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Original Research Article

Interspecific divergence of Adh enzyme, ethanol and acetic acid tolerance in three cosmopolitan drosophila species from India

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Interspecific divergence; Drosophila species; Adh polymorphism; Ethanol and acetic acid tolerance; Resource utilization; Natural selection.

ABSTRACT
The three cosmopolitan and domestic species of Drosophila collected along latitude28̊ 54 N from Rohtak of the Indian subcontinent for this study. The pattern of Adh genetic variability, ethanol and acetic acid tolerance in adult and in larval individual revealed significant genetic divergence in these three species. Adh (Alcohol Dehydrogenase) locus was found to be effectively polymorphic and was represented by two common allele and high heterozygosity in D. melanogaster and D. ananassae while D. busckii revealed one frequent and one rare allele and low heterozygosity. D. melanogaster revealed highest ethanol as well as acetic acid tolerance level as compared with the D. ananassae and D. busckii. Due to ethanol utilization, increased longevity periods were found to be 84.5 hrs, 165 hrs. and 300 hrs. in D. busckii, D. ananassae and D. melanogaster respectively. However, the increase in longevity on the basis of acetic acid utilization was found to be 66 hrs., 110 hrs., 216 hrs. in D. busckii, D. ananassae and D. melanogaster is significantly different. Thus, the interspecific differences for these metabolites' tolerance could be adaptively maintained by natural selection mechanism and patterns of resource utilization are species specific.

1. Introduction

The evolutionary potential of a species is a function of the amount of genetic variation it undergoes. Colonizing species populations offer the most suitable material for micro-evolutionary studies [1, 2]. Eight Drosophila species have been known as cosmopolitan while 21 drosophilids have been designated as widespread [3, 4]. Many species of the drosophilidae family feed on diverse types of fermenting and rotting fruits, vegetables, cacti, flowers and decaying organic food materials [5, 6]. Ethanol is the end product of fermentation and ethanol vapors provide a normal energy source in D. melanogaster [7].

Ethanol is converted into acetic acid via acetaldehyde and thus the concentrations of these two metabolites are generally found in natural habitats of the Drosophila species. Recently acetic acid has been found to be a resource similar to that of ethanol [8]. The parallel patterns of utilization of acetic acid and ethanol seem to be correlated with the concentration of these two metabolites found in nature [9]. The phenomenon of ethanol tolerance has been studied from the ecological, physiological and genetic viewpoints in D. melanogaster [7, 10].

Most studies on allozymic polymorphism have been made on American and Australian populations of D. melanogaster while Asian populations remain unexplored [11, 12, 13]. Adh is known to be involved in the utilization and detoxification of exogenous alcohols, the fermentation by products produced in the environment depends on the type of microflora (yeasts and microbes) involved in the decomposition of different types of organic matter [7]. The alcohol dehydrogenase of D. melanogaster converts a wide range of alcohols to aldehyde and more than 90% of the external alcohols are metabolized in a pathway initiated by Adh [14].

Natural populations of D. melanogaster, D. ananassae and D. busckii found to be polymorphic at the Adh locus and generally contained both the common electrophoretic alleles: Slow -Adh S and Fast-Adh F. Ethanol tolerance in D. melanogaster was found to be Adh genotype dependent i.e., Adh-FF homozygotes revealed higher ethanol tolerance [15, 16]. Among three sympatric Melbourne populations, the threshold ranking was found to be D. melanogaster > D.simulans > D. immigrans. At the intraspecific level, adult ethanol tolerance to ethanol vapours decreased towards the equator in Australian populations of D. melanogaster, D. simulans, D. immigrans [17, 18, 19]. Thus, diverse types of drosophilids reflect the interspecific differences in tolerance to different alcoholic resources. David and Van Herrewege, 1983 revealed that D. melanogaster and D. lebanonis are highly ethanol tolerant while other species are found to be ethanol sensitive. Ethanol tolerance analysis revealed significant divergence in D. melanogaster and D. simulans and thus provided a mechanism for niche separation for these sibling species [20, 21].

Most studies on ethanol tolerance have been made on D. melanogaster and D. simulans population from Europe, Africa [22] and Australia [10, 20, 24]. But the interspecific divergence of ethanol and acetic acid tolerance in three cosmopolitan

Drosophila species from temperate as well as tropical parts of the world are still lacking.

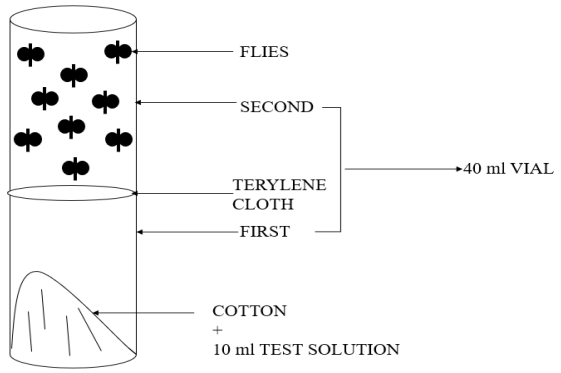

Figure 1: Adult ethanol/acetic acid testing apparatus.

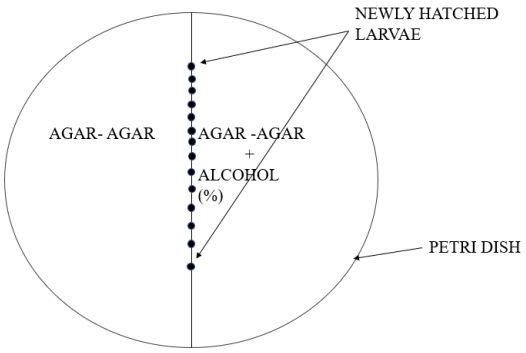

Figure 2: Larvae testing apparatus.

2. Materials and methods

Mass bred populations of three cosmopolitan species were used for ethanol and acetic acid utilization and Adh enzyme analysis from Rohtak (northern region 28° 54 N).

Data on the number of isofemale lines which were maintained for five to six generations in the lab is given in Table 1. Homogenates of single individual (one fly per isofemale line) were subjected to electrophoresis at 250V and 25mA at 4° C for 4 hrs. The gel slices were stained for Adh gene enzyme system by a standard staining procedure. Genetic control of Adh banding patterns was interpreted from the segregation patterns of enzyme electro morphs of parents, F1 andF2 progeny of several single pair mating.

The ethanol or acetic acid tolerance patterns of mass-cultures of D. melanogaster, D. ananassae and D. busckii were assessed following the procedure of Starmer et al. [30]. In order to test the ethanol utilization groups of 10 males or 10 females, grown on a killed yeast medium, were aged for 3 days on fresh food medium and then transferred to a set of 2 air tight plastic vials (40 ml, 4 inches), the flies were admitted to the upper vial which was separated by fine terylene cloth from the lower vial containing different concentrations of 10 ml ethanol or acetic acid (1 to 8%) absorbed on 1 gm cellulose wool (Figure 1). Such paired vials were sealed with the cellophane tape and all the experiments were conducted at 23°C. The alcoholic solutions were not changed during the experiments. The flies were not etherized during the experiment. The control vials contained 10 ml of distilled water absorbed on cellulose wool.

All the experiments were performed in four replicates at 23°C. For the control experiments water is used in place of test solution for each concentration of ethanol or acetic acid, 40 males and 40 females were treated with a range of 1-8 different concentrations of ethanol or acetic acid. The male and female individuals did not reveal any significant difference in ethanol or acetic acid tolerance and thus the data for two genders were averaged in all the experiments. Adult survivorship was monitored by daily observations of control and ethanol or acetic acid treatment experiments that is the effects of metabolic alcoholic vapours were assessed from the number of flies alive after various time intervals. The LT50 values were calculated as the number of hours at which 50% of the flies had died and were estimated by linear interpolation. The ethanol or acetic acid threshold values were used as indices that is if vapours were utilized as resource, then LT50 ethanol / LT50 control was found to be more than 1; if this ratio is less than 1then it is acted as stress. The threshold values were determined when LT50 ethanol / LT50 control =1 [7].

The larval behavior towards ethanol was analyzed by following the method of Gelfandand McDonald [29].The relative number of larvae out of a total of 10 on the two sectors of agar petri dishes (with and without ethanol or acetic acid) were noted after 20 min of each ethanol or acetic acid concentrations (Figure 2). Five replicates were tested at each ethanol or acetic acid concentration at 20°C for each species the threshold values between attraction and avoidance after 20 minutes were then calculated.

3. Results and discussion

1. Interspecific genetic basis of electrophoretic phenotype

The genetic basis of enzyme banding patterns was investigated from Mendelian segregation ratio of electrophoretic phenotypes in the progeny of genetic crosses i.e., of the parents and progeny. The double band represented the homozygous, triple band and four banded patterns represented the heterozygous genotype respectively.

D.melanogaster: The Adh enzyme revealed segregating two banded patterns both for faster and slower mobilities. The Adh electrophoretic data on parents and progeny of genetic crosses was found to be in agreement with the monogenic control of Adh patterns, Thus homozygous individuals showing two banded patterns represented electro morphs or allozymic variants (Figure 3).

D.ananassae: The Adh enzyme revealed a single cathodal zone of activity, segregating two banded patterns (of either faster or slower mobilities) and four banded patterns of Adh were observed in the individual's genetic crosses involving different two banded patterns resulted in four banded patterns in F1 individuals and 1:2:1 ratio of segregating two banded and four banded patterns in F2 progeny. Thus, Adh electrophoretic data of parents and progeny of genetic crosses were found to be in agreement with the monogenic control of Adh patterns. The homozygous individuals exhibit two banded patterns and four banded patterns are observed in heterozygotes (Figure 3).

D. busckii: The single Adh zone depicted segregating patterns of two banded and three banded phenotypes in single

individuals. Genetic crosses involving different two banded Adh patterns resulted in three banded patterns in F1 and segregation ratio of 1:2:1 in F2. Thus, this enzyme represented conformational electrophoretic phenotypes under the independent control of a single locus. There is occurrence of two banded electrophoretic phenotypes of Adh in homozygous strains of D. busckii (Figure 3).

The present observations on Adh electrophoretic phenotypes concurred with other reports in D. melanogaster that in NAD requiring dehydrogenase more than one electro morph (conformational isozymes) might arise due to post-translational differential binding of co-enzyme NAD in all the three species of Drosophila. Since Adh enzyme banding pattern was found to be identical in both the genders so the loci coding for this enzyme system is autosomal in all the three species of Drosophila.

The present observations correspond to what has been known for Adh for a long time in D. melanogaster and other species.

2. Interspecific Adhallozymic variation

The distribution of Adh genotypes, allelic frequencies observed and expected heterozygosityand log-likelihood chi-square test for fit to Hardy-Weinberg's expectations at the Adh locus in D. melanogaster, D. ananassae and D. busckii has been given in Table 1.

The Adh locus was found to be effectively polymorphic and was represented by two frequent alleles and revealed high heterozygosity in D. melanogaster and D. ananassae. But D. busckii revealed one frequent and one rare allele and low heterozygosity. All the three species revealed deviation from Hardy-Weinberg equilibrium at Adh locus.

3. Interspecific variation of ethanol tolerance in Larvae and adult

Larval behavior with ethanol: The data on mean number of larvae of each of the three Drosophila species choosing agar-agar plus various concentrations of ethanol for the experimental duration of 20 minutes have been shown in Figure 4(a) (Table 2). The larval ethanol tolerance response revealed significant variation between three different Drosophila species, i.e. D. busckii (3.2%), D. ananassae (4.2%) and D. melanogaster (10.1%). This shows that D. melanogaster larval forms revealed the highest ethanol tolerance levels as compared with the other two cosmopolitan Drosophila species. The ranking order of the tolerance of three species is D. melanogaster > D. ananassae > D. busckii. The longevity data of D. busckii revealed a minimum increase (84.5 hrs.) as compared to the other two species i.e., D. ananassae (165 hrs.) and D. melanogaster (300 hrs.).

The data of LT50 hrs. and LT50 maximum/LT50 control (as a measure of resource versus stress) are given in Figures 5(a) and (6a) (Table 2).

Adult ethanol tolerance: The adult ethanol threshold values were found to follow the similar ranking in order as observed for three species specific larval individual, i.e., D. melanogaster > D. ananassae > D. busckii.

But as compared to larval analysis the adult ethanol threshold values were found to be lower in D. busckii (2.3%) and D. ananassae (3.4%), but higher for D. melanogaster (13.0%).

The LC50 ethanol concentrations were calculated from mortality data of adults after 2 days in D. busckii; Fourth day in D. ananassae and after sixth day in D. melanogaster are given in Table 2. The LC50 values were found as 2% in D. busckii; 3.5% in D. ananassae and 10.8% in D. melanogaster (Figure 7(a)). Comparative survival data /longevity data of ethanol tolerance at 1% in D. busckii and D. ananassae and 6% in D. melanogaster have been shown in Figure 8(a).

4. Interspecific variation of acetic acid tolerance in Larvae and adult

Larval behavior to acetic acid: The data on mean number of larvae of each of the three Drosophila species choosing agar-agar plus various concentrations of acetic acid for the experimental duration of 20 minutes have been shown in Figure 4(b), (Table 2). The larval acetic acid tolerance response revealed significant variation between three different Drosophila species i.e., D. busckii (3%), D. ananassae (3.9%) and D. melanogaster (9%). Thus, D. melanogaster revealed the highest acetic acid tolerance levels as compared with the other two cosmopolitan Drosophila species. The ranking order of the species included: D. melanogaster > D. ananassae > D. busckii. The longevity data of D. busckii revealed a minimum increase (66hrs.) as compared with the other two species i.e., D. ananassae (110 hrs.) and D. melanogaster (216 hrs.). The data on LT50 hrs. and LT50 maximum/LT50control (as measures of resource versus stress) are given in Figures 5(b) and 6(b) (Table 2).

Adult behavior to acetic acid: The adult acetic acid values were found to follow the similar ranking order as observed for the species-specific larval individuals i.e., D. melanogaster > D. ananassae > D. busckii. As compared with larval analysis, the adult acetic acid threshold values were found to be lower in D.busckii (3.1%) and D.ananassae (4.6%) but higher for D. melanogaster (12.6%). The LC50 acetic acid concentrations were calculated from mortality data of adults after 2 days in D. busckii; 4^{th} day in D.ananassae and after 6^{th} day in D. melanogaster are given in Table 2.The LC50 values were found as 3% in D.busckii ; 4% in D.ananassae and 9% in D.melanogaster (Figure 7(b)). Comparative survival data/ longevity data of acetic acid tolerance at 1% in D.busckii and D. ananassae and 6% in D. melanogaster have been shown in Figure 8(b).

But interestingly the larval and adult stages of all the three Drosophila species could utilize acetic acid in parallel way to that of ethanol. The increased longevity data revealed parallel but lesser effect of acetic acid utilization i.e., 66 hrs. in D. busckii, 110 hrs. in D. ananassae and 216 hrs. in D. melanogaster. The comparative data on species specific acetic acid threshold values, mortality and longevity responses further supported that acetic acid was utilized as resource in the three Drosophila species (Table 2).

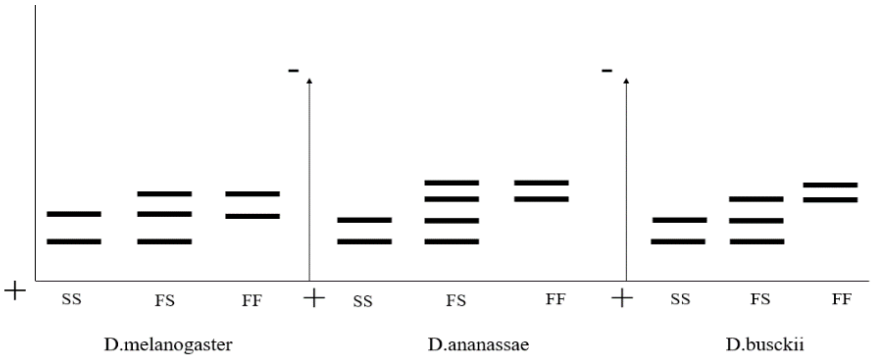

Figure 3: Starch gel electrophoretic banding pattern of Adh enzyme.

Table 1: Adh genotypes, allelic frequencies, heterozygosities (obs./exp.) and G-Values for log-likelihood chi square test for Hardy-Weinberg expectations in three cosmopolitan Drosophila species from Rohtak.

Species	Adh Genotypes			Sample size	Allelic frequency		Het. Obs./exp.	G-Values
	FF	SS	FS		F	S		
D.melanogaster	62	13	28	103	.74	.26	.27/.38	8.54*
D.ananassae	44	16	28	88	.66	.34	.32/.45	7.38*
D.busckii	4	92	4	100	.06	.94	.04/.11	18.39*

* Significant at 5% level.

Table 2: Comparison of ethanol as well as acetic acid tolerance indices (increase in longevity LT50 hrs.; LT50 maximum/LT50 control; adult and larval threshold concentrations and LC50) in three cosmopolitan Drosophila species from Rohtak.

Metabolites/Species	Increase in Longevity*		Threshold concentration**		LC50 values***
	LT50(hrs.)	LT50max/LT50control	Larval	Adult	
ETHANOL					
D.melanogaster	300.0	3.12	10.1	13.0	10.8
D.ananassae	165.0	2.39	4.2	3.4	3.5
D.busckii	84.5	1.76	3.2	2.3	2.0
ACETIC ACID					
D.melanogaster	216.0	2.25	9.0	12.6	9.0
D.ananassae	110.0	1.60	3.9	4.6	4.0
D.busckii	66.0	1.38	3.0	3.1	3.0

* Increase in longevity at 1% in D. busckiiandD. ananassae as well as at 6% in D. melanogaster.

** Threshold obtained by interpolation from graphs andtherefore without standard errors.

***LC50 was measured in D. busckii (on 2nd day); in D. ananassae (on 4th day) and in D. melanogaster (after 6th day).

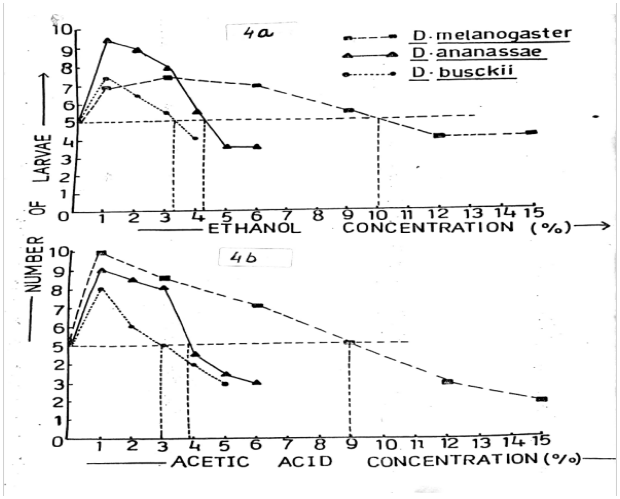

Figure 4(a): Mean number of larvae preferring different concentration of ethanol in three Drosophila species.
Figure 4(b): Mean number of larvae preferring different concentration of acetic acid in three Drosophila species.

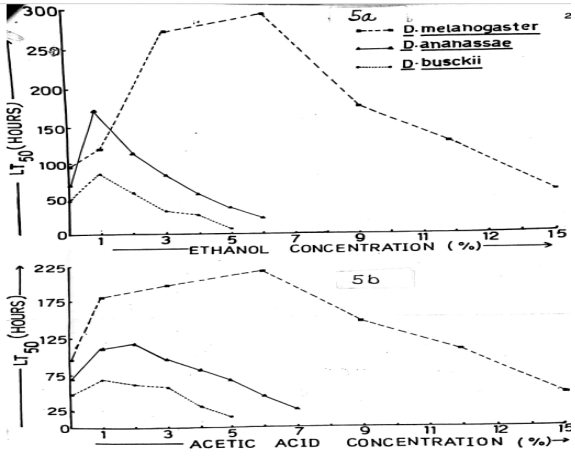

Figure 5(a): Comparative profile of adult survivorship expressed as LT50 hrs. at different ethanol concentrations in three Drosophila species.
Figure 5(b): Comparative profile of adult survivorship expressed as LT50 hrs. at different acetic acid concentrations in three Drosophila species.

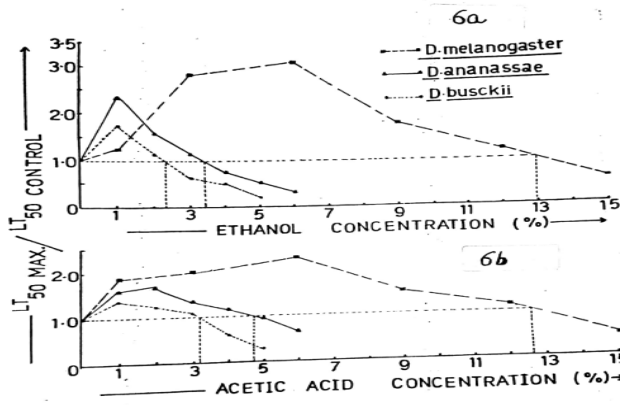

Figure 6(a): Comparative adult longevity data LT50 maximum / LT50 control at different ethanol conc. in three Drosophila species.
Figure 6(b): Comparative adult longevity data LT50 maximum / LT50 control at different acetic acid conc in three Drosophila species.

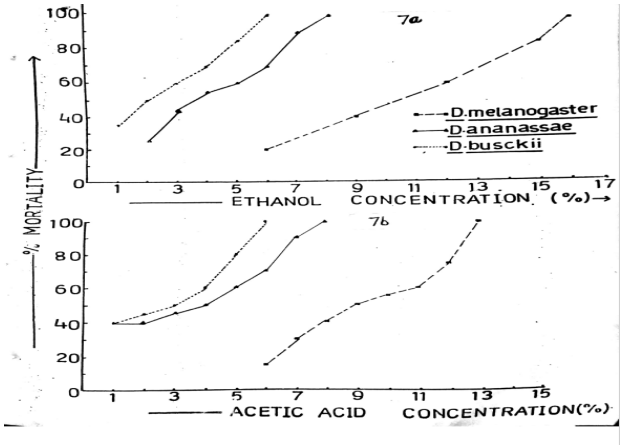

Figure 7(a): Comparative profiles of %mortality relationship at different concentrations of ethanol in three Drosophila species.
Figure 7(b): Comparative profiles of %mortality relationship at different concentrations of acetic acid in three Drosophila species.LC50 was measured on 7th day in D. melanogaster, on 4th day in D. ananassae and on 2nd day in D. busckii.

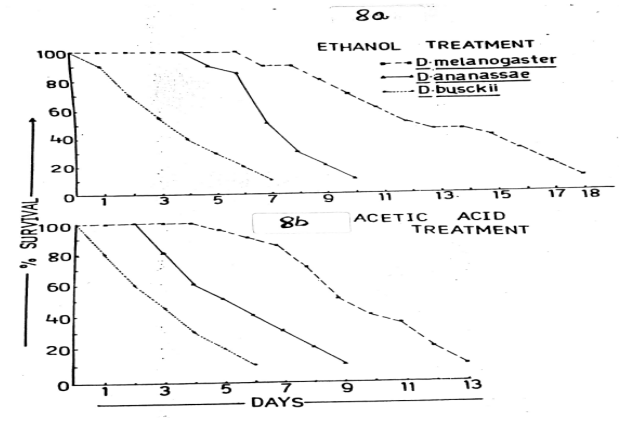

Figure 8(a): % survival data of three Drosophila species at different concentrations of ethanol i.e., at 6% in D. melanogaster,4% in D. ananassae and 2% in D. busckii.
Figure 8(b): % survival data of three Drosophila species at different concentrations of acetic acid i.e., at 6% in D. melanogaster, 4% in D. ananassae and 2% in D. busckii.

The cosmopolitan Drosophila species exploit a wide array of fermenting and decaying fruits and vegetables. As these Drosophila species are fruit niche species, they are known to utilize ethanol as a resource in nature [21].

The niche width of colonizing species such as D. melanogaster and D. ananassae was found to be much higher than D. busckii. The present data revealed that the threshold concentration at which ethanol ceases to be resource and becomes a stress in local cosmopolitan population that is Rohtak was found to be 13% in D. melanogaster, 3.4% in D. ananassae and 2.3% in D. busckii. Responses of newly hatched larvae also gave threshold between attraction and avoidance in the same sequence.

The threshold concentration at which acetic acid became a stress was found to be almost similar to that for ethanol for all the three cosmopolitan species. On the other hand, the values of LT50 maximum: LT50 control were not equivalent for the two metabolites that is such values for acetic acid were lower than that of ethanol. Thus, there were some correspondence between utilizations of ethanol and acetic acid. The threshold values for both metabolites were highest for D. melanogaster and lowest for D. ananassae while LT50 maximum: LT50 control values did not reveal a similar trend for acetic acid. The threshold values and values of LT50 max: LT50 control for both the metabolites were highest in D melanogaster as expected from its presence in domestic fermenting sites and lowest values were for D. ananassae.

The occurrence of low level of ethanol and acetic acid tolerance in D. busckii corresponds with the low level of polymorphism at Adh locus in this species. The larval individuals of all the three species revealed lower ethanol as well as acetic acid tolerance than that of adults.

So out of all the three cosmopolitan species D. melanogaster was found to be highly tolerant, D. busckii was ethanol and acetic acid sensitive while D. ananassae was found to be intermediate in tolerance to ethanol and acetic acid. Ethanol has been mainly considered as a resource for D. melanogaster in labs as well as field experiments [27]. The present observations concurrent with the suggested relationship between ethanol and acetic acid tolerance and larval habitat and their tolerance in several Drosophila species [28].

Thus, the three species seem to have adaptively partitioned their ecological niches in terms of concentrations of alcoholic resources available in man-made indoor fermenting and outdoor fermenting resources. Since the fermenting food sources as well as the biotic factors (temperature, humidity, rainfall) are markedly different in Indian subcontinent.

All these observations suggested that for ethanol tolerance regulatory genetic mechanisms seem to be more important than structural differences between Adhallozyme and further ethanol tolerance threshold values in larval and adult individuals are found to vary with species. The observed alcoholic utilization profiles of the three cosmopolitan and domestic species of D. melanogaster, D. ananassae, D. busckii reflected species specific characteristics in alcoholic metabolism that is ethanol and acetic acid are species specific and adaptive characteristics in Indian subcontinent.

4. Conclusions

It is predicted from this study that diverse types of drosophilids reflect inter specific variance in tolerance to ethanol, acetic acid and Adh genetic diversity in the Indian subcontinent and could be adaptively maintained by natural selection mechanism. Also, the present studies revealed that acetic acid also constitutes the resource in parallel to ethanol in all three types of Drosophila species which significantly differ in the alcoholic utilization profiles. This present study also provided a quantitative assessment of species-specific pattern of resource utilization.

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