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The effects of dibutyl phthalate (DBP) on the development and fecundity of *Drosophila melanogaster.*

Atli, Emel.   Hacettepe University, Faculty of Science, Department of Biology, Ankara, Turkey. E mail: eakkan@hacettepe.edu.tr.

Abstract

Dibutyl phthalate (DBP) is a plasticizer used in the manufacture of several industrial and household articles. They get easily released to the environment and may cause adverse effects to living organisms. In this study, effects of DBP on the development and fecundity of *Drosophila melanogaster* have been studied. 72 h larvae of *D. melanogaster* were exposed to 0.25 mL/L, 0.5 mL/L and 1 mL/L DBP. The percentages and times of transition from larvae to pupae and from pupae to adults were determined, and the daily mean egg number was examined using the female offspring, which had been exposed as larvae. No differences were found in the transition percentages from larvae to pupae and from pupae to adults ($p > 0.05$). It was found, however, that both the mean pupation and the mean maturation time were accelerated with 0.25 mL/L DBP exposures ($p < 0.05$). In addition, it was determined that 1 mL/L DBP exposure caused a delay in the mean pupation time ($p < 0.05$). Also in the 0.25 mL/L exposure group was found a statistically significant decrease in mean fecundity as compared to the control groups ($p < 0.05$). Keywords: Dibutyl phtalate, *Drosophila melanogaster*, developmental time, fecundity.

Introduction

In recent years there has been growing concern that certain chemical compounds released into the environment may have a harmful influence on the development and reproduction of several animal species. As widely used industrial chemicals, phthalate esters (PAEs) are mainly used in the polymer industry as external plasticizers in polyvinyl chlorides (PVCs). PAEs have also found application in the manufacture of children's toys, printing inks, nail polishes, varnishes, shampoos, sunscreens, skin emollients, garden hoses, building materials, lubricating oils, automobile parts, paints, glues, insect repellents, photographic films, perfumes, and food packaging (Heudorf *et al.*, 2007; Liu *et al.*, 2009). Their low melting point and high boiling point make them very useful as heat transfer fluids and carriers (Van Wezel *et al.*, 2000). The world wide production of phthalates approximates 5.2 million metric tons a year (Liu *et al.*, 2009). PAEs tend to migrate slowly out of the plastic, either into the air by volatilization or into water or other solvents by dissolution (Zhao *et al.*,

2009). Consisting of a large number of compounds with varying alkyl chain lengths, PAEs are often assumed to have a high potential to accumulate in biological organisms due to their lipophilicity and regarded as endocrine disrupting compounds (EDCs) that may interfere with the normal hormone-regulated physiological processes of humans and wildlife (Jobling *et al.* 1995; Sonnenschein and Soto 1998; Liu *et al.*, 2009).

Dibutyl phthalate (DBP) is one of PAEs, and it is used in PVC plastics, cellulose plastics, food wraps, adhesives, personal care products, perfumes and cosmetics (Heudorf *et al.*, 2007; Prasanth *et al.*, 2009). It is identified as priority controlled hazardous substances by the United States Environmental Protection Agency (US EPA) (Zhao *et al.*, 2009) and has been shown to have potential for endocrine disrupting effects on humans and wildlife (Jobling *et al.*, 1995; Sonnenschein and Soto, 1998).

The toxic effects of DBP were investigated in many organisms. It was found that DBP can cause reproductive tract malformations in male rats (Wölfle *et al.*, 2009), fertility reductions and ovarian malfunctions in female rats (Gray *et al.*, 2006). The developmental effects of DBP were found to be significantly toxic in the exposed larvae of abalone *Haliotis diversicolor supertexta.* DBP exposures caused embryonal malformations and low larval settlements (Zhihui *et al.*, 2009). In a similar study, the normal embryonic development of *H. diversicolor supertexta* showed a good dose-response decrease when exposed to DBP (Liu *et al.*, 2009). In another study, it is shown that DBP significantly prolonged the generation time of freshwater rotifer *Brachionus calyciflorus* (Zhao *et al.*, 2009). Also, Memmi and Atlı (2009) found that DBP exposure extended developmental time of *Drosophila melanogaster.*

Studies on the effects of EDCs have centered mainly on effects in vertebrates. From many studies reported in the literature about endocrine disruption (ED), only a minor fraction have investigated their effects in invertebrates; from these only 10% were conducted with terrestrial invertebrates (Lemos *et al.*, 2010). From this point of view, *Drosophila melanogaster* can be a suitable organism to determine adverse effects of these chemicals because of its short developmental cycle.

In this study, the developmental and reproductive effects of DBP were examined by taking into account the changes in developmental stages and the differences in daily mean egg number in *Drosophila melanogaster.*

Materials and Methods

The organism and environmental conditions
In this study, the wild type Oregon strain of *Drosophila melanogaster* was used. The flies were kept in a *Drosophila* culture room (Hacettepe University, Ankara, Turkey) at 25 ± 1°C and relative humidity of 50-60% and in 12 hrs light, 12 hrs dark periods on a standard cornmeal *Drosophila* medium.

Virgin Oregon females and males of the same age were crossed in culture bottles. Individuals were then removed from the culture bottles after 8 hours. 72 ± 4 hrs later, the third instar larvae were collected.

DBP exposures
DBP (CAS No: 84-74-2) was firstly diluted in 1 mL acetone and it was filled to 1 liter with 5% sucrose (Merck; Durmstadt, Germany) solution to prepare stock solutions. The treatment doses were 0.25 mL/L, 0.5 mL/L, and 1 mL/L. Each dose had two replicates. Acetone control group was

used in experiment, and all experimental groups except the control group were made up to the same concentration of acetone which was 1 mL per liter.

Oregon strain (w.t.) third instar larvae of *D. melanogaster* were exposed to 0.25 mL/L, 0.5 mL/L, and 1 mL/L DBP for six hours. During the DBP exposures, larvae were placed in glass tubes (2.5 × 7.5 cm) containing drying papers that had absorbed stock solutions. Dose selection was based on results from our previous studies.

Observation of developmental stages

DBP exposed and non-exposed (control groups) larvae were placed in 250 mL glass bottles that contained a standard *Drosophila* medium. The development of all experimental groups was observed at 24 h intervals by recording the number of individuals passing from larvae to pupae and from pupae to adults, and the transition periods.

Determination of the mean fecundity

In order to determine the effects of the DBP on the mean fecundity, virgin females, hatched from the exposed larvae were used. An exposed female and 3 non-exposed males of same age were crossed in empty glass culture bottles. Then spoons containing standard medium were placed in these culture bottles. These spoons were changed for every 24 h and the eggs were counted for a period of 10 days.

Statistical Analysis

The statistical analysis of the results was carried out using the SPSS 11.5 programme. The determination of the significance of the transition percentages from larvae to pupae and from pupae to adults were obtained by an analysis of variance, and the comparison of the transition period from larvae to pupae and from pupae to adults, were done by a two-variable t-test. The daily mean egg number was calculated with the ANOVA test. For all statistical analysis, the criteria for significance was $p < 0.05$.

Results

Effect of the DBP on the transition percentages from larvae to pupae

The pupated larvae from those exposed and non-exposed to the DBP were counted separately and their transition percentages were determined (Table 1). The result of a one variable variance analysis showed that DBP exposures did not have any effect in terms of the number of pupated larvae ($p > 0.05$).

Effect of the DBP on the mean pupation time

Table 2 and Figure 1 show the effect of different DBP doses on mean pupation time. The mean pupation time was 85.2 hours for the control group and 84.5 hours for the acetone control group. The mean pupation time decreased to 78.3 hours in 0.25 mL/L DBP exposure group ($p < 0.05$), but it increased to 89 hours in 1 mL/L DBP exposure group ($p < 0.05$) compared to the control groups. In the other groups, there was no statistically significant change ($p > 0.05$).

Table 1. The changes of the transition rate from larvae to pupae depending on DBP exposure.

Groups	Number of larvae	Number of pupae	Rate ± S.E.	S.D.	p
Control	100	95	95 ± 0.03	0.097	
Acetone Control	100	98	98 ± 0.02	0.063	
0.25ml/l (DBP1)	100	98	98 ± 0.01	0.042	0.386
0.5ml/l (DBP2)	100	97	97 ± 0.02	0.048	
1 ml/l (DBP3)	100	93	93 ± 0.02	0.067	

DBP: Dibutyl pthalate, S.E.: Standard error, S.D.: Standard deviation, $p > 0.05$

Table 2. The changes of mean pupation time depending on DBP exposure doses.

Group No.	Groups	Mean Pupation Time (hour)	S.D.	Significant Differences of the Means
1	Control	85.2	27.85	1-3*
2	Acetone Control	84.5	27.87	2-3*
3	0.25ml/l (DBP1)	78.3	31.34	1-5*
4	0.5ml/l (DBP2)	86.0	27.45	2-5*
5	1 ml/l (DBP3)	89.0	25.40	

DBP: Dibutyl pthalate , S.D.: Standard deviation, * : $p < 0.05$

Table 3. The changes of the transition rate from pupae to adult depending on DBP exposure.

Groups	Number of pupae	Number of adult	Rate ± S.E.	S.D.	p
Control	95	92	96.8 ± 0.03	0.103	
Acetone Control	98	97	98.9 ± 0.02	0.067	
0.25ml/l (DBP1)	98	94	95.9 ± 0.16	0.052	0.196
0.5ml/l (DBP2)	97	93	95.9 ± 0.21	0.067	
1 ml/l (DBP3)	93	91	97.9 ± 0.18	0.057	

DBP: Dibutyl pthalate, S.E.: Standard error, S.D.: Standard deviation, $p > 0.05$

Table 4. The changes of mean maturation time depending on DBP exposure doses.

Group No.	Groups	Mean Maturation Time (hour)	S.D.	Significant Differences of the Means
1	Control	112.0	39.63	1-3*
2	Acetone Control	111.0	40.20	2-3*
3	0.25ml/l (DBP1)	106.0	42.99	
4	0.5ml/l (DBP2)	112.2	40.12	
5	1 ml/l (DBP3)	113.1	39.08	

DBP: Dibutyl pthalate, S.E.: Standard error, S.D.: Standard deviation * : $p < 0.05$

Table 5. The effect of DBP on daily mean egg production of *Drosophila melanogaster*.

Group No.	Groups	Number of female	Number of egg	Daily mean egg production per female ± S.E.	S. D.	Significant differences of the means
1	Control	24	1450	6.04 ± 0.23	3.59	1-3*
2	Acetone Control	24	1474	6.14 ± 0.26	3.99	2-3*
3	0.25ml/l (DBP1)	24	841	3.50 ± 0.23	3.51	
4	0.5ml/l (DBP2)	24	1371	5.63 ± 0.26	3.97	
5	1 ml/l (DBP3)	24	1315	5.48 ± 0.27	4.18	

DBP: Dibutyl pthalate, S.E.: Standard error, S.D.: Standard deviation * : $p < 0.05$
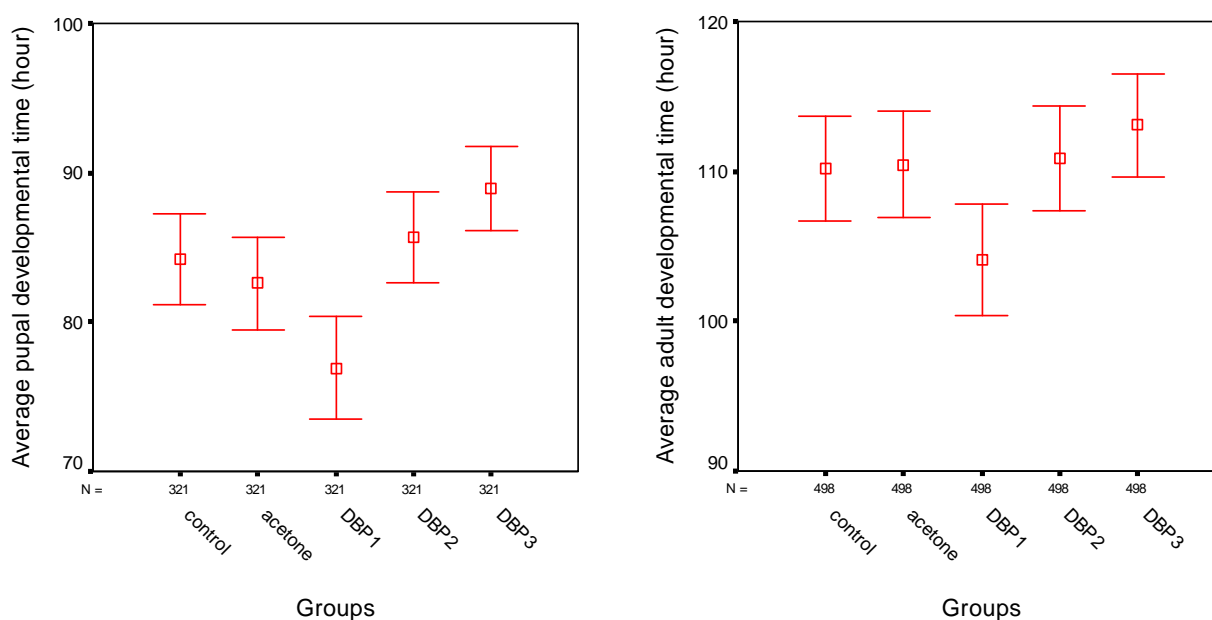

Figure 1 (Above, left). The changes of mean pupation time depending on DBP exposure doses.

Figure 2 (Above, right). The changes of mean maturation time depending on DBP exposure doses.

Effect of the DBP on the transition percentages from pupae to adult

The transition rates of DBP exposed and non-exposed flies from pupae to adult were determined and then compared statistically (Table 3). A variance analysis showed that the differences between the groups were statistically insignificant at a level of $p > 0.05$.

Effects of the DBP on the mean maturation time

The mean maturation time was 112 hours for the control and 111 hours for the acetone control group. It was found that the mean maturation time was accelerated ($p < 0.05$) at 0.25 mL/L DBP exposure group (Table 4 and Figure 2).

Effects of the DBP on daily mean egg numbers

Table 5 shows the effect of different DBP exposure doses on the daily mean egg number. The daily mean egg number per female was 6.04 for the control group and 6.14 for the acetone control group. The daily mean egg number, however, was 3.50, 5.63, and 5.48 in the 0.25 mL/L, 0.5 mL/L and 1 mL/L exposure group, respectively.

As seen in Table 5, there was a statistically significant reduction in the daily mean egg number in the 0.25 mL/L DBP exposure group compared to the control and the acetone control groups (p < 0.05). The other deviations observed in the other exposure groups were not statistically significant (p > 0.05).

Discussion

DBP is one of the endocrine disrupting chemicals that, acting as xenoestrogens, interfere with the endocrine system (Yang *et al*., 2009). It was found that DBP affects developmental and reproductive parameters in some organisms (Gray *et al*., 2006; Zhihui *et al*., 2009; Liu *et al*., 2009; Zhao *et al*., 2009; Memmi and Atlı, 2009). In this study, developmental and reproductive effects of DBP were examined by using *D. melanogaster.* Under the experimental conditions, the factors that might have affected the development and fecundity were kept stable. Thus, the differences in the results were interpreted as being caused by the DBPs used. The results showed that 0.25 mL/L and 1 mL/L DBP exposures elicited developmental and reproductive toxic effects.

In publications dealing with the developmental and reproductive effects of DBP, its effects have not always occured in the same direction. While in some it was reported that DBP exposures increased fecundity (Huang *et al*., 1999; Zhao *et al*., 2009), in some it was reported that they decreased fecundity (Mylcherest *et al*., 1998; Huang *et al*., 1999) and caused developmental delay (Liu *et al*., 2009; Memmi and Atlı, 2009).

The toxic effects of DBP have been investigated by using different features in various organisms different results have been obtained. In previous studies, 0.001-1 mL/L DBP caused statistically significant reductions in pupation time of *D. melanogaster* (Memmi and Atlı, 2009). Similarly to our findings, the researchers found that DBP significantly influenced all the life-table demographic parameters of *Brachionus calyciflorus*. Compared to the solvent control, DBP at 500 μg/L significantly increased the net reproductive rate and prolonged the generation time and the life expectancy at hatching of the rotifers, but DBP at 50 μg/L markedly decreased the intrinsic rate of population increase of the rotifers (Zhao *et al*., 2009). In another study, it was found that the development and reproduction of *Daphnia magna* was affected by DBP. At low concentration (0.5 mg/L), DBP stimulated the reproduction of *D. magna* while DBP inhibited it at high concentrations (1.0, 2.0, and 4.0 mg/L) (Huang *et al*, 1999).

Liu *et al*. (2009) examined the toxicity of seven phthalate esters (PAEs), dimethyl phthalate (DMP), diethyl phthalate (DEP), dibutyl phthalate (DBP), butylbenzyl phthalate (BBP), di-n-hexyl phthalate (DnHP), di-(2-ethylhexyl) phthalate(DEHP), and di-n-octyl phthalate (DOP) to embryogenesis and larval development of the marine univalve *Haliotis diversicolor supertexta*. The normal embryonic development of *H. diversicolor supertexta* showed a good dose-response decrease when exposed to DMP, DEP, DBP, BBP, and DnHP. Similarly, the toxicity of dimethyl phthalate (DMP), diethyl phthalate (DEP), dibutyl phthalate (DBP), and di (2-ethylhexyl) phthalate (DEHP) on embryogenesis and larval development of the marine univalve *Haliotis diversicolor supertexta* was investigated. The results show that the malformation of embryos appeared during the experiment, such as embryos died or lysed, small transparent flocculent rings studded on the periphery of the

embryo, and the larvae could fail to hatch. In embryo toxic test, embryos incubated at the highest concentration of DMP, DEP, and DBP solutions showed significantly high abnormal rate compared with the control, while DEHP solutions displayed no significant difference. In larval toxic test, in all concentrations of DMP, DEP, and DBP solutions, larval settlement rates were significantly lower than that of the control. Similarly, DEHP solutions show nearly no effect on the larval settlement. The order of toxicity on embryos and larvae is DBP>DEP>DMP>DEHP (Yang *et al*., 2009). In another study, lethal and sublethal effects of di(2-ethylhexyl) phthalate (DEHP) and dibutyl phthalate (DBP) on adult individuals of the collembolan *Folsomia fimetaria* were investigated. When exposed to DEHP, adults and juveniles were unaffected at all test concentrations, that is, up to 5,000 mg/kg. But DBP caused increased adult mortality at 250 mg/kg and juvenile mortality at 25 mg/kg. For DBP, adult reproduction was a more sensitive endpoint than was survival, with an EC10 and EC50 of 14 and 68 mg/kg, respectively (Jensen *et al*., 2001).

In our experiments, it was seen that DBP exposures did not have any effect on the pupation and maturation rate. But mean pupation time was affected 0.25 and 1 mL/L DBP exposures, and the mean maturation time was accelerated with 0.25 mL/L DBP exposure. The developmental effect might have resulted from ecdysteroid hormone disruption. Ecdysteroids have essential roles in developmental changes in the life of *D. melanogaster* (Li *et al*., 2001). Ecdysteroids (ecdysone and 20-hydroxyecdysone) are steroid hormones. 20-hydroxyecdysone (20-E) triggers major developmental transitions in arthropods (*Drosophila*, *Chironomus*, and so forth) (Kozlova and Thummel, 2000). Molecular studies have demonstrated that the ecdysteroid receptor of *Drosophila* has homologies to the steroid hormone receptors of vertebrates in that the ecdysteroid receptor and other steroid receptors share a set of conserved features corresponding to the hormone and DNA-binding domains (Zou and Fingerman, 1997). EDCs capable of binding to steroid hormone receptors can also bind to ecdysteroid receptors of invertebrates (Watts *et al*., 2001). In our study, DBP might attach to ecdysteroid receptors and interact with them, changing the developmental processes.

Many external (temperature, humidity, nutrition, population density, and so forth) and internal factors (genetic structure, age, and so forth) affect fecundity of *Drosophila melanogaster* (McMillan *et al*., 1970; David *et al*., 1975; Ashburner, 1989; Huey *et al*., 1995). It was reported that there were considerable changes in the *Drosophila* reproductive functions under external stress factors and that ecdysteroid hormones (ecdysone and 20-hydroxyecdysone) were involved in these events. It was reported that there is negative feedback mechanism between 20-hydroxyecdysone quantity and egg production. Therefore, any effect for increasing the quantity of 20-hydroxyecdysone will reduce the egg production. For instance, it was shown that 1-day-old females exposed to 38°C showed a one-day delay in egg laying and a reduced fertility during 6 days. This situation was attributed to increasing concentration of 20-hydroxyecdysone. Likewise, there was an increase at 20-hydroxyecdysone level among the females left hungry (Rauschenbach *et al*., 2000).

In the studies dealing with the effects of EDCs, it is emphasized that exposure to EDCs could induce stress (Roy *et al*., 1997; Obata and Kubota, 2000; Bindhumol *et al*., 2003; Cho *et al*., 2008). In our study, 0.25 mL/L DBP exposure caused a statistically significant reduction in the egg production of *Drosophila melanogaster.* The reason for this reduction may be the increased proportion of ecdysteroid hormones because of stress conditions caused by DBP.

In this study, the effects of dibutyl phtalate (DBP) on the development and fecundity of *D. melanogaster* were investigated. This investigation has shown that DBP affect developmental time and cause decreases in the number of offspring in *D. melanogaster.* Invertebrates comprise approximately 95% of all terrestrial and aquatic animal species, so it is clearly necessary to determine the potential developmental and reproductive hazards posed by EDCs. It is well known that the effects of these chemicals on the development and reproduction of invertebrates are of great

importance in the protection of the natural population's health. Therefore, more studies should be done to clarify the effect mechanisms.

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Assessing effects of sex, mating status, and a white-eye mutation in a co-isogenic background, on circadian locomotor activity in *Drosophila melanogaster*.

Breitbart, Sophie[1], Alysia Hildebrandt[2], and Bernard Possidente[2,3]. [1]Saratoga Springs High School; [2]Skidmore College Biology Department, Saratoga Springs, NY 12866 USA, [3]bposside@skidmore.edu.

Introduction

A co-isogenic strain of *Drosophila melanogaster* was used to determine how white eye color, sex, and mating status affect circadian locomotor activity. This approach allowed us to test the effects of a white eye mutation against a constant genetic background. Changing the eye pigmentation may alter the input to the circadian clock in the brain driving the circadian locomotor rhythm. We also asked whether mating experience changes locomotor activity by comparing virgin