, 472-479.

Taraschi, T. F. and Rubin, E. (1985). Effects of ethanol on the chemical and structural properties of biologic membranes. *Lab. Invest.* **52**, 120-131.

Thewke, D., Kramer, M. and Sinensky, M. S. (2000). Transcriptional homeostatic control of membrane lipid composition. *Biochem. Biophys. Res. Commun.* **273**, 1-4.

Tiku, P. E., Gracey, A. Y., Macartney, A. I., Beynon, R. J. and Cossins, A. R. (1996). Cold-induced expression of *delta 9-desaturase* in carp by transcriptional and posttranslational mechanisms. *Science* **271**, 815-818.

Upson, R. H., Haugland, R. P. and Malekzadeh, M. N. (1996). A spectrophotometric method to measure enzymatic activity in reactions that generate inorganic pyrophosphate. *Anal. Biochem.* **243**, 41-45.

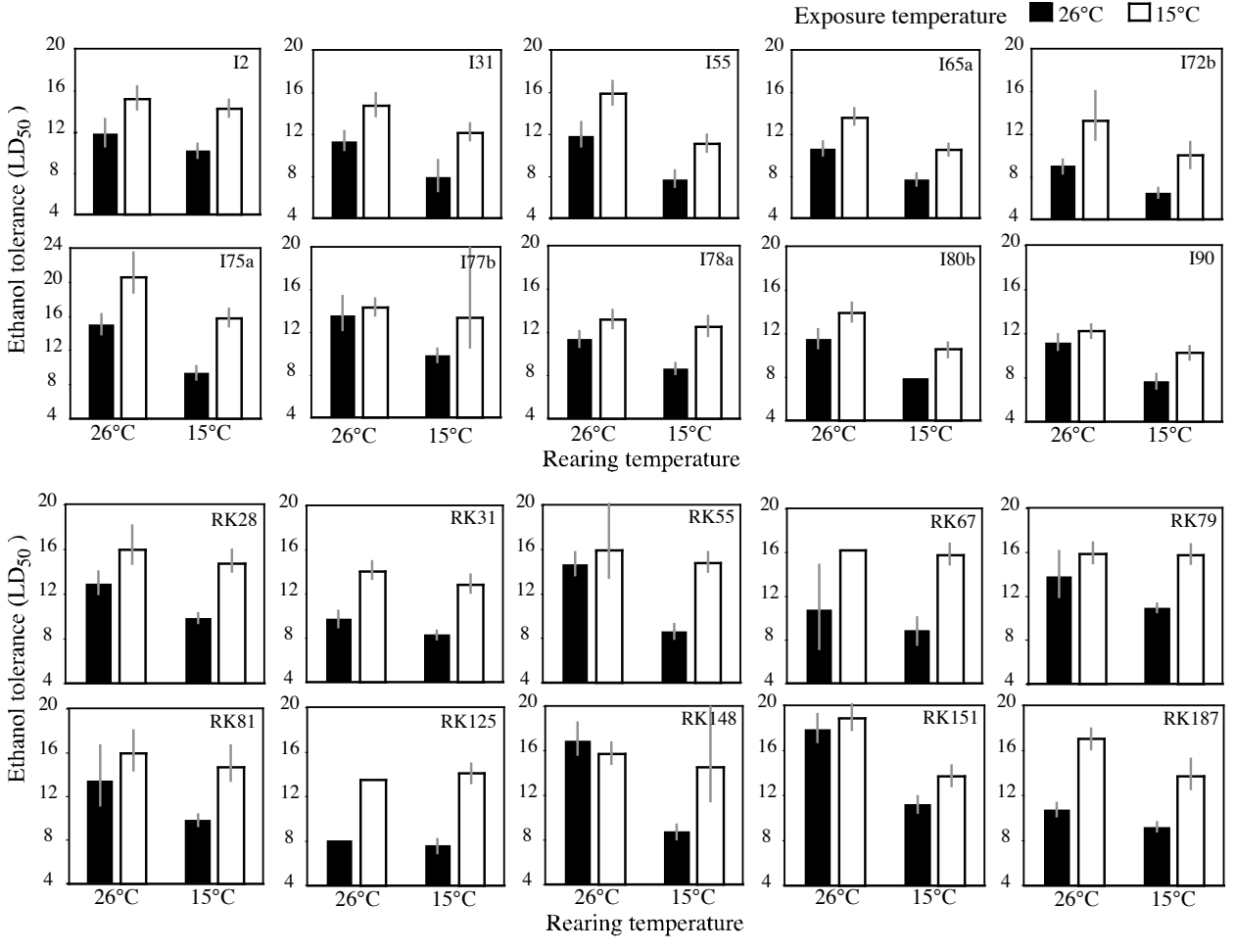
van Delden, W. and Kamping, A. (1997). Worldwide latitudinal clines for the alcohol *dehydrogenase* polymorphism in *Drosophila melanogaster*: what is the unit of selection? *Exs* **83**, 97-115.

van Delden, W., Kamping, A. and van Dijk, H. (1975). Selection at the *alcohol dehydrogenase* locus in *Drosophila melanogaster*. *Experentia* **31**, 418-420.

Vigh, L., Maresca, B. and Harwood, J. L. (1998). Does the membrane's physical state control the expression of heat shock and other genes? *Trends Biochem. Sci.* **23**, 369-374.

You, K. M., Rosenfield, C. L. and Knipple, D. C. (2003). Ethanol tolerance in the yeast *Saccharomyces cerevisiae* is dependent on cellular oleic acid content. *Appl. Environ. Microbiol.* **69**, 1499-1503.

Zehmer, J. K. and Hazel, J. R. (2003). Plasma membrane rafts of rainbow trout are subject to thermal acclimation. *J. Exp. Biol.* **206**, 1657-1667.


Exposure temperature
26°C
15°C
Ethanol tolerance (LD50)
I2
I31
I55
I65a
I72b
I75a
I77b
I78a
I80b
I90
26°C
15°C
Rearing temperature
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RK67
RK79
RK81
RK125
RK148
RK151
RK187

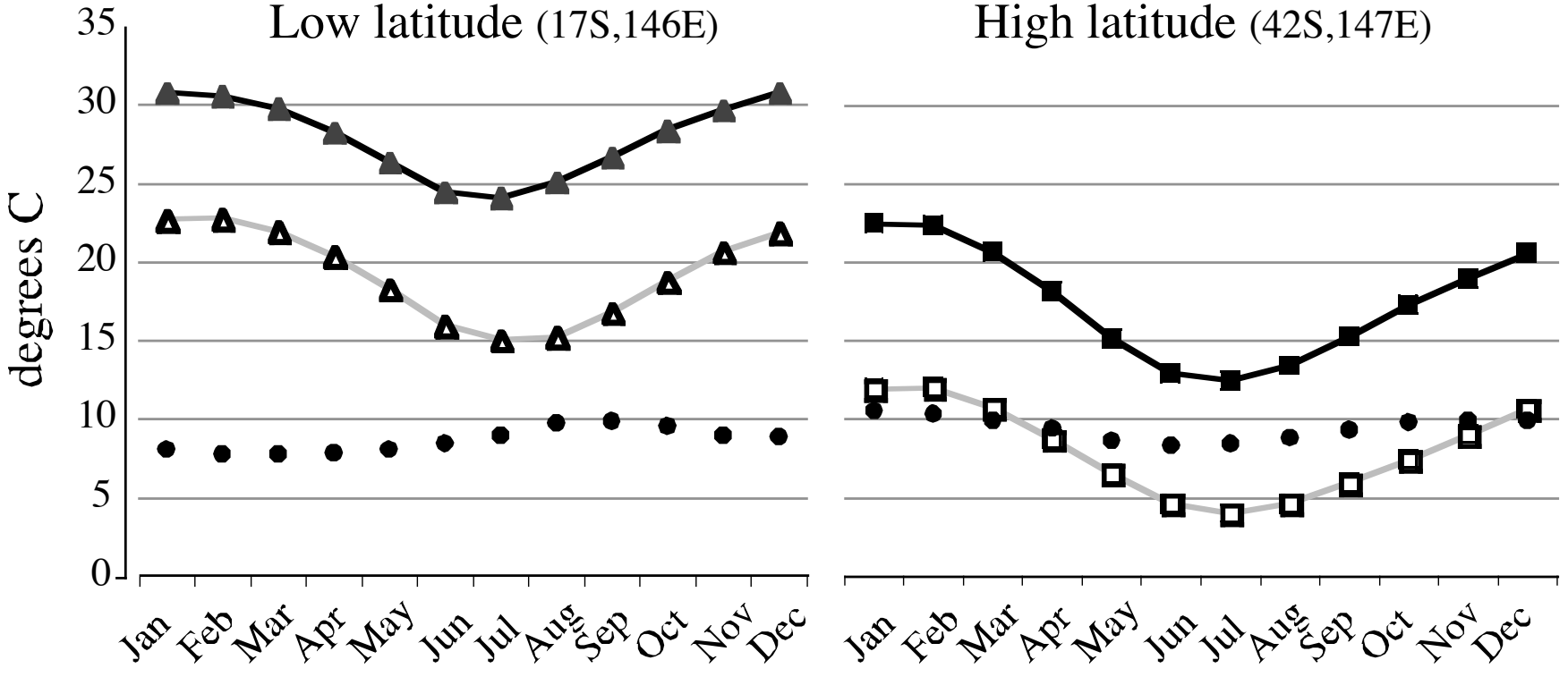
Low latitude (17S,146E)
High latitude (42S,147E)
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Table S1. *Primers, probes and $MgCl_2$ concentrations used to assay gene expression by qRT-PCR*

Protein	EC	Gene (CG)	F primer (5′–3′) R primer (5′–3′)	Probe (5′Fam – 3′MGB)	$MgCl_2$ (mmol l^{-1})*
Acetyl-CoA synthetase	6.2.1.1	*AcCoAS* (9390)	CGGAAACCGGTGGTCATG CCGCACTCATCCAGCAAAG	CGGGATCTGCTTCATT	3.75
		CG6432	TCCGCACGCTTTACTTTCAA GATATAGCCACGTTCGTCCTTGT	AGTTTCCGGGTTACTATGA	3.50
		CG8732	GCCTGGGTGATAAGTCCTTTGA CGGGAACCTCATATTTTTGCA	TGCGCGGAAATGTAAA	3.50
Acetaldehyde dehydrogenase	1.2.1.3	*Aldh* (3752)	GTCCAGCCCACGGTGTTC GGCGGCCAATCCGTACT	TTGCAAGGGAGGAGAT	3.50
Alcohol dehydrogenase	1.1.1.1	*Adh* (3481)	GACCAACAAGAACGTGATTTTCG GGGTTCTCAATGCGGTCGA	CTGAAGAACCTGGTGATC	3.25
CDP-ethanolamine diglyceride transferase	2.7.8.1	*Cdpet* (6016)	CCGCCAAGGTCACCAATAA CGTGAACCACAGTAGCCAGATCT	TGATCGCTCACATGAC	3.50
Fatty acid desaturase	1.14.19.1	*desat1* (5887)	CGCCTTCGGTTACCTCCAT CCTAGGCCAGAAATGACGTATAGAA	CGTGCATCTTAGCTTATT	3.50
Phosphoethanolamine cytidylyltransferase	2.7.7.14	*Pect* (5547)	AGCGCGTGCTCAGTGTTTT GCGTCCATGACAGACAACATC	CCTGCAAGTTTGTCAAT	3.50
Phosphatidate phosphatase	3.1.3.4	*wunen* (8804)	TGGCAGGATCGCTTATTGG GCTTCGTGTTGGGCTTTTGA	TCGTGGCCAACTAT	3.75
Phospholipase D	3.1.4.4	*Pld* (12110)	CGCATGAATGGCAAGAAGTATC CCGACTGGAACCTTCGCTTT	AGAACACTTAGGCCTCC	3.50
Ribosomal protein L32	NA	*RpL32* (7939)	AGGCCCAAGATCGTGAAGAA GACGCACTCTGTTGTCGATACC	AGCTGTCGCACAAAT	3.50
Sphinganine-1-phosphate lyase	4.1.2.27	*Sply* (8946)	CCTGAATGCGCTGCAGTTT CGACTCCGGGCTGTGTGT	CCATCTGGTATCCACC	3.50
Sterol regulatory element binding protein	NA	*Srebp/HLH106* (8522)	GAGAAGTTCCAGACCGATTTGAA GCGACAAACTGCCTCGTACA	TACCGAACGCCCAATC	3.50

*Final concentration of $MgCl_2$ in a 50 ml reaction containing 200 μmol l^{-1} dATP, dCTP and dGTP, 400 μmol l^{-1} dUTP, 900 nmol l^{-1} each primer, 250 nmol l^{-1} probe and 0.025 i.u./μl AmpliTaq Gold; PCR, 10 min hold at 95°C followed by at least 30 cycles of 15 s at 95°C and 60 s at 60°C.

Table S2. *Adh genotypes for the F/S amino acid and ∇1 indel polymorphisms*

	Innisfail									
Line	2	31	55	65A	72B	75A	77B	78A	80B	90
F/S*	H‡	S	S	S	S	H	H	H	S	H
∇1f/∇1s†	H	∇1s	∇1s	∇1s	∇1s	H	H	H	∇1s	H
	Tasmania									
Line	28	31	55	67	79	81	125	148	151	187
F/S	F	F	H	F	H	H	F	H	F	H
∇1f/∇1s	∇1f	∇1f	H	∇1f	H	H	∇1f	H	∇1f	H

*Forward primer, agctccctggcggtaagttgat; Reverse primer, acgagggctgggtgggatgag; PCR concentrations, 1.5 mmol l^{-1} $MgCl_2$, 0.2 mmol l^{-1} dNTPs, 0.3 μmol l^{-1} each primer, 0.02 i.u./μl *Taq*; PCR, 30 cycles of 50 s at 94°C, 50 s at 65°C and 30 s at 72°C; restriction digest, 1 μg of PCR product with 2.5 i.u. of *Hpy*CH4IV at 37°C for 1.5 h.

†Common reverse primer, agggctccgttagttgttggtttc, in combination with the control forward primer, gccaagtgcggaaataaaatgacag, and either the ∇1f forward primer, caagcatcaggcatataatata, or the ∇1s forward primer, 5′-caagcatcaggcatagttgggc; ∇1f PCR concentrations, 2.5 mmol l^{-1} $MgCl_2$, 0.2 mmol l^{-1} dNTPs, 0.3 μmol l^{-1} control forward primer, 0.3 μmol l^{-1} ∇1f forward primer, 0.6 μmol l^{-1} reverse primer and 0.025 i.u./μl *Taq*; ∇1f PCR, 30 cycles of 50 s at 94°C, 50 s at 58°C and 50 s at 72°C; ∇1s PCR concentrations, 4.5 mmol l^{-1} $MgCl_2$, 0.2 mmol l^{-1} dNTPs, 0.3 μmol l^{-1} control forward primer, 0.3 μmol l^{-1} ∇ 1s forward primer, 0.6 μmol l^{-1} reverse primer and 0.015 i.u./μl *Taq*; ∇1s PCR, 30 cycles of 50 s at 94°C, 50 s at 68°C and 50 s at 72°C.

‡H, both alleles were present in a sample of 20 flies.