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Evaluation of the Lifespan of Fruit Fly *Drosophila melanogaster* Exposed to Dioxins

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ABSTRACT
The polychlorinated dibenzo-*p*-dioxins (PCDDs) formed during combustion processes and as by-products of industrial processes are persistent organic pollutants. In the present study, the PCDDs of 2,3,7,8-TCDD, 1,2,3,7,8-PeCDD, 1,2,3,7,8,9-HxCDD and 1,2,3,4,6,7,8,9-OCDD (1, 2.5, 5 and 10µg/mL medium) were evaluated for their possible toxicity on the survival rate of *Drosophila melanogaster, in vivo.* The effects of different concentrations of dioxins were separately administered to female and male populations of *D. melanogaster*. In all application groups, both the survival rate and each population's longevity decreased, depending on the concentration of dioxins (p<0.05). In conclusion, the toxic effect for the survival rate and longevity was observed in the following order: 2,3,7,8-TCDD> 1,2,3,7,8-PeCDD> 1,2,3,7,8,9-HxCDD> 1,2,3,4,6,7,8,9-OCDD.

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Dioksinlere Maruz Bırakılan Meyve Sineği *Drosophila melanogaster*'in Ömür Uzunluğunun Değerlendirilmesi

ÖZET
Poliklorlu dibenzo-p- dioksinler (PCDDs) yanma prosesi sırasında ve endüstriyel süreçlerin yan ürünleri olarak ortaya çıkan kalıcı organik kirleticilerdir. Bu çalışmada, 2,3,7,8-TCDD, 1,2,3,7,8-PeCDD, 1,2,3,7,8,9-HxCDD ve 1,2,3,4,6,7,8,9-OCDD'nin (1, 2.5, 5 ve 10µg/mL medium) *Drosophila melanogaster*'in yaşam oranı ve ömür uzunluğu üzerine olan olası toksik etkileri *in vivo* olarak araştırılmıştır. Dioksinlerin ömür uzunluğu üzerine etkisi, *D. melanogaster*'in dişi ve erkek populasyonlarında ayrı ayrı çalışılmıştır. Tüm uygulama gruplarında, dioksinlere maruz kalan hem dişi hem de erkek populasyonlarda ömür uzunluğu konsantrasyon artışına paralel olarak azalmıştır (p<0.05). Sonuç olarak, yaşam oranı ve ömür uzunluğu üzerine dioksinlerin toksik etki sıralamasının 2,3,7,8-TCDD> 1,2,3,7,8-PeCDD> 1,2,3,7,8,9-HxCDD> 1,2,3,4,6,7,8,9-OCDD şeklinde olduğu gözlenmiştir.

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Anahtar Kelimeler
Dioksinler,
Drosophila melanogaster,
Ömür uzunluğu,
Larval ölüm oranı,
Oksidatif stres

Araştırma Makalesi

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INTRODUCTION
Polychlorinated dibenzo-p-dioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs) are persistent organic pollutants (Fiedler 2007; Altarawneh et al., 2009). PCDD/Fs are unintentional by-products of combustion processes and many industrial activities, such as waste incineration, metal production activities, power and heating facilities and chemical manufacturing processes (Hung et al., 2015). PCDD/Fs pose potential risks to environmental and human health globally because of their toxicity, persistence, and long-range transport (Weber et al., 2008; Holt et al., 2010). These health risks include chloracne, immunotoxic, endocrine disruptor, neurological disorders and carcinogenicity (Schecter et al., 2006; Yang et al., 2015; Rosińczuk et al, 2018).

2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), a member of the polychlorinated dibenzo-p-dioxin (PCDD) group and has become the prototypical model for investigating the toxicity of these environmentally relevant organochlorinated compounds (Humblet et al., 2008). There have been several studies in the

literature reporting a relationship between the exposure to TCDD and mortality, reproductive and developmental toxicity (Flesch-Janys et al., 1995; Pesatori et al., 1998; Terrell et al., 2011).

In this study, 2,3,7,8- Tetrachlorodibenzo-p-dioxin (TCDD), 1,2,3,7,8- Pentachlorodibenzo-p-dioxin (PeCDD), 1,2,3,7,8,9- Hexachlorodibenzo-p-dioxin (HxCDD), and 1,2,3,4,6,7,8,9- Octachlorodibenzo-p-dioxin (OCDD) of dioxin members, in the first class of carcinogenic substances, investigated the effects of larval survival rate/ larval mortality rate and longevity of fruit fly *Drosophila melanogaster*.

MATERIALS and METHODS

Insect Rearing

The flies used in the experiments were Oregon-R wild-type (w.t.) strain of *Drosophila melanogaster* Meigen (Diptera; Drosophilidae). This stock had been maintained for many years in the Laboratory at the Department of Biology of the Atatürk University in Erzurum in Turkey. Therefore, it was highly inbred with little genetic variation.

Laboratory Condition

The flies were kept at a constant temperature of 25±1 °C on a standard *Drosophila* medium (SDM) composed of maize-flour, agar, sucrose, dried yeast and propionic acid. The flies were kept in darkness, except during the transfers onto fresh medium. The humidity of the experimental chamber was 40-60%. The females used in this experiment were virgins.

Chemicals

2,3,7,8- Tetrachlorodibenzo-*p*-dioxin (CAS No. D-404S), 1,2,3,7,8- Pentachlorodibenzo-*p*-dioxin (CAS No. D- 501S), 1,2,3,7,8,9- Hexachlorodibenzo-*p*-dioxin (CAS No. D- 605S) and 1,2,3,4,6,7,8,9-Octachlorodibenzo-*p*-dioxin (CAS No. D- 801S) were purchased from Accu Standard (USA). Prior to use, the compounds were dissolved in 1% dimethyl sulphoxide (DMSO) (DMSO; Sigma 67-68-5).

Application of Chemicals

The first stage of our study, twenty pairs of adult *D. melanogaster* (20 ♀♀ × 20 ♂♂) were placed into culture bottles. The adults lay their eggs removed by waiting over 8 h for individuals. The larvae with 72±4 h developing from the eggs were transferred to culture vials contained the medium with different concentrations of dioxins (1, 2.5, 5 and 10 µg/mL medium), and then female and male offspring developing from the larvae were counted. In the second stage, two experiment sets were prepared; application groups contained SDM and different concentrations of 2,3,7,8-TCDD, 1,2,3,7,8- PeCDD, 1,2,3,7,8,9- HxCDD, and 1,2,3,4,6,7,8,9-OCDD (1, 2.5, 5 and 10µg/mL medium), and control groups contained only SDM and SDM with DMSO. On average, 100 individuals were collected from among the same aged female and male flies which were not mated and obtained from the pupa. Then, the gathered individuals were put into the empty culture vials and starved for 2h before the dioxin application. Afterward, the gathered fruit flies get into the application vials were left for 2h. Following the application, 100 individuals put into one vial for an application (separately applied for female and male flies) were placed into the culture vials containing only SDM as 25 × 25. The experiments for both control and application groups were started synchronically. All the vials were kept in appropriate thermal cabins. During the experiments, the food was replaced with fresh food twice a week. The number of individuals was counted both at the beginning and at the end of each application day, and the dead individuals were registered and then removed from the culture vials. The application was conducted until the last individual died.

Statistical Analyses

Statistical calculations were performed by using SPSS 15.0 software. To be able to determine the statistical significance of the results, Duncan's one-way range test was applied. The differences between groups were considered significant at $p<0.05$ level.

RESULTS

In this study, four different concentrations (1, 2.5, 5 and 10µg/mL medium) of 2,3,7,8-TCDD, 1,2,3,7,8-PeCDD, 1,2,3,7,8,9- HxCDD, and 1,2,3,4,6,7,8,9-OCDD were investigated in *Drosophila melanogaster* for effects on larval survival rate and longevity of male and females.

In the first part of our study, it was observed that the highest larval survival rate was in the control and DMSO control groups when compared to all application groups. According to the results obtained from the control and dioxin application groups, it was determined that the survival rate indicating the number of adult individuals who developed from larvae significantly decreased compared to the control group. In addition, it was reported that dioxins larval mortality ranking of was as OCDD< HxCDD< PeCDD< TCDD (Table 1-4).

Table 1. Comparison of the survival rate and longevity in each 2,3,7,8-TCDD concentration

Experiment Groups (μg mL^{-1}) (No)	Female population				Male population				Survival rate (%)		
	N	ML_1	ML_2±SE	*P*	N	ML_1	ML_2±SE	*P*	♀ Adult	♂ Adult	Total Adult
Control (1) DMSO	100	79	58.86±1.28		100	78	57.56±1.31		51	49	100[a]
Control (2)	100	78	58.48±1.26		100	77	56.98±1.31		50	50	100[a]
1.0 (3)	100	67	48.72±1.46	1-2*	100	67	44.22±1.53	1-2*	47	42	89[b]
2.50 (4)	100	64	40.34±1.41	4-5*	100	65	43.12±1.53	3-4*	43	39	82[bc]
5.0 (5)	100	57	37.75±1.28		100	57	40.13±1.42	4-5*	31	26	57[d]
10.0 (6)	100	43	24.59±1.09		100	42	24.83±1.13		14	11	25[e]

N: Number of individuals, ML_1: Maximum lifespan, ML_2: Mean lifespan, SE: Standard error, P: Probability level, *: The mean difference is not significant at the 0.05 level, [a-e]: Different letters in the same column indicate statistically significant differences at 0.05 level.

Table 2. Comparison of the survival rate and longevity in each 1,2,3,7,8- PeCDD concentration

Experiment Groups (μg mL^{-1}) (No)	Female population				Male population				Survival rate (%)		
	N	ML_1	ML_2±SE	*P*	N	ML_1	ML_2±SE	*P*	♀ Adult	♂ Adult	Total Adult
Control (1) DMSO	100	79	58.86±1.28		100	78	57.56±1.31		51	49	100[a]
Control (2)	100	78	58.48±1.26		100	77	56.98±1.31		50	50	100[a]
1.0 (3)	100	68	49.28±1.42	1-2*	100	68	48.11±1.65	1-2*	47	43	90[b]
2.50 (4)	100	64	42.00±1.37	4-5*	100	65	43.50±1.61	4-5*	43	41	84[bc]
5.0 (5)	100	59	39.77±1.41		100	58	40.33±1.45		33	31	64[d]
10.0 (6)	100	48	32.90±1.25		100	50	31.15±1.31		17	15	32[e]

N: Number of individuals, ML_1: Maximum lifespan, ML_2: Mean lifespan, SE: Standard error, P: Probability level, *: The mean difference is not significant at the 0.05 level, [a-e]: Different letters in the same column indicate statistically significant differences at 0.05 level.

Table 3. Comparison of the survival rate and longevity in each 1,2,3,7,8,9- HxCDD concentration

Experiment Groups (μg mL^{-1}) (No)	Female population				Male population				Survival rate (%)		
	N	ML_1	ML_2±SE	*P*	N	ML_1	ML_2±SE	*P*	♀ Adult	♂ Adult	Total Adult
Control (1) DMSO	100	79	58.86±1.28		100	78	57.56±1.31		51	49	100[a]
Control (2)	100	78	58.48±1.26		100	77	56.98±1.31		50	50	100[a]
1.0 (3)	100	70	50.38±1.38	1-2*	100	71	49.58±1.55	1-2*	48	45	93[b]
2.50 (4)	100	65	45.69±1.65	5-6*	100	65	46.00±1.52	3-4*	45	44	89[b]
5.0 (5)	100	61	38.71±1.40		100	60	42.43±1.50	4-5*	37	37	74[c]
10.0 (6)	100	52	34.87±1.34		100	53	35.99±1.50		34	33	67[d]

N: Number of individuals, ML_1: Maximum lifespan, ML_2: Mean lifespan, SE: Standard error, P: Probability level, *: The mean difference is not significant at the 0.05 level, [a-d]: Different letters in the same column indicate statistically significant differences at 0.05 level.

Table 4. Comparison of the survival rate and longevity in each 1,2,3,4,6,7,8,9-OCDD concentration

Experiment Groups (μg mL^{-1}) (No)	Female population				Male population				Survival rate (%)		
	N	ML_1	ML_2±SE	*P*	N	ML_1	ML_2±SE	*P*	♀ Adult	♂ Adult	Total Adult
Control (1)	100	79	58.86±1.28		100	78	57.56±1.31		51	49	100[a]
DMSO Control (2)	100	78	58.48±1.26		100	77	56.98±1.31	1-2*	50	50	100[a]
1.0 (3)	100	71	50.18±1.49	1-2* 3-4*	100	71	44.45±1.90	3-4*	48	47	95[b]
2.50 (4)	100	66	46.11±1.65		100	65	40.87±1.84	4-5*	46	46	92[b]
5.0 (5)	100	61	37.56±1.45		100	60	38.91±1.69	4-6* 5-6*	43	42	85[bc]
10.0 (6)	100	57	33.13±1.59		100	54	36.70±1.58		40	38	78[d]

N: Number of individuals, ML_1: Maximum lifespan, ML_2: Mean lifespan, SE: Standard error, P: Probability level, *: The mean difference is not significant at the 0.05 level, [a-d]: Different letters in the same column indicate statistically significant differences at 0.05 level.

The survival percentage of TCDD application group was determined to be lower than the other dioxin application groups. For example, the survival rate in TCDD female and male population decreased from 51-14% to 49-11%, respectively; the survival rate in PeCDD female and male population decreased from 51- 17% to 49- 15%, respectively; the survival rate in HxCDD female and male population decreased from 51- 34% to 49- 33%, respectively and the rate in OCDD female and male population decreased from 51- 40% to 49- 38%, respectively (Table 1- 4).

In terms of sex ratio, there is no statistical difference between survival rate and longevity of control and application groups.

In the second part of our study, in all application groups, each population's longevity decreased, depending on the concentration of dioxins (Table 1- 4 and Figure 1- 8). It was observed that the maximum lifespan of the control group was 79 days, DMSO control group 78 days for the females and 78, 77 days for the males, respectively.

However, the maximum lifespan for the lowest (1.0μg) and highest (10.0μg) application groups among the adult populations of *D. melanogaster* subjected to dioxins were observed to be 67-43 in TCDD, 68-48 in PeCDD, 70-52 in HxCDD and 71-57 in OCDD days for ♀♀, respectively and 67-42 in TCDD, 68-50 in PeCDD, 71-53 in HxCDD and 71-54 in OCDD days for ♂♂, respectively (Table 1-4 and Figure 1-8). The difference between the groups in longevity was statistically significant (p<0.05).

It was shown that there was a negative correlation between the mean lifespan of application groups and changing dioxin concentrations. These values were to R=-0.665 in TCDD, R=-0.573 in PeCDD, R=-0.540 in HxCDD and R=-0.545 in OCDD for ♀♀, respectively and R=-0.595 in TCDD, R=-0.531 in PeCDD, R=-0.461 in HxCDD and R=-0.434 in OCDD for ♂♂, respectively. In addition, we observed that there was no statistically significant between the average lifespan of group sex (p>0.05).

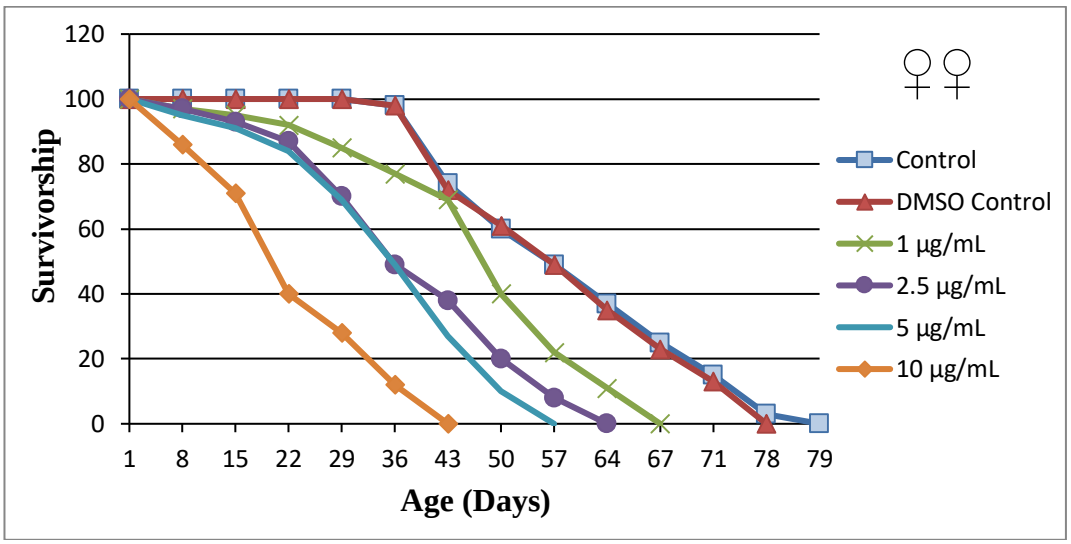

Figure 1. Exposure of 2,3,7,8-TCDD in female adult *D. melanogaster* leads to lifespan reduction

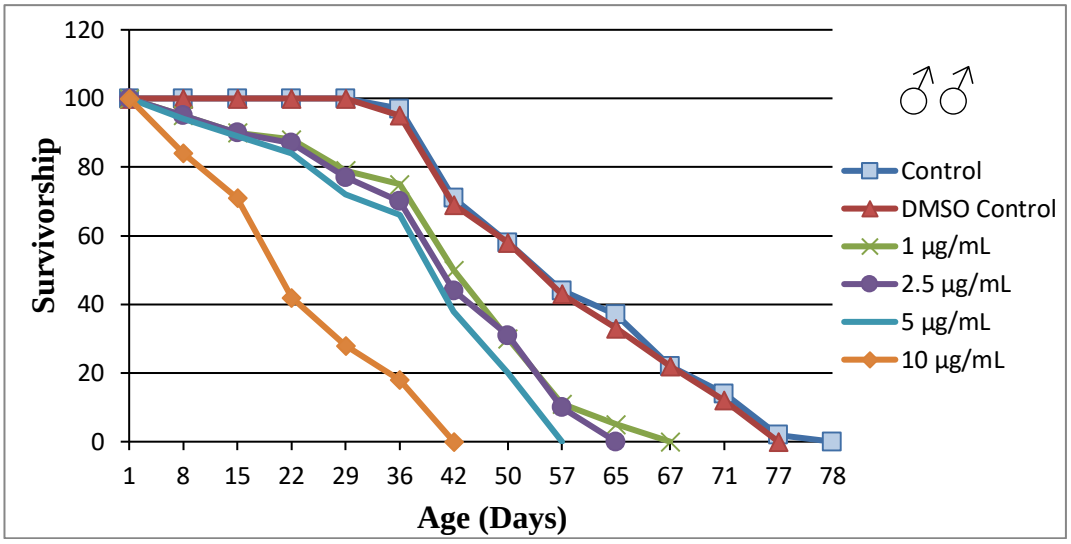

Figure 2. Exposure of 2,3,7,8-TCDD in male adult *D. melanogaster* leads to lifespan reduction

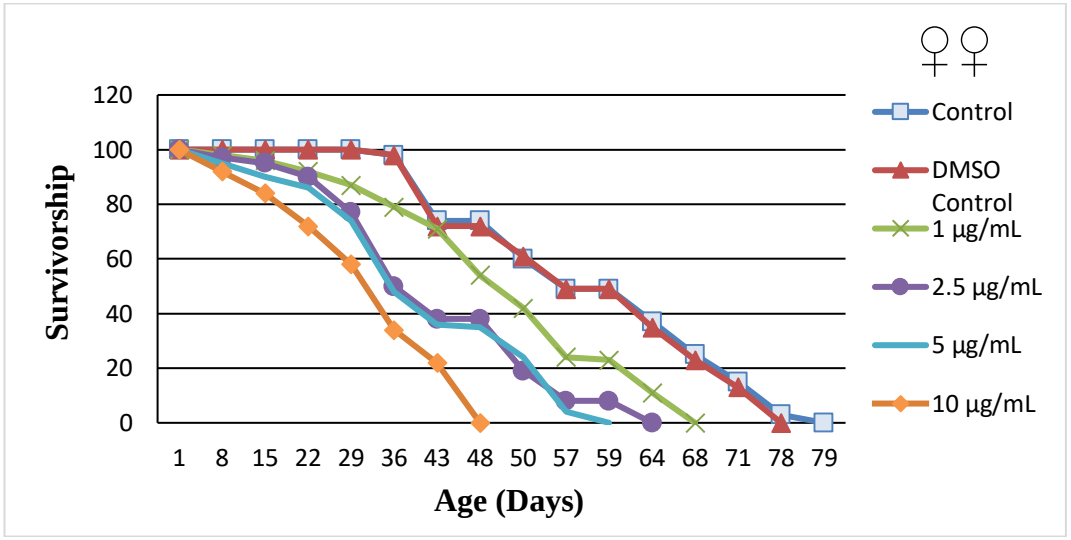

Figure 3. Exposure of 1,2,3,7,8- PeCDD in female adult *D. melanogaster* leads to lifespan reduction

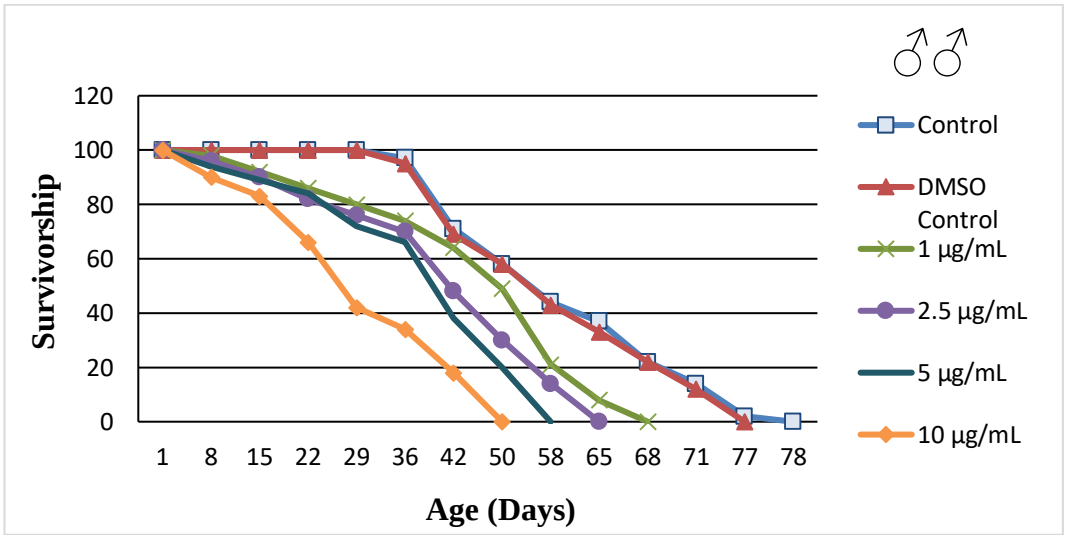

Figure 4. Exposure of 1,2,3,7,8- PeCDD in male adult *D. melanogaster* leads to lifespan reduction

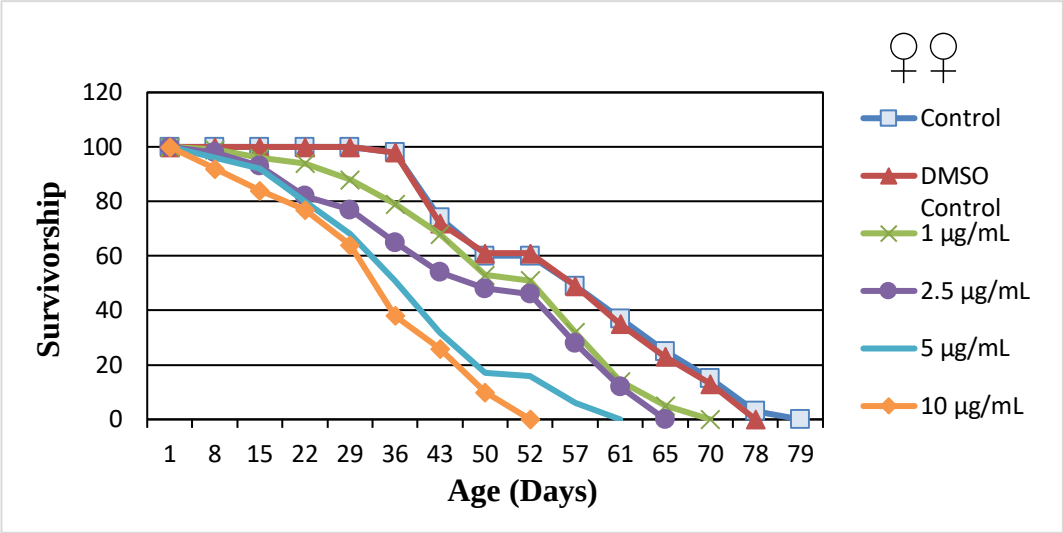

Figure 5. Exposure of 1,2,3,7,8,9- HxCDD in female adult *D. melanogaster* leads to lifespan reduction

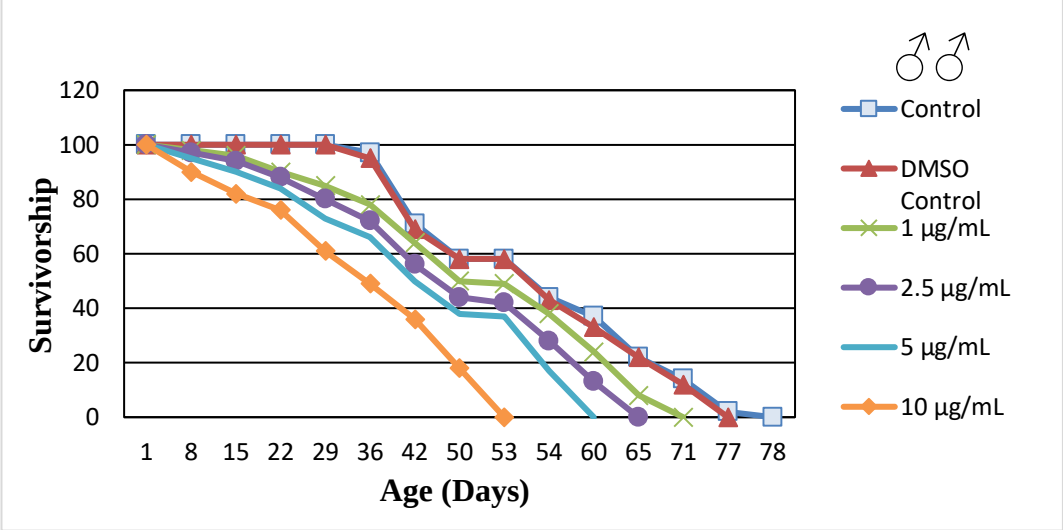

Figure 6. Exposure of 1,2,3,7,8,9- HxCDD in male adult *D. melanogaster* leads to lifespan reduction

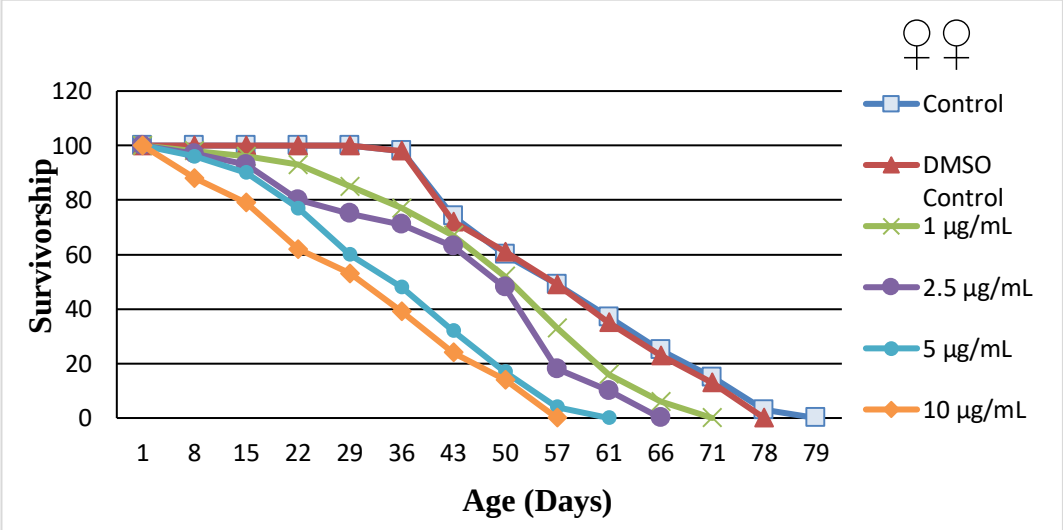

Figure 7. Exposure of 1,2,3,4,6,7,8,9-OCDD in female adult *D. melanogaster* leads to lifespan reduction

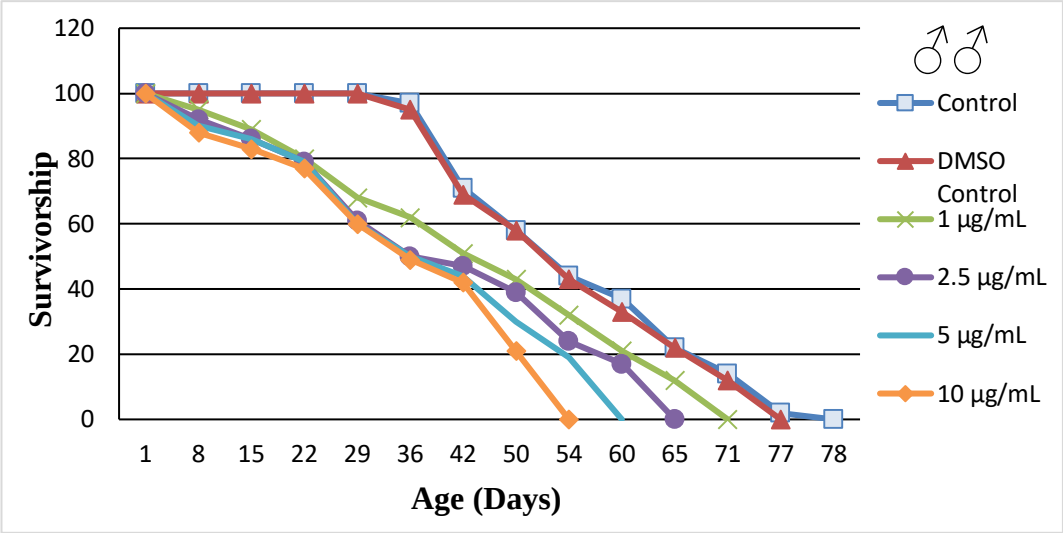

Figure 8. Exposure of 1,2,3,4,6,7,8,9-OCDD in male adult *D. melanogaster* leads to lifespan reduction

DISCUSSION and CONCLUSIONS

Studies on laboratory animals such as rat, mouse, zebrafish, rhesus monkeys showed that dioxins are toxic even at low concentrations (IARC, 1997; Theobald et al., 2003; Arima et al., 2009; Baker et al., 2014). TCDD reveal its biological effects in a wide range including the metabolic pathway changes, immunotoxicity, neurotoxicity, cardiotoxicity, reproductive and developmental abnormalities and cancer (EPA, 2000). Dioxins and dioxin-like chemicals demonstrate high-affinity binding to the aryl hydrocarbon receptor (AhR), a ligand-activated the transcription factor, which mediates most, if not all, of the toxic responses of these agents (Schecter et al., 2006). There is much evidence suggesting that Ah receptor is an important factor in developmental and homeostatic processes. The aryl hydrocarbon receptor [Ah receptor (AhR)] is a founding member of the basic-helix-loop-helix (bHLH)– Per-ARNT-Sim (PAS) superfamily of transcriptional regulators (Hahn, 1998). The *Drosophila melanogaster* AhR, as well as other invertebrate AhR homologs from *Mya arenaria* and *Caenorhabditis elegans*, do not bind the prototypical vertebrate AHR ligands and TCDD. This property distinguishes invertebrate from vertebrate AhRs (Butleri et al., 2001). Mammalian Ahr and its *Drosophila* homologous protein, Spineless (Ss), are highly similar in the bHLH and PAS-A domains. Spineless (Ss), is not able to bind dioxins probably because its PAS-B domain, which contains the dioxin-binding domain, is highly divergent from vertebrates (Duncan et al., 1998; Emmons et al.,1999; Hahn, 2002; Qin et al., 2006; Céspedes et al., 2010).

It was shown that the toxic effect caused by dioxins was also created by mechanisms not involving AhR (Ishida et al., 2005). In animal experiments exposed to polychlorinated dibenzo-*p*-dioxins, it was observed that the formation of reactive oxygen species due to increased oxidative stress and lipid accelerated peroxidation. Significantly, increase the number of DNA damage because of these have been found to occur (Zhang et al., 2012). In spite of many studies showing a lack of direct genotoxicity, oxidative DNA damage was detected *in vivo* and *in vitro* after exposure to TCDD as follows (Yoshida and Ogawa, 2000). Oxidative stress probably contributes to many other toxic responses produced by TCDD (Stohs, 1990). It is believed that the formation of reactive oxygen species caused by increasing the molecular oxygen transport, oxidative stress and lipid peroxidation may lead to these toxic effects of the dioxin compounds observed in *Drosophila*.

In animal experiments, exposure to dioxin during pregnancy and lactation induce various functional effects on offspring at very low doses. The types of effects observed in the offspring of animals exposed to 2,3,7,8-TCDD include structural malformations, functional alterations-damage to the immune system and impaired development of the reproductive system, decreased growth, and fetal/newborn mortality (Theobald et al., 2003). The timing of TCDD administration is important in the occurrence of lethality (the day of gestation on which dosing occurred is an important factor). For example, when 24µg/kg of TCDD was administered once to pregnant C57BL/6 mice on day 6 of gestation (GD6), the number of stillbirths increased. However, when the administration took place on GD8, GD10, GD12 or GD14, there were no effects (Couture et al., 1990). An intraperitoneal TCDD dose of 25, 100, 250, 500, 750, 1000 and 2000/3000µg/kg BW were injected into female and male Golden Syrian hamsters. It was observed that higher doses than 500µg/kg of body weight caused death. It was also reported that high doses can lead to premature death (Olson et al., 1980).

Two mixtures of polychlorinated biphenyls (PCB), one with 30 percent chlorine (Clophen 30) and the other with 50per cent (Clophen 50) were fed to adults or

larvae of *Drosophila melanogaster.* These concentrations caused a delay of the hatching without causing any noticeable lethality (Nilsson and Ramel, 1974). The literature is consistent with increasing larval mortality and decreasing longevity data obtained from our study results. In another a study using the female C57BL/6J inbred mouse, it was showed that intraperitoneal treatment of 5 micrograms TCDD per kilogram on 3 consecutive days produces a striking, prolonged oxidative stress response (Shertzer et al., 1998). Similarly, it was reported that an increase in the production of reactive oxygen species (ROS) in the brain of female B6C3F1 mice following subchronic exposure to TCDD at doses as low as 0.45ng/kg/day (Hassoun et al., 1998).

In a fifteen day study with female pregnant Sprague-Dawley rats which were orally treated with TCDD (10, 100 or 200 ng/kg body weight) resulted in that body size and sex ratio between the pregnant period of rats were not altered (Rebourcet et al., 2010). In many studies with laboratory animals, it has been reported that the toxicity of TCDD is very potent according to the other dioxins (Sutter et al., 2006). Experimental mice in a study conducted on that vary according to the gender of TCDD toxicity and toxic effects in male rats was lower, more accumulation in the tissues of the female of dioxins and stated that due to the longer half-life (USEPA, 2004; Pohjanvirta, 2009). The literature supports our results. In spite of many studies showing a lack of direct genotoxicity, oxidative DNA damage was detected *in vivo* and *in vitro* after exposure to TCDD as follows (Yoshida and Ogawa, 2000).

In conclusion, the survival rate and longevity reduced because increased oxidative stress caused dioxin toxicity in healthy flies. Hence, it can be said that there is a negative relation between dioxin exposure and larval survival rate and longevity of *D. melanogaster.*

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