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RESEARCH ARTICLE

Fitness differences due to allelic variation at *Esterase-4* locus in *Drosophila ananassae*

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Abstract. Esterases are known to play essential role in metabolism, reproductive physiology and behaviour of *Drosophila*. Esterases are highly polymorphic enzymes in *Drosophila*, but the polymorphism of these enzymes is not well studied in *Drosophila ananassae*. Recent studies on esterase polymorphism in *D. ananassae* revealed that *Est-4* locus comprises *Est-4 active* and *Est-4 null* alleles depending on enzymatic activity. For the *in vivo* functional characterization of this locus, homozygous lines of genotypes *Est-4 active* and *Est-4 null* were derived from the flies collected from Gangtok, Sikkim, in 2006. Mating propensity, mating pattern, fecundity, fertility and productivity of female, life span and triglycerides level were investigated in the flies bearing either *Est-4 active* or *Est-4 null* genotypes. Results showed that mating occurred randomly with nonsignificant difference in mating propensity between *Est-4 active* and *Est-4 null* flies. However, a significant difference in fecundity and strong dependency between genotypes and the rate of fertility was found. The median values of progeny produced per female were 24 and 20 for *Est-4 active* and *Est-4 null* genotypes, respectively. The life span assay showed a significant difference in the survivorship between the two genotypes. Triglycerides level was higher in *Esterase-4 active* larval haemolymph as well as in mature flies' homogenate than that of *Esterase-4 null*. Thus, *Esterase-4* locus of *D. ananassae* has its role in fecundity, fertility and productivity of female, life span control and lipid metabolism.

Keywords. esterases; null allele; reproductive fitness; natural selection; *Drosophila ananassae*.

Introduction

The primary aim of the population genetics is to determine the adaptive significance of genetic polymorphism. Genetic polymorphism for the enzyme loci was first introduced by Lewontin and Hubby (1966) in *Drosophila pseudoobscura*, followed by Ayala *et al.* (1970) in sibling species of *D. willistoni*. Esterases are one of the most studied enzymes and found to be highly variable in *Drosophila*. Approximately, 22 soluble esterase isozymes were identified through electrophoretic analysis in *D. melanogaster*, of these 10 were carboxylesterases (Healy *et al.* 1991). Then again, computational annotation of fly genome lists 35 genes encoding carboxylesterase and of them 10 genes are from the *α*-*Esterase* cluster (Tweedie *et al.* 2009; www.flybase.org). There is considerable support for the diverse function of *α*-*Esterase* genes. *α*-*Esterase1*, *α*-*Esterase2* and *α*-*Esterase8* are prominently expressed in adult fly heads (Campbell *et al.* 2003; Chintapalli *et al.* 2007). *α*-*Esterase1* and *α*-*Esterase8* are transcriptionally upregulated in heads of adult males of fly lines selected for aggressive behaviour (Dierick and Greenspan 2006), *α*-*Esterase2* acts as mating responsive gene (Ellis and Carney 2010). In addition to this, being the members of fat body lipid droplet proteome, *α*-*Esterase2* and *α*-*Esterase7* may have function in lipid metabolism (Beller *et al.* 2006). Recently, study by Birner-Gruenberger *et al.* (2012) revealed that *α*-*Esterase7* has important functions in insecticide tolerance, lipid metabolism and life span control. Moreover, *Esterase-6* was found as most analysed locus in *D. melanogaster* whose product is primarily an adult male enzyme and the majority of its activity was localized to the anterior ejaculatory duct of male reproductive tract. *Esterase-6* is transferred to the female within the first minute after the initiation of copulation (Richmond and Senior 1981). The presence of enzyme in a male's seminal fluid has effects on the duration of copulation (Gilbert and Richmond 1981), short-term and long-term remating of females (Gilbert *et al.* 1981), sperm use and progeny production (Gilbert and Richmond 1982). Gilbert and Richmond (1982) demonstrated that at low temperature, *D. melanogaster* males carrying active *Esterase-6*, mate sooner, copulate for shorter time and produce more progeny than *Esterase-6* null males, deducing selection is operating on *Esterase-6*. The Mendelian pattern of inheritance of *Esterase-6* was explained by Wright (1963). *Esterase-5* of *D. pseudoobscura* is an orthologue of *Esterase-6* of *D. melanogaster*, which is mainly expressed in the eye. On the other hand, *Esterase-6* of *D. melanogaster* was expressed in male reproductive tissue (Oakeshott *et al.* 1993). Consequently, esterases are well known to hold functional diversity.

Regarding the study of genetic polymorphism in genus *Drosophila*, *D. ananassae* has received much attention (Singh 2013). Chromosomal polymorphisms have extensively been examined in this species (Singh 2010). However, studies on protein polymorphisms needs to be explored. In our laboratory, surveys on enzyme polymorphism in *D. ananassae* are set off by Kumar and Singh (2014). While studying allozyme polymorphism in *D. ananassae*, it was observed that *Esterase-4* locus consists of active and null alleles in this species (Krishnamoorti and Singh 2013). Individuals, hemizygous, homozygous, or heterozygous for null alleles at loci coding for the production of enzymes are expected to have reduced fitness and the depression in fitness is correlated with the importance of the enzyme functions to overall metabolism (Voelkar *et al.* 1980).

The present study is an attempt to investigate the *in vivo* functions of *Est-4* locus of *D. ananassae*. It is assumed that this locus may have direct or indirect role in mating propensity, mating preference, fecundity, fertility and productivity, longevity and lipid metabolism. Thereby, above parameters were assayed in the flies which are either homozygous for *Est-4 active* or *Est-4 null* alleles.

Materials and methods

Drosophila stocks

Drosophila ananassae stocks used in the present study were derived from the flies of natural population of Gangtok, India, collected in 2006. Several isofemale lines were set up. Arrangements of gene in all the chromosomes of every isofemale line were examined, following the map of salivary gland chromosomes of *D. ananassae* prepared by Ray-Chaudhuri and Jha (1966). Isofemale lines having standard gene arrangement in all the chromosomes were isolated through the process of selection. Subsequently, by crossing these isolated lines, a karyotypically homozygous stock was established, namely, GT-ST / ST. Virgin females and males were collected from this stock to set large number of pair mating to see the pattern of esterase variants in their progeny. Esterase profiling of progeny was carried out through native PAGE using enzyme specific substrate (1-naphthylacetate AR) and stain (fast blue RR). On the basis of enzymatic activity, active and null alleles of *Est-4* locus were identified. Various homozygous lines for *Est-4 active* (+/+) and *Est-4 null* (−/−) were isolated through selection process (Aslund and Rasmuson 1978). For the *in vivo* functional characterization of *Est-4* locus, homozygous stocks of *Est-4 active* (+/+) and *Est-4 null* (−/−) genotypes were established by crossing different homozygous lines of each genotype to randomize the genetic background, so that the observed differences were attributable to the variation at *Est-4* locus alone (figure 1). It was found that the heterozygotes (+/−) of *Est-4 active* and *Est-4 null* express enzyme activity, indicating *Est-4 active* allele behaving as a dominant allele and these two alleles follow the Mendelian pattern of segregation. Maintenance and experimentations of *D. ananassae* stocks were carried out in a temperature controlled laboratory (∼24°C) with 60–80% relative humidity (RH) and 12-h L/D cycle.

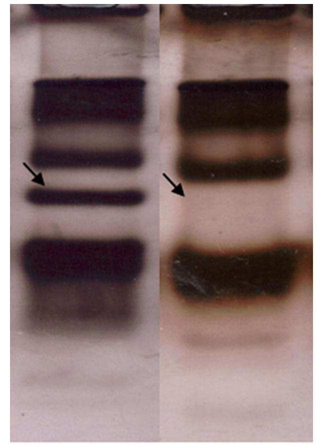

Figure 1. Alpha-esterase patterns observed in native polyacrylamide gel, arrows indicating *Est-4 active* and *Est-4 null* alleles in *D. ananassae*.

Mating propensity and mate choice

Virgin flies of *Est-4 active* and *null* were collected and aged for 6–7 days. To assay the mating propensity of these two genotypes, 20 males and 20 females of the same genotype were introduced into Elens–Wattiaux mating chamber (Elens and Wattiaux 1964) without etherization and observed for 60 min. When a pair commenced mating, it was aspirated out and number of matings was recorded.

To test whether there is preferential or random mating between these two genotypes, male-choice method was employed in which 15 males of one type, i.e. either *Est-4 active* or *null* and 15 females of both the genotypes were placed in Elens–Wattiaux mating chamber. To identify the females of either genotype, *Est-4 null* females were marked by wing clipping method (Som and Singh 1998; Nanda and Singh 2008). The total number of flies in a mating chamber was 45 and sex-ratio was 1 male : 2 females. Mating was observed for 1 h. When a pair started mating, it was aspirated out and kept in a separate empty vial. Later, the type of mating, i.e. homogamic or heterogamic was identified. Each experiment was conducted in five replicates. The experiments were carried out between 7:00 and 11:00 am.

Fecundity

Six to seven days aged single virgin female and male flies of the respective genotypes ($n = 21$) were kept in food vial seeded with active yeast. After 24 h, each pair was transferred to fresh food vials and eggs were counted using binocular. This process was repeated, for consecutive observations of egg production, for the next 10 days.

Fertility and productivity

Virgin female flies of the respective genotypes were individually mated with male of the same genotype ($n = 148$ for *Est-4 active* and $n = 150$ for *Est-4 null* in three replicates of cohorts of ~50 flies). The females that were able to produce progeny were considered as fertile females. Then, productivity of fertile females ($n = 97$ for *Est-4 active* and $n = 71$ for *Est-4 null*) was observed up to 12 days. Flies were transferred to fresh food vials every 4th day. Thus, three transfers were made during 12 days observation. F_1 progeny of fertile females were scored.

Longevity

Virgin female and male flies of both genotype ($n = 30$) were kept individually in separate food vials to observe their survivorship. They were transferred to fresh food vials every week, while the surviving flies were counted every day.

Triglycerides assay

Triglyceride was measured in the larval haemolymph and seven-days-aged flies of *Est-4 active* and *Est-4 null* using Autospan-diagnostic kit for triglycerides assay.

Haemolymph collection from larvae: Third instar larvae were slit with fine needle on a slide containing 0.1% saturated PTU in ethanol. Five to six speared larvae were put in a 0.5 mL perforated microfuge tube. Then, such perforated microfuge tubes were placed into 1.5 mL tubes without lid. Further, it was centrifuged at 10,000 rpm for 2 min and then 0.5 mL tubes were discarded. The heamolymph accumulated in 1.5 mL tube was collected in another tube and kept at –20°C.

Adult fly homogenate preparation: Flies ($n = 5$) were homogenized in 100 μL PBS + 0.1% Tween, centrifuged at 10,000 rpm for 2 min; supernatant was collected and heated for 5 min at 65°C to inactivate lipase and then used for triglyceride assay. The triglyceride assay was made in three replicates of cohorts for both the samples.

Statistical analyses

JMATING software, which is the first complete and versatile software for analysing sexual selection and sexual isolation from mating frequency data was used for the analysis of mate choice experiment (Coyne *et al.* 2005; Carvajal-Rodriguez and Rolan-Alvarez 2006). It is freely available on http://www.uvigo.es/webs/c03/webc03/XENETICA/XB2/JMsoft.htm and requires the Java runtime environment.

Unpaired *t*-test was employed to test the difference in mating propensity, fecundity and triglycerides level using Sigma-Stat 2.0 software. Mann–Whitney rank sum test was used to see the level of difference in productivity. For survivorship assay, log-rank test was applied.

Results

Mating propensity and mate choice

During the 1 h observation, of the 20 possible matings, an average mating success was found to be 8.4 and 7 for *Est-4 active* and *Est-4 null* genotypes, respectively. Unpaired *t*-test indicated that there is no significant difference in the mean mating success of the two genotypes of *Est-4* ($P = 0.447$; table 1).

Results of mate choice experiment presented in table 2 show the number of homogamic or heterogamic matings between the genotypes of *Est-4*. Theoretically, two main mechanisms can produce deviations from random mating, sexual selection and sexual isolation due to discrepancy in mating propensity and mate choice. Therefore, heterogeneity G test for sexual selection effects (GS), sexual isolation effects (GI) and for the combined effects (GT = GI + GS) were assessed (table 3). GT, GI and GS values are not statistically significant, providing evidence for random mating. However, value of GS is very close to the value of $P = 0.05$, suggesting that a moderate sexual selection might be operating. Estimates of PTI coefficients for each mating pair combination and I_{PSI} coefficient are shown in table 4. The PTI coefficients correspond to the combined sexual selection and sexual isolation effects; whereas I_{PSI} coefficient is the estimate of global sexual isolation. PTI coefficients are not statistically significant for all the possible mating pair combinations except for one, i.e. *Est-4 active* male + *Est-4 null* female combination. An estimate of I_{PSI} coefficient is also not significant.

Fecundity

The mean fecundity of females of *Est-4 active* and *Est-4 null* genotypes are presented in table 1, which demonstrates significant difference for the fecundity in females of the two genotypes ($P = 0.007$).

Table 1. Results of mating propensity, fecundity and triglycerides measurement of *Est-4 active* and *null* flies of *D. ananassae*.

	Est-4 active (mean ± SE)	*Est-4 null* (mean ± SE)	*t*	*Df*	*P*
Mating propensity	8.4 ± 1.28	7.0 ± 1.18	0.80	8	0.447
Fecundity	141.47 ± 8.67	103.57 ± 10.023	2.856	40	0.007*
Triglycerides in larvae (heamolymph) (mg/dL)	47.43 ± 3.95	20.45 ± 5.52	3.972	4	0.017*
Triglycerides in female (mg/dL/fly)	138.36 ± 23.92	70.05 ± 16.91	2.332	4	0.080
Triglycerides in male (mg/dL/fly)	44.65 ± 6.0	23.28 ± 5.74	2.576	4	0.062

*Significant.

Table 2. Results of male choice experiments involving *Est-4 active* and *Est-4 null* flies of *D. ananassae.*

Crosses		Homogamic matings		Heterogamic matings	
Female	Male	*n*	%	*n*	%
Est-4 active + *Est-4 null*	*Est-4 active*	25	33.33	17	22.66
Est-4 active + *Est-4 null*	*Est-4 null*	24	32	31	41.33

n, Total number of females mated.

Table 3. Heterogeneity G test for sexual selection and sexual isolation effects in the male choice experiments involving *Est-4 active* and *Est-4 null* flies of *D. ananassae*.

GS	GI	GT (GI + GS)
4.08	0.1	4.17^{NS}

GS is a G test for the sexual selection effects, GI is a G test for sexual isolation effects and GT is the G test for both combined effects (GT = GI + GS). NS, not significant.

Table 4. Results for PTI coefficients (estimates of mating preferences) and their standard deviations (in parentheses) for each mating pair combination.

	Male	
Female	*Est-4 active*	*Est-4 null*
Est-4 active	1.028 (0.177) $P = 0.93$	1.277 (0.19) $P = 0.154$
	0.0333 (0.1044) **$P = 0.7436$**	
Est-4 null	0.702 (0.155) $P = 0.05$*	0.993 (0.176) $P = 0.91$

The I_{PSI} coefficient (estimating sexual isolation) and its standard deviation are in bold. *Significant.

Fertility and productivity

Present study revealed that there is strong dependency between genotypes and the rate of fertility. The flies of *Est-4 active* are more fertile than *Est-4 null* flies ($P < 0.01$). The median value of progeny produced per female for *Est-4 active* and *Est-4 null* is 24 and 20, respectively (Mann–Whitney rank sum test), but the difference is not statistically significant ($P = 0.081$; table 5).

Longevity

Life spans of *Est-4 active* and *Est-4 null* female and male flies were observed and the results are depicted in figure 2. Survival curves showed a reduction in the life span of *Est-4 null* compared to the *Est-4 active* flies in both the sexes. Further, reduction in the life span of *Est-4 null* females compared to the *Est-4 active* females is significant ($P < 0.05$) indicating role of *Est-4* locus in the regulation of life span.

Triglycerides assay

To test the role of *Est-4* locus in lipid metabolism, triglycerides level in the 3rd instar larval haemolymph and in seven-day-aged females and males homogenate was measured. Triglycerides level in haemolymph of *Est-4 active* larvae is found to be significantly more than in *Est-4 null* larvae ($P = 0.017$). Triglycerides content was higher in adult flies of *Est-4 active* than *Est-4 null* in both the sexes as well. However, the difference is not significant ($P = 0.080$ in case of females and $P = 0.062$ in males; table 1).

Discussion

Apparent fitness differences among allozyme genotypes have been reported for various organisms and enzymes (Kojima and Yarbrough 1967; Birley and Beardmore 1977; Aslund and Rasmuson 1978). Studies on esterases in *Drosophila* have proven its functional diversity. *Esterase-6* plays a role in the physiological and behavioural dynamics of sex pheromone (cis-vaccenyl acetate; cVA) response in *D. melanogaster* males and it also acts as an odorant degrading enzyme (ODE) in male antennae (Chertemps *et al.* 2012). Despite the knowledge of representative of *Esterase-6* of *D. melanogaster*, in *D. ananassae*, it was speculated that if *Esterase-4* locus of *D. ananassae* have similar

Table 5. Results showing dependency between the rate of fertility and genotypes of *Est-4* locus (using R × C contingency table) and productivity of females of *Est-4 active* and *null* flies of *D. ananassae*.

Genotypes	Total no. of crosses	No. of crosses in which progeny appeared (fertile)	No. of crosses in which progeny not appeared (infertile)	χ^2	*Df*	*P*	Median value of F1 progeny/fly	*P*
Est-4 active	148	97	51	10.04	1	<0.01*	24	0.081
Est-4 null	150	71	79				20	

* Significant.

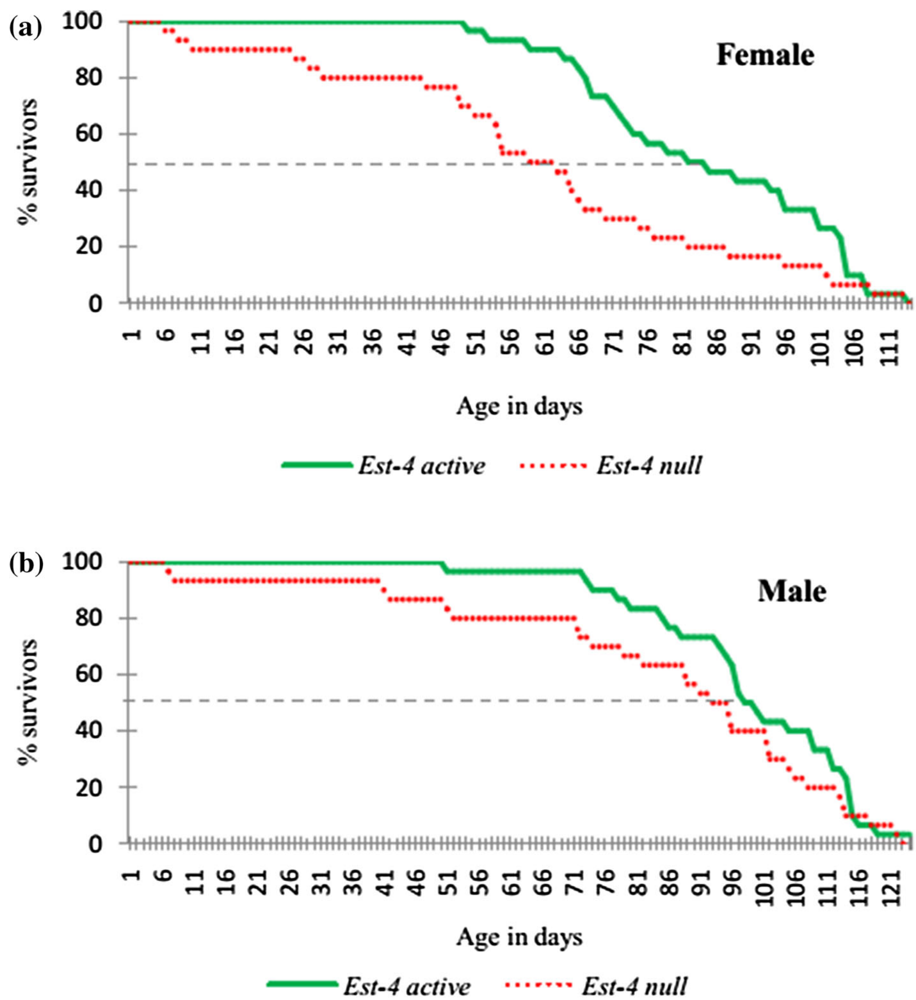

Figure 2. Survival curves showing median life span reductions more in genotype *Est-4 null* than genotype *Est-4 active.* (a) Survivorships in females of both the genotypes, log-rank $P < 0.05$*. (b) Survivorships in males of both the genotypes, log-rank $P > 0.05$. *Significant.

kind of function, then there could be the difference in mating propensity or activity and mate choice between the two genotypes, i.e. *Esterase-4 active* and *Esterase-4 null*. Nevertheless, in the present study, we could not get significant deviation in mating propensity and mate choice, indicating *Est-4 locus* of *D. ananassae* does not play role concerning these aspects.

Henceforth, it was thought that *Esterase-4* locus of *D. ananassae* may have some postmating functions like fecundity, fertility and productivity, etc. Concurrently, findings of the present study support our assumptions since we found significant difference for fecundity (egg laying capacity) and rate of fertility (female's ability to produce progeny) between *Esterase-4 active* and *Esterase-4 null* flies. Moreover, *Esterase-4 active* females produce more progeny than *Esterase-4 null*. Thus, present study revealed that there is positive association for fecundity, rate of fertility and productivity with the presence of *Esterase-4 active* allele in *D. ananassae*. In *D. melanogaster*, *Esterase-6* is a characterized enzyme which has effects on reproductive functions (Gilbert *et al.* 1981; Gilbert and Richmond 1982; Saad *et al.* 1994). In *D. melanogaster*,*Esterase-6* is concentrated in male anterior ejaculatory duct which is transferred to female during copulation that lead to rapid uses of sperm stored in storage organs. Rapid depletion in sperm storage organs is positively correlated with frequent remating, which is further associated with productivity.

Drosophila exhibits robust genetic variance for lifespan. Quantitative trait locus analyses (Mackay *et al.* 2006) and artificial selection regimes (Harshman and Hoffmann 2000) have demonstrated that flies derived from the natural populations harbour allelic variation that affects lifespan. Natural allelic variation has been characterized at a handful of loci, which are identified as ageing genes, for example, allelic variation in *Catsup*, *Ddc*, *Dox-A2*, *Lim3*, *ms(2)35Ci*, *stc* and *tup*, associated with variation in longevity in *D. melanogaster* (Paaby and Schmidt 2009). Allelic variation at G-protein coupled receptor *mth* in *D. melanogaster* showed significant differences in lifespan, fecundity and resistance to oxidative stress (Paaby and Schmidt 2008). Similarly, in the present study, *Esterase-4* locus is identified as one of the candidates causing natural genetic variation in longevity of *D. ananassae*. It is shown here that *Est-4 active* females have significantly higher life span than *Est-4 null* females. *Est-4 active* males also have higher life span than *Est-4 null* males, although the difference is statistically insignificant. The present study provides evidence that allelic variation at individual locus affects longevity.

Many fundamental aspects of cellular functioning including lipid metabolism require esterase activity. Insects store energy reserves in the form of glycogen and triglycerides in the adipocytes, the main fat body cells. Therefore, triglycerides level was measured in 7-day-aged flies homogenate and larval haemolymph of *Est-4 active* and *Est-4 null*. Results illustrated that there is a difference in triglycerides level in 7-day-aged flies as well as in larval haemolymph of *Est-4 active* and *Est-4 null*. Further, triglycerides level present in larval heamolymph of *Est-4 active* is significantly more than *Est-4 null*, indicating the role of *Est-4* locus in lipid metabolism in *D. ananassae*.

Thus, *Est-4* locus causes disparity in fecundity, rate of fertility and productivity, lifespan and triglyceride metabolism in *D. ananassae*. Reproduction, fat metabolism and lifespan are interconnected (Hansen *et al.* 2013). Various studies in different organisms manifested that increased life span is associated with reduced reproduction, but markedly increased lipid storage (Chippindale *et al.* 1993; Gems *et al.* 1998; Tatar *et al.* 2001; Judd *et al.* 2011). Reproduction is an energetically expensive process, which has profound effects on the metabolism of fat. Thus, there is inverse relationship between reproduction and fat storage that reflect an energetic trade-off. As a result of depletion of energy reserves to support reproduction (cost of reproduction), organisms compromise its ability to scaffold somatic maintenance and survival (Williams 1966), so that individuals with reduced reproduction survive better and live longer than those with higher reproductive effort (Bell and Koufopanou 1986; Partridge *et al.* 2005). However, such direct correlations are not found in our case, regardless of the evidence that *Est-4* locus has effect on reproduction, life span and triglycerides metabolism.

Considering the adaptive significance and frequency of nulls in natural populations, earlier workers proposed that the biological effects on the carriers and null homozygotes may be negligible and nulls are less likely to have strongly deleterious effects at loci coding for enzyme function in intermediary metabolism because of enzyme redundancy and alternate pathways (Voelkar *et al.* 1980). Surveys of null alleles at allozyme loci have demonstrated that they appear in natural populations due to mutation–selection balance (Voelkar *et al.* 1980; Langely *et al.* 1981). However, in this report, *Est-4* is identified as an important locus and it must be involved in metabolic pathways affecting fecundity, fertility, life span and triglyceride storage. While, lack of sexual isolation and trivial difference in sexual selection between the two genotypes of *Est-4* of *D. ananassae* might be one of the plausible reasons for the maintenance of *null* alleles in natural population despite of its low fitness value. Future studies related to characterization of this locus will reveal its structural homology with *D. melanogaster* esterases.

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