Adaptation to environmental stress in different life stages of Drosophila melanogaster

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Chapter 4

Effect of the Presence of Ethanol on ADH Activity and ADH Expression in Adults and Larvae of Drosophila melanogaster

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Abstract.

The effect of the presence of ethanol in the feeding medium on adult and larval ADH activity is investigated in selection lines of Drosophila melanogaster. The lines were selected for increased adult alcohol tolerance (ADU-SS and ADU-FF, homozygous for AdhS and AdhF respectively), juvenile tolerance (LAR-SS and LAR-FF) or were selected for increased ethanol tolerance during the whole life cycle (WHO-SS and WHO-FF). The increase in adult resistance to ethanol was accompanied by an increase in adult ADH activity in AdhF selected lines on standard medium without ethanol, and on ethanol medium. In AdhSS selected lines, adult ADH activity increased on standard medium but not after one day on ethanol medium. The increase in egg-to-adult survival in LAR-SS compared to the AdhSS control line was not linked with an increase in larval ADH activity on standard medium and on ethanol medium. Adult and larval Adh expression did not show differences between selected and control lines for both Adh genotypes, on standard medium and on ethanol medium. The role of Adh induction in the increase in alcohol tolerance is discussed according to the life stage.
INTRODUCTION

In holometabolous insects, the modes of life are very different between the juvenile and adult life stages. Consequently, a particular environmental stress can have different selective effects and can lead to a complex form of adaptation which can be specific to the life stage. Alcohol tolerance in *Drosophila melanogaster* provides an excellent model system to study that important evolutionary aspect in insects.

*D. melanogaster* adults feed on fermenting fruit, females lay their eggs in this substrate and the larvae develope inside the decaying fruits. Consequently, both adults and larvae of *D. melanogaster* can become exposed to toxic concentrations of alcohol during the evolutionary process and exhibit a high degree of tolerance to this environmental stress (McKenzie and Parsons, 1972; David and van Herrewege; 1983; Geer et al., 1990). More than 90 per cent of the ethanol metabolised by *D. melanogaster* is degraded by a biochemical pathway involving alcohol dehydrogenase (ADH), an enzyme encoded by a single gene. This *Adh* gene is located on the second chromosome and possesses two promoters. The proximal promoter is essentially used during the juvenile stages, from mid-embryogenesis through the mid-third larval instar, and the distal promoter is used essentially during the adult stage (Benyajati et al., 1983; Savakis and Ashburner, 1985; Savakis et al., 1986; Heberlein and Tjian, 1988). Natural populations generally contain two common electrophoretic alleles, *Adh-fast* (*AdhF*) and *Adh-slow* (*AdhS*). The two alleles differ in one base pair, leading to an amino acid difference in the ADH protein: lysine at position 192 in *AdhS* is substituted by threonine in *AdhF*. Individuals homozygous for the *AdhF* allele show a higher *in vitro* ADH activity than individuals homozygous for the *AdhS* allele, and have generally a higher resistance to ethanol. Heterozygous individuals show an intermediate ADH activity and an intermediate resistance (van Delden, 1982; Geer et al., 1990; Chambers, 1991; Heinstra, 1993; van Delden and Kamping, 1997).

The presence of ethanol in the feeding medium increases the ADH level in larvae (McKechnie and Geer, 1984; Kerver and van Delden, 1985), due to an increase in transcription from the proximal promoter (Geer et al., 1988; Kapoun et al., 1990). The presence of ethanol also induces, though to a lesser extent, transcription from the distal promoter (Geer et al., 1988). This relative insensibility of the distal promoter to ethanol...
explains the initial failure in the detection of induction of Adh in adults by environmental ethanol (McKechnie and Geer, 1984; Kerver and van Delden, 1985). Boulétreau and David (1981) claimed that the stay of adults on the fermenting fruits was too short to lead to a metabolic adaptation, and concluded that the selective pressure of ethanol mainly occurs during the juvenile stages. It becomes more and more evident that induction, differently according to the life stage, may have played a primordial role in the differential alcohol tolerance in adults and larvae of D. melanogaster (Chapter 3 of this thesis).

We have selected two lines of D. melanogaster, one homozygous for the Adh\(^S\) allele and the other homozygous for Adh\(^F\), for increased alcohol tolerance. The selection was performed in three different ways according to the life stage: increased adult tolerance (ADU), increased larval tolerance (LAR), and increased tolerance during the whole life (WHO). Evidence for life stage specificity of some genes involved in alcohol tolerance were shown. The lines selected at the adult stage increased their adult alcohol tolerance but not their juvenile tolerance, while lines selected at the juvenile stages increased effectively the egg-to-adult survival on ethanol medium but had a limited increase in adult tolerance on ethanol medium (Chapters 2 and 3 of this thesis). The lines selected during their whole life exhibited an increase in both juvenile and adult tolerance to ethanol.

In the present paper, the role of the induction of Adh by ethanol in this increase in tolerance is studied for both life stages, juvenile and adult. ADH activity in D. melanogaster can be considered as a quantitative character (Ward, 1975) with several polymorphisms involved in the level of Adh expression (Stam and Laurie, 1996; Wu and Gibson, 1998; Wu et al., 1998; Parsh et al., 2000). Different factors may affect the quantity of ADH in D. melanogaster (Clarke et al., 1979), and the present goal is to determine the exact role played by the induction process according to the life stage, and to observe more specifically an eventual difference in strategy between the two life stages, suggested by the difference in induction ability of the two promoters. Larvae are not mobile like adults, and they have to cope with the alcohol present in their feeding medium. In absence of alcohol, Adh expression is limited, reducing the cost necessary to produce this enzyme, while when the alcohol concentration increases in the medium, Adh expression in larvae increases as well as alcohol tolerance. On the contrary, adults
are able to move when alcohol concentration is too high with respect to their level of *Adh* expression. ADH activity and *Adh* expression were examined in adults and larvae of all the selected and control lines. Measurements were made with adults and larvae from various environments with or without ethanol. The role played by ADH in the increase in juvenile and in adult alcohol tolerance is discussed, especially the role played by induction of *Adh* in the presence of alcohol in the medium.
MATERIAL AND METHODS

Experimental stocks

Five lines homozygous for $Adh^S$ and five lines homozygous for $Adh^F$ were derived from a polymorphic population founded in 1983 with 403 females from a fruit market in Groningen, The Netherlands. The two lines used for the experiments, one homozygous for $Adh^S$ and the other for $Adh^F$, were obtained from all possible intercrosses of the five lines with the proper $Adh$ genotype in order to increase the genetic variability. All lines were homozygous for the $\alpha$-glycerophosphate dehydrogenase fast allele ($\alpha Gpdh^F$), as $\alpha Gpdh$ variants show epistatic interactions with $Adh$ with respect to ethanol tolerance (Cavener and Clegg, 1981; van Delden, 1984, McKechnie and Geer, 1988, Izquierdo and Rubio, 1989).

The two lines kept on regular medium (18g agar, 54g sucrose, 32g dead yeast and 100mg ampiciline per 1000ml water), with a new generation every two weeks, were used as control strains (CON-SS and CON-FF lines). All selected and control strains were kept at 25°C (50 per cent RH and 24 hours light regime), in two replicates of five bottles (30 ml of food per bottle) with 300 eggs per bottle. At each generation, the flies emerged from the 5 bottles were mixed before starting a new generation and the two sets of five bottles were kept as two independent lines.

Selection procedures

1. The adult selection procedure (ADU):
Flies were allowed to mate and females laid eggs into five bottles with 30 ml of standard medium. The density was maintained at about 300 eggs per bottle to avoid crowded conditions. Larvae were grown in the medium without alcohol, and the emerging adults were transferred and kept on normal food for one week. To avoid egg retention in females and to keep the flies in perfect condition, the food was refreshed 24 hours before the flies were transferred into bottles with food supplemented with ethanol, 12 per cent for the $Adh^{SS}$ flies (ADU-SS) and 18 per cent for $Adh^{FF}$ flies (ADU-FF). When approximately a quarter of the flies were dead, the survivors were transferred
again into bottles with standard medium for an egg-laying period of 24 hours to start a new generation.

2. The larval selection procedure (LAR):
In this selection procedure, only the juvenile stages were in contact with the food supplemented with ethanol. Newly emerged flies were kept on standard medium for one week. Twenty-four hours before starting a new generation, medium was refreshed to allow females to lay eggs and avoid egg retention. Then, flies were transferred to the egg-laying vials. These vials (55 mm high, 50 mm diameter) were provided with a lid containing a thin layer of normal food and a little drop of yeast to stimulate egg laying. Females were allowed to lay eggs during a period of four hours and the eggs were then transferred to five bottles containing ethanol food [300 eggs per bottle with 30 ml of food containing 10 per cent ethanol for $Adh^{SS}$ flies (LAR-SS) and 12 per cent for $Adh^{FF}$ flies (LAR-FF)]. Eggs were always transferred within 8 hours after egg laying because egg-to-adult survival on ethanol medium depends on the age at which the eggs are transferred (Bijlsma-Meeles, 1979; Kerver and Rotman, 1987). Larvae developed in this medium, and emerging adults were daily transferred into new bottles with fresh standard food, and kept on this medium for one week before starting the next generation.

3. The whole life cycle selection procedure (WHO):
Flies were kept continuously on food supplemented with ethanol [10 per cent for the $Adh^{SS}$ flies (WHO-SS) and 12 per cent for $Adh^{FF}$ flies (WHO-FF)] during the whole life cycle. Females laid eggs on ethanol food, larvae grew in this medium and emerging adults were kept in the bottles for one week. Then, the flies were transferred to bottles with ethanol-supplemented food to allow the females to lay eggs (about 300 eggs per bottle) to initiate a new generation.

Experiments

1. Experimental protocol
The lines LAR and WHO were selected for 40 generations while ADU was selected for 45 generations. Both selected and control lines were raised on standard medium for one
generation before experiments to avoid phenotypic effects. Virgin adult males and females were collected and kept in vials (75 mm high, 25 mm diameter containing 9 ml of standard medium) for one week. Then groups of 10 adults were transferred to test vials containing standard medium for one day or on ethanol-supplemented medium (10% for \( Adh^{SS} \) and 12% for \( Adh^{FF} \)) for either one or three days. Additional one-week-old females were allowed to mate and to lay eggs on standard medium. Eggs were then transferred to test vials containing standard or ethanol-supplemented medium (10% for \( Adh^{SS} \) and 12% for \( Adh^{FF} \)) for either three or four days and developed into larvae.

RNA was extracted from five groups of ten flies or larvae directly after the different treatments and kept at –80° C for later northern hybridization. Groups of ten adults or ten larvae were directly kept at –20° C for later ADH activity measurement. The complete experimental procedure is summarized in figure 1.

2. ADH activity assays
Directly after the experiment, samples of ten one-week-old non-mated adults (males or females) or ten three-day-old larvae were frozen at –20° C. The samples were homogenized in 0.5 ml (adults) or in 0.3 ml (larvae) of cold buffer (50 mM glycine-NaOH, 1 mM EDTA, pH 9.5). After centrifugation (5 min at 11000 g) the supernate was kept on ice for immediate assay. ADH enzyme activity was measured following a modification of the procedure described by Oudman et al. (1991). Shortly, 170 µl buffer at 30°C, 10 µl homogenate and 20 µl reagent buffer (glycine-NaOH buffer containing 5 mM NAD\(^+\) and 200 mM 2-propanol) were mixed. The reaction rate was measured after 30 sec, during 90 sec at 30°C and 340 nm (extinction of NAD) using a multi samples spectrophotometer Spectra Max Plus. For each selection regime, \( Adh \) genotype and environment, ten replicates were measured twice. ADH enzyme activity was expressed as µmole of NADH by minute and by µg of protein.

Protein content was measured according to Bradford (1976), using the Biorad Protein Assay kit. To 0.1 ml defrosted homogenate, 5 ml reagent was added. After 15 minutes, the absorbance was measured at 595 nm. Total protein content was calculated in micrograms using bovine serum albumin (BSA) as a standard.
3. RNA extraction and Northern hybridization

Total RNA was isolated from larvae and adults using the RNeasy kit from Qiagen. Approximately 10 µg RNA (as determined by spectrophotometry) was vacuum-dried and dissolved in 20 µl sample buffer (50% v/v formamide, 2.2 M formaldehyde in 1xMOPS buffer (200 mM 3-(N-morpholino) propanesulfonic, 80 mM sodiumacetate, 10 mM EDTA; pH 7.0). From each sample 5 µl was loaded onto a 1% agarose gel (electrophoresis buffer 1xTBE) containing 0.2 mM guanidine thiocyanate and 25 µg/100 ml ethidium bromide, run for 2 hrs at 10V/cm and photographed, to visualise the amount of RNA loaded on the northern gel. Then 15 µl (7.5 µg) was loaded onto a 1% agarose gel (electrophoresis buffer 1xMOPS) containing 2.2 M formaldehyde, run for 4 hrs at 10 V/cm and transferred onto a nylon membrane (Hybond-N, Amersham) in 20xSSC. RNA was bound to the membrane at 80 °C and hybridized to a 32P labeled (random primed DNA labeling kit, Roche) Adh probe. This probe was obtained as the PCR product using primers 1 and 2 (Figure 2) from D. melanogaster DNA. Hybridization was carried out in 50% (v/v) dextran sulfate, 10% (v/v) formamide, 1% SDS, 1M NaCl at 42°C. The filters were washed twice for 15 min in 2xSSC at room temperature and once for 15 min at 60°C in 2xSSC, 1% SDS. Autoradiography was carried out for 48 hrs at -80°C using intensifying screens. Filters were stripped by pouring a boiling solution of 0.1% (w/v) SDS onto the blot and allowing it to cool to room temperature. Then the operation of hybridization was repeated with a tubuline probe for calibration, tubuline is supposed to be expressed in the same way in all the samples (Abel et al., 1992). For each selection regime, Adh genotype, environment and sex for the adults, two replicates were effectued.

Statistical analysis

ANOVAs and Tukey tests for multiple comparison of means were performed for ADH activities by using Statistix 4.0 Analytical Software. The level of significance for the Tukey tests was 5 per cent.
RESULTS

1. Adult ADH activity

Environment (i.e. standard medium, ethanol medium for one day or ethanol medium for three days), selection procedure (CON, ADU, LAR or WHO) and sex had significant effects on adult ADH activity for both Adh genotypes (Table 1). Interactions between these three factors were always significant, except for the interaction between selection procedure and sex in AdhSS flies (Table 1).

Standard medium:
On standard medium, without ethanol, ADH activity of adults was generally higher in the selected lines compared to the correspondent control line, for both sexes and both Adh genotypes (Figure 3). Table 2 presents the details of mean adult ADH activities for all selected and control lines. The values presented for standard medium are identical with those from Table 4 in Chapter 3. The increase compared to control was significant in males and females of ADU-SS as well as of ADU-FF. For the two other selection procedures, the increase compared to the control was significant in males for both Adh genotypes, but not in females (Table 2).

One day on ethanol medium:
After one day on ethanol medium, adult ADH activity in the two control lines CON-SS and CON-FF was similar to the enzyme activity measured on standard medium (Figure 3). The increase observed in CON-SS males compared to medium without ethanol (Table 2) was not significant (P=0.08).

After one day on ethanol medium, the results for the selected lines were different for the two Adh genotypes. In AdhSS lines, adult ADH activity of the three selected lines was not significantly different from the control line CON-SS for both sexes (Table 2). On the contrary in AdhFF lines, adult ADH activity was significantly higher in the three selected lines compared to the control line CON-FF for the females, and significantly higher in ADU-FF and in WHO-FF for the males. The increase in LAR-FF males was not significant (Table 2). For males ADU-FF, the result was significantly higher than in the two other selection procedures LAR-FF and WHO-FF.
Three days on ethanol medium:
After three days on ethanol medium, adult ADH activity was decreasing in all cases, for both sexes and for both \textit{Adh} genotypes (Figure 3). In \textit{Adh}^{SS} lines the differences in enzyme activity between each selected line and the control line were not significant for both sexes, excepted for the males LAR-SS (Table 2). In \textit{Adh}^{FF} lines, as observed already after one day on ethanol medium, adult ADH activity was significantly higher for all three selected lines compared to CON-FF for both sexes (Table 2).

Relative adult ADH activity of selected lines:
Figure 4 shows adult ADH activity for each selection procedure relatively to the corresponding control for both \textit{Adh} genotypes and both sexes in the three different environments. A result higher than one indicates an increase for the selected line compared to the control line on the same environment.
Figure 4 underlines the difference between the two \textit{Adh} genotypes in adult ADH activity after the different selection procedures. Effectively, in \textit{Adh}^{FF} selected lines, values are higher than one, for the three selection procedures, in the different environments and for both sexes. It means that after selection, ADH activity in adult \textit{Adh}^{FF} generally increased compared to the control line. On the contrary, in \textit{Adh}^{SS} selected lines, values were generally close to one. On standard medium, without ethanol, males of the three selected lines exhibited a higher ADH activity than males of CON-SS. For the females, only ADU-SS was higher than CON-SS. On ethanol medium, after one day and after three days, the values were close to one, which indicates that ADH activity of selected lines was close to ADH activity of the control line. Furthermore this figure shows that the presence of ethanol in the feeding medium did not clearly increase the ADH activity of adults for both sexes and both \textit{Adh} genotypes.
2. Larval ADH activity

Environment, selection procedure and the interaction between these two factors had significant effects on larval ADH activity for both Adh genotypes (Table 3).

On standard medium:
In AdhSS lines, on standard medium without ethanol, all three selected lines and the control line exhibited similar larval ADH activities (Figure 5). The differences observed were not significant between the selected lines and CON-SS (Table 4). In AdhFF lines on the contrary, larval ADH activity of all three selected lines together showed a increase compared to the control line (Figure 5). Table 4 reveals that the increase was significant in WHO-FF, but not in ADU-FF and LAR-FF.

On ethanol medium:
On medium supplemented with ethanol, a general increase in ADH activity was observed in larval ADH activity, for the selected lines as well as for the control lines of both Adh genotypes, excepted for WHO-FF (Figure 5, Table 4). In AdhSS lines, LAR-SS and WHO-SS exhibited similar larval ADH activities compared to CON-SS, while in ADU-SS larval ADH activity was significantly higher (Table 4). For the AdhFF lines, the selected lines ADU-FF, LAR-FF and WHO-FF presented a similar ADH activity, which was significantly higher than CON-FF for LAR-FF and WHO-FF.

Relative larval ADH activity of selected lines:
Figure 6 presents larval ADH activity of each selected line relatively to the correspondent control line. As for the adults, a value higher than one indicates a larval ADH activity higher in the selected line compared to the control line.

For the AdhSS larvae, on standard medium, the ADH activity of the three selected lines was close to the ADH activity of the control line and then the three values are close to one. On ethanol medium, the increase for LAR-SS and WHO-SS compared to standard medium was parallel to the increase in CON-SS and the values are still close to one. On the contrary for ADU-SS the increase in larval ADH activity was clearly more drastic (Figure 6).
For the \( Adh^{FF} \) larvae, the three selected lines exhibited higher larval ADH activity than CON-FF on standard medium, without ethanol, the increase was significant only for WHO-FF. On medium supplemented with ethanol, the increase observed in ADU-FF and LAR-FF compared to standard medium was parallel to the increase in CON-FF, while WHO-FF exhibited a surprising decrease in larval ADH activity on ethanol medium compared to the standard medium (Table 4), and reached an activity level comparable to the two other selection procedures (Figure 6). However, larval ADH activity on ethanol medium in LAR-FF is significantly higher than in CON-FF (Table 4).

3. Adult \( Adh \) expression

Figures 7 and 8 show results for \( Adh \) expression and tubuline expression for respectively \( Adh^{SS} \) and \( Adh^{FF} \) adults. Histograms present \( Adh \) expression relatively to tubuline expression for each mRNA sample. Because only two replicates were effected, comparisons between samples were not statistically tested.

On standard medium, there was no apparent difference in control expression level between \( Adh^{SS} \) and \( Adh^{FF} \) genotypes (Figure 7 and 8), in accordance with the fact that the difference of activity is not primarily due to differences in mRNA levels between the two genotypes (Parsh et al., 2000). Compared to controls, selection for ethanol tolerance had no clear effect on adult \( Adh \) expression for either genotype or selection regime.

After one day exposure to ethanol, \( Adh \) expression increased for both selected and control lines, for both genotypes and both sexes (Figure 7 and 8). No difference was observed in \( Adh \) expression between the control lines CON-SS and CON-FF and the three selected lines of the correspondant \( Adh \) genotype. After three days on ethanol medium, in all cases and for both \( Adh \) genotypes, \( Adh \) mRNA level was back to the level observed on standard medium, or even slightly lower.
4. Larval Adh expression

Results for larval Adh expression on standard and ethanol medium for both Adh genotypes are shown in figure 9. As for the adult expression, only two replicates were effected and comparisons between samples were not statistically tested.

On standard medium, without ethanol, the quantity of Adh mRNA was always lower in the control lines CON-SS and CON-FF compared to all three selected lines of the correspondent Adh genotype. Generally, samples from Adh<sup>FF</sup> lines contained more Adh mRNA than samples from Adh<sup>SS</sup> lines. However, results of CON-FF were similar to the results of the different Adh<sup>SS</sup> selected lines. The level of Adh expression was higher in LAR-SS than in the two other selected lines ADU-SS and WHO-SS. In Adh<sup>FF</sup> lines, level of expression was similar in all three selected lines.

On ethanol medium, the level of Adh mRNA of CON-SS increased more than in all three Adh<sup>SS</sup> selected lines, and reached the same level. Sample two of CON-SS was discarded because of a technical problem. Then, larval Adh expression in all Adh<sup>SS</sup> lines, selected or not, was similar on ethanol medium. In Adh<sup>FF</sup> lines, the increase in Adh mRNA was more or less parallel in all samples, and the difference still existed between the control and the three selected lines.
DISCUSSION

The lines selected for increased ethanol resistance showed a positive response. The increase in adult tolerance was more pronounced when the adult stage was included in the selection regime. For egg-to-adult survival, a positive response was observed only when the larval stage was included in the selection regime (Chapters 2 and 3 of this thesis).

- \textit{Adh} expression and ADH activity of adults.

Measurements of ADH activities on standard medium, without ethanol, do not fully correlate with the clear increase in alcohol tolerance observed in the selected lines. The increase in adult tolerance was effectively linked to an increase, limited but significant, in adult ADH activity, especially in the lines selected at the adult stage (ADU) for both \textit{Adh} genotypes.

After one day on ethanol medium, the level of \textit{Adh} expression is higher than on standard medium in all samples. This result is in accordance with previous work about inducibility of the distal promotor of \textit{Adh} (Geer \textit{et al.}, 1988). This increase in \textit{Adh} expression is limited and not related to a significant increase in ADH activity in our results, because of the relative insensibility of the distal promotor compared to the proximal promotor. Previously, McKechnie and Geer (1984) and Kerver and van Delden (1985) failed to show an increase in ADH activity in adults placed on ethanol medium.

However, a difference exists in the results between the two different \textit{Adh} genotypes. In \textit{Adh}^{SS} lines, the differences observed in adult ADH activity between selected and control lines are not significant after one day exposure to ethanol. Furthermore, the level of ADH activity after one day on ethanol medium is similar to that on standard medium. These results indicate that induction in \textit{Adh}^{SS} adults does not play an essential role in the increase in adult alcohol tolerance in the selected lines. On the contrary in \textit{Adh}^{FF} lines, while adult ADH activity of the control line CON-FF after one day on ethanol medium is similar to ADH activity on standard medium, the females of the three selected lines and the males of ADU-FF exhibit higher adult ADH activity compared to the standard.
medium. This increased ADH activity is reflected in the Adh expression level. It seems that the increase in adult alcohol tolerance in AdhFF is not only linked to an increase in adult ADH activity on standard medium, but also to an increase in the induction ability of the distal promoter.

The other test concerned a stay of three days on ethanol medium. The measurements of Adh expression and ADH activity were made on the surviving adults. The level of Adh expression is comparable, similar or even lower than the level of expression on standard medium. This decrease is accompanied by a decrease in adult ADH activity. This is certainly due to the toxic effects of alcohol and the products (acetate and acetaldehyde) of the metabolic pathway of degradation of ethanol, mediated by ADH. The high concentration of ethanol in the feeding medium combined with high ADH activity lead to high intracellular concentration of these two products which can have toxic effects on the flies (Heinstra et al., 1989; Emans and Eisses, 1992; Chakir et al., 1993; Chakir et al., 1994; Chakir et al., 1996; van’t Land, 1997; Karan et al., 1999; Oppentocht, 2001). In fact, the survivors after three days on ethanol medium, tested in our experiment, may be the adults with an initial lower ADH activity which produce less acetaldehyde. It is also possible that one effect of the different toxic compounds is to reduce the general metabolism of the flies, and thus, among others, the Adh expression.

- Adh expression and ADH activity in larvae.

On standard medium, without ethanol, the quantity of Adh mRNA in larvae seems to be slightly higher in the selected lines than in the control lines for both Adh genotypes, and more specifically in LAR-SS for AdhSS lines and in LAR-FF and WHO-FF for the AdhFF lines. In these three lines the larval stage was included in the selection regime. It seems possible that the presence of ethanol in the feeding medium is favorable for the larvae with higher Adh expression. However, on the one hand WHO-SS does not exhibit such higher Adh expression compared to CON-SS, even if ethanol was present in the feeding medium during the selection procedure. On the other hand, results of larval ADH activity among the different lines do not perfectly reflect the observed differences in Adh expression. Effectively, in AdhSS lines larval ADH activity is similar between all the lines, selected and non-selected. It indicates that Adh expression and ADH activity
in larvae on standard medium without ethanol is far from the only factor involved in the increase in juvenile alcohol tolerance for LAR-SS and WHO-SS.

As in adults, results are quite different for the AdhFF lines. The three selected lines exhibit higher larval ADH activity on standard medium compared to CON-FF. These results have to be linked with the increase in juvenile alcohol tolerance in the selected lines, and suggest an important role played by ADH in this increase. However, larval ADH activity in ADU-FF is very close to larval ADH activity in LAR-FF while a large difference exist in egg-to-adult survival on ethanol medium between these two lines (0.42 and 0.58 respectively), and 0.31 for CON-FF (Chapter 3). Thus, the increase in larval ADH activity on standard medium, probably linked to the increase in juvenile tolerance, is certainly not the only factor involved in this increase. The higher larval ADH activity in ADU-FF may be linked to the adult selection procedure and, at least partly, with the increase in adult ethanol tolerance.

Larval ADH activity in WHO-FF on standard medium is very high compared to all the other lines, while egg-to-adult survival on ethanol medium is close to LAR-FF (0.64 and 0.58 respectively; see chapter 3). This difference may be also linked to the adult ethanol tolerance, WHO-FF showing a significant increase in adult tolerance compared to CON-FF for both sexes, while for LAR-FF the increase is not significant for males and females.

On ethanol medium the level of Adh mRNA increases in all samples compared to the level on standard medium, confirming the ability of induction of the proximal promoter of Adh (Geer et al., 1988). In AdhSS lines, exposure to ethanol diminished the increased Adh expression in the selected lines compared to control. Larval ADH activities, higher on ethanol medium than on standard medium, do not show differences between the two selected lines LAR-SS and WHO-SS, and the control line CON-SS. Nevertheless the two AdhSS selected lines show a significant increase in egg-to-adult survival on ethanol medium. It indicates that the increase of juvenile alcohol tolerance in these two lines is not due to a higher induction capacity of Adh. Surprisingly, larval ADH activity on ethanol medium for ADU-SS is significantly higher than larval ADH activity on ethanol medium for CON-SS (Table 4), while the egg-to-adult survival on ethanol medium is similar for these two lines (Table 7 of Chapter 3). Then, opposite to what could be
expected, an increase in larval ADH activity seems sometimes to be linked to an increase in adult alcohol tolerance and not to an increase in juvenile tolerance.

In $Adh^{FF}$ lines, all four lines presented an increase in $Adh$ expression. This increase is parallel to an increase in larval ADH activity on ethanol medium compared to standard medium, with the exception of WHO-FF. In the latter line surprisingly larval ADH activity is lower on ethanol medium than on standard medium. It is probably linked to the very high enzyme activity measured on standard medium. However, the three selected lines show similar larval activities on ethanol medium, significantly higher than the control line CON-FF for LAR-FF and WHO-FF. The increase in enzyme activity on ethanol medium compared to regular medium is similar between the two selected lines, ADU-FF and LAR-FF, and the control line CON-FF, and thus is not specifically linked to a particular selected line, and then not linked to the increase in juvenile tolerance observed in LAR-FF.

- Conclusions.

In a natural environment, the mobility of the flying adults allow them to move to an other place when the alcohol concentration is too high. The larvae on the contrary have to cope with their environment, and thus with the alcohol concentration existing in the feeding substrate. This essencial difference may explain the higher ability of ethanol to induce higher $Adh$ transcription from the proximal promoter, which is used during the juvenile stages. In absence of ethanol, $Adh$ expression is limited to a low level, reducing the cost necessary to produce the enzyme, while in presence of ethanol the $Adh$ expression increases, accompanied by increased alcohol tolerance and egg-to-adult survival.

In the larval selection procedure, the larvae selected for their high alcohol tolerance could have been the larvae with the higher capacity to increase ADH production when alcohol is present in the feeding medium. However, our results show that it is not the case, and the increase in larval $Adh$ expression and larval ADH activity on ethanol medium is comparable for all selected and non-selected lines for both $Adh$ genotypes.
The specific increase in juvenile tolerance is not due to an increase in the ability of induction of the proximal promoter of \textit{Adh}.

For the lines selected for increased adult alcohol tolerance, ADU-SS and ADU-FF, the increase in resistance, specific to the life stage, is linked to an increase in adult ADH activity on a medium without alcohol. The presence of ethanol in the feeding medium does not seem to have an important effect on adult \textit{Adh} expression, and the specific increase in adult tolerance is not due to an increase in the capacity of induction of the distal promoter, important for the \textit{Adh} expression in the adults. ADH activity in adults certainly plays an important role in the increase in adult alcohol tolerance in the two selected lines ADU-SS and ADU-FF, but the increase in enzyme activity in adults is also true for LAR-SS and LAR-FF. Then, we can expect that other factors are also important for adult alcohol tolerance.

In juvenile tolerance, larval ADH activity is not involved in the \textit{Adh}^SS lines, while for \textit{Adh}^FF lines an increase is observed. However, ADU-FF and LAR-FF exhibit comparable larval ADH activities while a large difference in juvenile ethanol tolerance exist between these two lines. This indicates that not only in \textit{Adh}^SS lines but also in \textit{Adh}^FF lines ADH activity is not, as for the adult alcohol tolerance, the only factor involved in the increase in juvenile alcohol tolerance. Other physiological systems are the basis for increased ethanol resistance (see Geer \textit{et al.}, 1993;), and both lipid metabolism and membrane structure have been put forward in this respect (Miller \textit{et al.}, 1993; Swanson \textit{et al.}, 1995; Eanes, 1999).

We observed clear differences between adult male and female responses. Females seem to display more pronounced direct and indirect responses to selection than males. This could be the consequence of the fact that females need the energy to produce eggs, they cannot stop feeding on ethanol supplemented food and have to oviposit on the medium. Males, however, can sustain longer periods of refraining from feeding, and may ingest smaller amounts of ethanol.

It may be no surprise that no clear correlation between ethanol tolerance and \textit{Adh} gene expression was observed in this study. Previous research has demonstrated that \textit{Adh} mRNA levels are not necessarily associated with higher ADH activity or, indeed,
alcohol tolerance (Laurie et al., 1991; Choudhari and Laurie, 1991). Moreover, the regulation of ADH activity can be effectuated at all possible levels. The two promoter system allows life time specific regulation at the transcription level, enhancer elements allow regulation at the transcription level within life stages, regulatory elements present within exons and at the 3’ region of the mRNA are involved in post transcriptional regulation (Stam and Laurie, 1996). Recently it has been observed that codon bias plays an important role in translation efficiency of the Adh gene, pointing at the importance of tRNA availability for the efficient translation of the Adh mRNA (Carlini, 2004). The establishment of resistance to environmental ethanol is a complex trait, with different but overlapping components in the larval and adult life stages, for which Adh is not the only gene involved.
Table 1: Summary of ANOVAS for adult ADH activity for each *Adh* genotype. Environment (standard medium, ethanol medium for one day and ethanol medium for three days), selection procedure (CON, ADU, LAR and WHO) and sex are the main factors.

<table>
<thead>
<tr>
<th></th>
<th><em>Adh</em>&lt;sup&gt;SS&lt;/sup&gt;</th>
<th><em>Adh</em>&lt;sup&gt;FF&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Environment (A)</td>
<td>***</td>
<td>***</td>
</tr>
<tr>
<td>Selection procedure (B)</td>
<td>*</td>
<td>***</td>
</tr>
<tr>
<td>Sex (C)</td>
<td>***</td>
<td>***</td>
</tr>
<tr>
<td>A x B</td>
<td>**</td>
<td>***</td>
</tr>
<tr>
<td>A x C</td>
<td>*</td>
<td>***</td>
</tr>
<tr>
<td>B x C</td>
<td>ns</td>
<td>*</td>
</tr>
<tr>
<td>A x B x C</td>
<td>*</td>
<td>***</td>
</tr>
</tbody>
</table>

ns: not significant; * P<0.05; ** P<0.01; *** P<0.001
Table 2: Mean adult ADH activity (μmol/min/μg protein) and standard errors in the three environments. Significant differences at the 5% level between the selection procedures and within genotype, sex and environment are indicated by a different Latin letter. Significant differences at the 5% level between environments and within selection procedure, genotype and sex are indicated by a different Greek letter.

<table>
<thead>
<tr>
<th></th>
<th>Standard medium</th>
<th>1 day on ethanol medium</th>
<th>3 days on ethanol medium</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>SE</td>
<td>Mean</td>
</tr>
<tr>
<td><strong>Males</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CON-SS</td>
<td>0.156</td>
<td>0.004</td>
<td>0.185</td>
</tr>
<tr>
<td>ADU-SS</td>
<td>0.198</td>
<td>0.010</td>
<td>0.126</td>
</tr>
<tr>
<td>LAR-SS</td>
<td>0.194</td>
<td>0.009</td>
<td>0.158</td>
</tr>
<tr>
<td>WHO-SS</td>
<td>0.203</td>
<td>0.010</td>
<td>0.182</td>
</tr>
<tr>
<td><strong>Females</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CON-SS</td>
<td>0.111</td>
<td>0.002</td>
<td>0.113</td>
</tr>
<tr>
<td>ADU-SS</td>
<td>0.152</td>
<td>0.016</td>
<td>0.130</td>
</tr>
<tr>
<td>LAR-SS</td>
<td>0.129</td>
<td>0.006</td>
<td>0.147</td>
</tr>
<tr>
<td>WHO-SS</td>
<td>0.120</td>
<td>0.004</td>
<td>0.144</td>
</tr>
<tr>
<td><strong>Males</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CON-FF</td>
<td>0.587</td>
<td>0.022</td>
<td>0.579</td>
</tr>
<tr>
<td>ADU-FF</td>
<td>0.788</td>
<td>0.029</td>
<td>0.903</td>
</tr>
<tr>
<td>LAR-FF</td>
<td>0.760</td>
<td>0.041</td>
<td>0.721</td>
</tr>
<tr>
<td>WHO-FF</td>
<td>0.855</td>
<td>0.037</td>
<td>0.749</td>
</tr>
<tr>
<td><strong>Females</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CON-FF</td>
<td>0.339</td>
<td>0.023</td>
<td>0.375</td>
</tr>
<tr>
<td>ADU-FF</td>
<td>0.457</td>
<td>0.031</td>
<td>0.653</td>
</tr>
<tr>
<td>LAR-FF</td>
<td>0.398</td>
<td>0.021</td>
<td>0.609</td>
</tr>
<tr>
<td>WHO-FF</td>
<td>0.389</td>
<td>0.020</td>
<td>0.565</td>
</tr>
</tbody>
</table>
Table 3: Summary of ANOVAS for larval ADH activity for each *Adh* genotype. Environment (standard medium or ethanol medium) and selection procedure (CON, ADU, LAR and WHO) are the main factors.

<table>
<thead>
<tr>
<th></th>
<th><em>Adh</em>&lt;sup&gt;SS&lt;/sup&gt;</th>
<th><em>Adh</em>&lt;sup&gt;FF&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Environment (A)</td>
<td>***</td>
<td>*</td>
</tr>
<tr>
<td>Selection procedure (B)</td>
<td>*</td>
<td>***</td>
</tr>
<tr>
<td>A x B</td>
<td>**</td>
<td>**</td>
</tr>
</tbody>
</table>

* P<0.05; ** P<0.01; *** P<0.001
Table 4: Mean larval ADH activity (μmol/min/μg protein) and standard errors in the environments. Significant differences at the 5% level between the selection procedures and within Adh genotype and environment are indicated by a different Latin letter. Significant differences at the 5% level between environments and within selection procedure and genotype are indicated by a different Greek letter.

<table>
<thead>
<tr>
<th></th>
<th>Standard medium Mean</th>
<th>SE</th>
<th>Ethanol medium Mean</th>
<th>SE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CON-SS</td>
<td>0.154α</td>
<td>0.011</td>
<td>0.211αβ</td>
<td>0.020</td>
</tr>
<tr>
<td>ADU-SS</td>
<td>0.151α</td>
<td>0.006</td>
<td>0.328bβ</td>
<td>0.033</td>
</tr>
<tr>
<td>LAR-SS</td>
<td>0.168α</td>
<td>0.012</td>
<td>0.222α</td>
<td>0.026</td>
</tr>
<tr>
<td>WHO-SS</td>
<td>0.175α</td>
<td>0.012</td>
<td>0.216α</td>
<td>0.022</td>
</tr>
<tr>
<td>CON-FF</td>
<td>0.725α</td>
<td>0.021</td>
<td>0.866αβ</td>
<td>0.041</td>
</tr>
<tr>
<td>ADU-FF</td>
<td>0.923α</td>
<td>0.060</td>
<td>1.106a,b,β</td>
<td>0.060</td>
</tr>
<tr>
<td>LAR-FF</td>
<td>0.915α</td>
<td>0.069</td>
<td>1.198bβ</td>
<td>0.115</td>
</tr>
<tr>
<td>WHO-FF</td>
<td>1.350bβ</td>
<td>0.071</td>
<td>1.167α</td>
<td>0.041</td>
</tr>
</tbody>
</table>
Figure 1: Experimental protocol; See text for details.
Effect of the presence of ethanol on ADH activity and Adh expression

**Figure 2:** Schematic view of the Adh gene of *Drosophila melanogaster* and the position of the different enhancers and transcription factor binding sites. Non-protein coding exon regions are indicated in dark grey and protein coding exon regions in black. PL, proximal (larval) promoter; PA, distal (adult) promoter. \( \text{∇}_1 \) and \( \text{∇}_2 \) indicates positions of insertion/deletion polymorphisms. S/F is the position of the slow/fast polymorphism. Protein factor binding sites: ADF-1, horizontal striped box; ADF-2, double hatched box. Binding sites in the adult enhancer: BBF-2, white triangle; AEF-1, white box; C/EBP, white oval; FTZ-F1, white rhombus. →1 and ←2 indicate the positions of the primers used. After J. Oppentocht (2001).
Figure 3: Adult ADH activity (μmol/min/μg protein) of the control (CON) and the three selected lines (ADU, LAR and WHO) for both Adh genotypes and both sexes on standard medium (0%), after one day (1 day) or after three days (3 days) on ethanol medium.
Effect of the presence of ethanol on ADH activity and Adh expression

Figure 4: Adult ADH activity of the three selected lines relatively to the activity measured in the control line with the correspondent Adh genotype and the correspondent sex. A value of one indicates equal ADH activities in the selected line and the control line. A * indicates a significant difference at the 5% level between the selected and the control line. Results are given for both Adh genotypes and both sexes on standard medium (0%), after one day (1 day) and three days (3 days) on ethanol medium.
Figure 5: Larval ADH activity (μmol/min/μg protein) of the control (CON) and the three selected lines (ADU, LAR and WHO) for both Adh genotypes on standard medium and on ethanol medium.
Effect of the presence of ethanol on ADH activity and $Adh$ expression

Figure 6: Larval ADH activity of the three selected lines relatively to the activity measured in the control line with the correspondent $Adh$ genotype. A value of one indicates equal ADH activities in the selected line and the control line. A * indicates a significant difference at the 5% level between the selected and the control line. Results are given for both $Adh$ genotypes on standard medium and on ethanol medium.
Figure 7: Northern blot analysis of Adh expression of AdhSS adults on standard medium, after one day and after three days on ethanol medium. RNA was isolated from one-week-old males or females of each selected and control line. These RNAs were fractionated on an agarose gel, transferred to nitrocellulose and probed with radioactive DNA probes from –first- Adh, and –second- tubuline. The level of Adh mRNA was calculated relatively to the tubuline control by densitometry of autoradiograms. Relative levels of Adh mRNA are shown in the histograms. Two replicates were analysed per line, sex and environment.
Effect of the presence of ethanol on ADH activity and Adh expression

**Figure 8:** Northern blot analysis of Adh expression of Adh\(^{FF}\) adults on standard medium, after one day and after three days on ethanol medium. RNA was isolated from one-week-old males or females of each selected and control line. These RNAs were fractionated on an agarose gel, transferred to nitrocellulose and probed with radioactive DNA probes from –first- Adh, and –second- tubuline. The level of Adh mRNA was calculated relatively to the tubuline control by densitometry of autoradiograms. Relative levels of Adh mRNA are shown in the histograms. Two replicates were analysed per line, sex and environment.
Figure 9: Northern blot analysis of Adh expression of larvae on standard medium and on ethanol medium. RNA was isolated from three-day-old larvae (standard medium) or four-day-old larvae (ethanol medium) of each selected and control line. These RNAs were fractionated on an agarose gel, transferred to nitrocellulose and probed with radioactive DNA probes from –first- Adh, and –second- tubuline. The level of Adh mRNA was calculated relatively to the tubuline control by densitometry of autoradiograms. Relative levels of Adh mRNA are shown in the histograms. Two replicates were analysed per line and environment.