Hydrogen peroxide, toluene, and dimethyl sulfoxide affect some development and reproductive parameters in *Drosophila melanogaster*.

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Abstract

Hydrogen peroxide (H_2O_2) is a compound used in numerous daily use products and exposure to them has been related to disturbing effects on human and animal reproduction and the increase of reactive oxygen species (ROS). Because high levels of Cyp450s enzymes have been correlated with an increase in ROS, we treated third instar larvae of flare (*flr*^{3}) and OR(R)-flare (OR(R)) strains (P_0), that have inducible and high levels of Cyp450s enzymes, respectively, to search the *in vivo* effects of hydrogen peroxide (H_2O_2, 20 mM) treatments on development (total and daily emergence, sexual proportion) and reproductive parameters (total fecundity, reproductive performance, and fertility percentage) in *D. melanogaster*. In addition, the emergence of the F_1 was tested to search for a putative hereditary effect. H_2O_2 did not alter the reproductive parameters but it modified development ones. Toluene (TOL, 10 mM), used as a positive control, affected significantly the development and reproductive traits with significant differences between the strains, confirming Cyp450s' participation in TOL's metabolism. DMSO, TOL's solvent, altered the sexual proportion and the total fecundity. No effect was observed on the F_1 in any of the traits studied. Our results agree with the concern that chronic exposure to these compounds could affect some development and reproductive processes in *D. melanogaster*. Keywords: oxidative stress, toxicity, cryoprotectant, chronic exposure.

Introduction

People and other living beings may subject themselves to an increased oxidation stress through exposure to chemicals that generate Reactive Oxygen Species (ROS) or increase free radicals; therefore, it is possible that constant exposure to such chemicals in high enough or chronic concentrations may cause disorders related to infertility (Agarwal *et al*., 2006). Hydrogen peroxide (H_2O_2) increases free radicals as hydroxyl radical ($^{\bullet}OH$) (Halliwell and Gutteridge, 2007) and is used as an active compound in numerous daily use products (Watt *et al*., 2004). The major source of human exposure to H_2O_2 is through the use of hair dyes. More than 60% of women and 5% to 10% of men dye their hair an average of 6-8 times a year (Benaiges, 2007) and H_2O_2 represents between 6% and 12% of the oxidant solution in the product. Heikkinen *et al.* (2015) suggested an association between the uses of hair dyes with breast cancer incidence. Oehninger *et al.* (1995) reported that H_2O_2 directly affects the human spermatic functions. Pandey *et al.* (2010) found that H_2O_2 acts as a signaling molecule and modulates diverse aspects of the oocyte physiology, such as meiotic cell cycle arrest. Cui *et al.* (2011) observed that H_2O_2 moderately increases the ROS content in porcine oocytes. In other contexts, humans are exposed to toluene (TOL) through products where it is used as a solvent. Exposure to TOL increases the generation of ROS (Kodavanti *et al*., 2015; Sarma *et al*., 2011) that may lead to neurodevelopmental disabilities (Grandjean and Landrigan, 2014; Win-Shwe *et al*., 2007). Long term exposure to TOL can induce apoptosis (Al-Ghamdi *et al*., 2004; El-Nabi Kamel *et al*., 2008) and exposed paint workers are at genotoxic risk (Priya *et al*., 2015); it has been suggested that its genotoxicity effects increase the risk of cancer (Kang *et al*., 2013). Bowen and Hannigan (2013) showed some negative effects of TOL in the reproductive performance of rats, while Webb *et al.* (2014) showed that similar effects are also observed in humans. Kawamoto *et al*. (1995) correlated the human cytochrome P450s (CYP450s), CYP1A1 and CYP2E1, with the metabolism of TOL, and Nakajima *et al*. (1997) described CYP2E1 as the main enzyme in producing benzyl alcohol, with other CYP450s enzymes participating in the production of *o*- and *p*-cresol, but Kim *et al*.

(2015) reported that they only found significant correlations between TOL´s metabolism and glutathione S-transferase (GSTM1 or GSTT1) enzymes. Singh *et al.* (2009) evaluated the effect of TOL in *Drosophila melanogaster* larvae, with inducible Cyp450s enzymes, as a response to oxidative stress. The solvent dimethyl sulfoxide (DMSO) is widely used in laboratories (Hallare *et al*., 2004; Turan *et al*., 2008) and direct exposure to it is limited. But in medicine, DMSO is also widely used as a vehicle for many pharmaceuticals (Santos *et al*., 2003), because it easily penetrates the skin, as an analgesic for muscle and joint pain (Ruso and Santarelli, 2016), and also is used as a chemically related nutritional supplement for the treatment of osteoarthritis (Brien *et al*., 2008). It has been reported to have radioprotective effect against the ROS produced by radiation (Cigarran *et al*., 2004; Jia *et al*., 2010), but it is concerning that it is also used as a cryoprotectant agent in embryo vitrification (Oikonomou *et al*., 2017). Taking in account that in *D. melanogaster* it had been demonstrated that Cyp450s' activity is analogous to the vertebrate liver and immune system (Yang *et al*., 2007), and there is a 80-90% global identity with mammals, for nucleotides and proteins' sequences of functional domains (Bhan and Nichols, 2011), we tested *in vivo* the capacity of H_2O_2, TOL, and DMSO to alter development and reproductive processes through chronic treatments in two *D. melanogaster* strains that differ in their Cyp450s levels: the flare (flr^3) strain with basal o inducible levels of Cyp450s, and the insecticide resistant Oregon-flare (OR(R)) strain with higher levels of these xenobiotic enzymes (Graf and van Schaik, 1992; Saner *et al*., 1996).

Methods

D. melanogaster

Eggs from flr^3 (flr^3/TM3, Bd^{Ser}) and OR(R) (*ORR*(1);*ORR*(2);flr^3/TM3, Bd^{Ser}) strains (full descriptions of the genetic characteristics of these strains may be found in Lindsley and Zimm, 1992) were collected 8 h separately by shaking the flies into bottles of 250 mL (Graf and van Schaik, 1992); the eggs collection bottles were kept undisturbed at 25°C and a relative humidity (RH) of 65 %. Three days later, the 72 ± 4 h, third instar larvae were collected through a fine-meshed stainless steel strainer and thoroughly washed free of yeast (Guzmán-Rincón and Graf, 1995).

Chemicals

H_2O_2 30% (w/w) stabilized solution (Sigma-Aldrich, Steinheim, Germany), CAS No. 7722-84-1; TOL 99.5% (J.T. Baker, México), CAS No. 108-88-3 (10 mM); DMSO 99.9% (Fluka Chemie, Switzerland), CAS No. 67-68-5 (0.3%); *Drosophila Instant Media* (DIM) (Carolina Biological Supply Co. Burlington N.C. USA).

Mortality assay with H_2O_2

Four independent experiments for each strain were carried out with three replicates for each of the following aqueous H_2O_2 concentrations: 5, 25, 50, 100, 150, and 200 millimolar (mM) (Courgeon *et al*., 1988). Third instar larvae were transferred to each vial and incubated at 25°C and 65% of RH until emergence of imagoes which, in turn, were recovered to quantify survival from 0-100% for each treatment. Finally, these data were plotted against the previously mentioned concentrations to calculate LC_{50} and LC_{20} with the correlation equation.

Chronic exposure

Third instar larvae from both strains were divided into four groups, each one consisting of five vials; two independent experiments were done. Each group was exposed to either: 0.5 g of DIM with 2 mL of milli-Q water (W) as negative control; H_2O_2 [20 mM] dissolved in W; TOL [10 mM] as positive control (Singh *et al*., 2009) dissolved in DMSO (0.3%); DMSO (0.3%) as solvent control (Nazir *et al*., 2003) dissolved in W. The concentration of H_2O_2 used corresponded to subtoxic LC_{20} and was chosen according to our results in the survival assay. All the vials remained approximately during 48 h in the incubator at 25°C and 65% of RH until emergence of imagoes.

Development Parameters

Total and daily emergence

The total emergence was calculated according to Arellano (2002), with some modifications:

$$Total\ emergence = \frac{F(X_i) + M(X_i)}{F(0) + M(0)}$$

$F(X_i) = Number\ of\ emerged\ females\ in\ the\ corresponding\ treatment.$

$M(X_i) = Number\ of\ emerged\ males\ in\ the\ corresponding\ treatment.$

$F(0) = Number\ of\ emerged\ females\ in\ the\ negative\ control.$

$M(0) = Number\ of\ emerged\ males\ in\ the\ negative\ control.$

This calculation was carried out in the exposed generation (P_0) to test for an acute effect and the descendants (F_1) to test for a possible hereditary effect in emergence values (Espinoza-Navarro *et al*., 2009). To obtain the daily emergence data, the number of imagoes from each group was daily counted between the 10th and 12th day after egg laying.

Sexual proportion

After emergence, imagoes from P_0 were segregated by sex and quantified. The sexual proportion (SP) for females (F) and males (M) corresponding to each treatment were calculated according to Arellano (2002):

$$SP = \frac{F\ (X_i)}{F\ (X_i) + M\ (X_i)}$$

$$SP = \frac{M\ (X_i)}{F\ (X_i) + M\ (X_i)}$$

Reproductive parameters

The reproductive parameters were calculated according to Gayathri and Krishnamurthy (1981) with some modifications:

Total fecundity

After emergence, virgin female and male flies (P_0) obtained from the incubation of chronically exposed larvae were segregated by sex and isolated in vials for 6 h. Five pairs of flies obtained from each treatment and strain were then transferred to individual plastic vials with media (Martínez-Castillo *et al*., (2012). For a period of ten days, the flies were daily transferred to new vials containing media to count the number of eggs they laid. The number of laid eggs was used in turn to determine the total fecundity (total laid eggs/ 10 days).

Reproductive performance and fertility percentage

The reproductive performance was calculated as the total number of hatched imagoes along 10 days. The fertility percentage was calculated based on the number of hatched flies in 10 days/ total laid eggs in 10 days × 100.

Statistical analyses

The development and reproductive parameters were individually analyzed by factorial ANOVA with *D. melanogaster* strain, sex, day, and treatment as factors, and also all the possible interactions between them. In the case of an ANOVA's difference ($p < 0.05$) the Fisher's Least Significant Difference (LSD) test was then used to determine whether or not there were differences ($p < 0.05$) between the factors. All the statistical analyses were performed using Minitab 17 software. Box plots were done with data where the box represents

50% with median as a horizontal line; the other 50% is represented by vertical lines and asterisk represents data out of range.

Results

Mortality assay with H_2O_2

Mortality assay's results (LC_{50}) were analyzed as a single data group, since only differences between the concentrations were observed (concentrations: $F = 37.54$, $p = 0.0$). Quadratic curves (*flr*^{3} strain: $r^2 = 0.8158$; OR(R) strain: $r^2 = 0.8638$) indicated that the LC_{50} of H_2O_2 was 38 mM for the *flr*^{3} strain and 48 mM for the OR(R) strain. Although the lineal slope of H_2O_2 yielded higher values in the OR(R) strain than in the *flr*^{3} strain, a *t*-test revealed that for this compound there was no difference between the strains. Table 1 shows sensitivity and lineal slope data obtained from the fitted curves. With these results we calculated the concentration that could yield 20% of mortality (LC_{20}) as a treatment for both strains, to evaluate the H_2O_2's effect on development and reproductive parameters.

Table 1. Values of sensivity, lineal slope, r^2 and equation of polinomic tendency for flies from flr^{3}and OR(R) strains treated with H_2O_2 (0, 5, 25, 50, 100, 150 and 200 mM).

Strain	Sensivity	Lineal Slope	r^2	Equation
flr^3	-0.003	0.9932	0.8158	$y = -0.003x^2 + 0.9932x + 16.241$
OR(R)	-0.003	1.0325	0.8638	$y = -0.003x^2 + 1.0325x + 8.0362$

Development parameters

Total emergence of P_0: ANOVA analyses of total emergence of flies (P_0) obtained from treated third instar larvae, indicated differences in treatments; the LSD test showed that the differences are in H_2O_2 *vs.* W and H_2O_2 *vs*. DMSO (Table 2 and Figure 1) with more dispersion and a lower median in the *flr*^{3} strain treated with H_2O_2, but not statistically different from OR(R) strain. Because, for this parameter, we did not obtain differences in DMSO *vs.* W or TOL *vs*. DMSO, we conclude that the total emergence was affected only by H_2O_2 and in a similar manner in both strains.

Table 2. Summary of the ANOVA and LSD results obtained from data's experiments.

		‡Factorial ANOVA			‡LSD Values				
		Strain factor (flr^3, OR(R)	Treatment factor (W, H_2O_2, TOL, DMSO)	Treatment-strain interaction	H_2O_2 vs. W	TOL vs. W	DMSO vs. W	H_2O_2 vs. TOL	H_2O_2 vs. DMSO
Development Parameters	Total emergence	ns	$p = 0.048$*	ns	$p = 0.012$	ns	ns	ns	$p = 0.018$
	Sexual proportion	ns	$p = 0.023$*	ns	$p = 0.011$	$p = 0.006$	$p = 0.006$	ns	ns
Reproductive Parameters	Total fecundity	ns	$p = 0.006$*	ns	ns	$p = 0.044$	$p = 0.004$	$p = 0.035$	$p = 0.003$
	Fertility percentage	ns	ns	$p = 0.036$*	ns	§.	ns	ns	ns

‡Statistically significant differences when $p \leq 0.05$. *See the corresponding LSD values in the second column on the right: §LSD values: TOL-flr^3 vs. W-flr^3 ($p = 0.018$); TOL-flr^3 vs. TOL-OR(R) ($p = 0.011$); TOL-flr^3 vs. DMSO-flr^3 ($p = 0.013$); TOL-flr^3 vs. DMSO-OR(R) ($p = 0.007$) after comparing between treatment and strain factors; ns = not significant values.

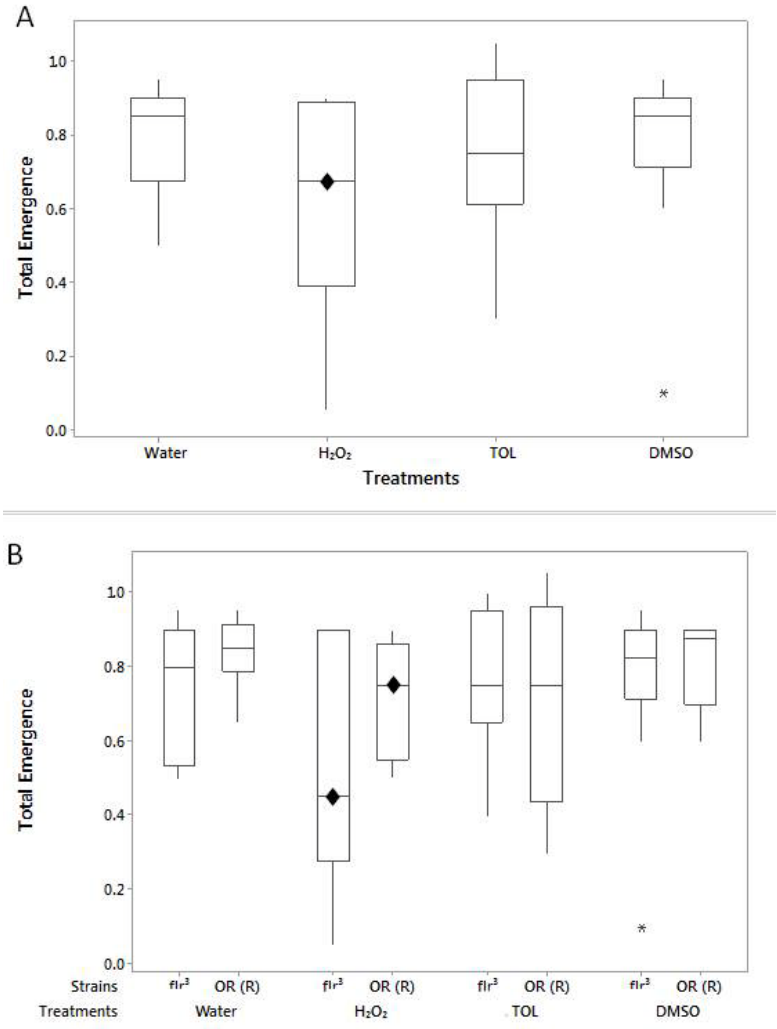

Figure 1. Medians and dispersions of (A) total emergence per treatment (Water, H_2O_2, 20 mM; TOL, 10 mM; DMSO, 0.03%) and (B) total emergence per treatment and strains (*flr*^{3}, OR(O)). ♦LSD test statistically significant differences ($p < 0.05$).

Total emergence of F_1: The comparison of the F_1´s hatches, all of them derived from progenitors (P_0) exposed to controls and treatments, showed similar medians and no statistical significant differences between them. This means that total emergence was affected only in P_0.

Daily emergence of P_0: Strain-treatment, strain-day, or strain-treatment-day interaction factors did not show differences in females, nor in males, but for males we found differences in day factor (Table 3 and Figure 2). Also, differences were found when comparing H_2O_2 *vs*. W treatments in males' emergence with a delay, and lower medians that are almost half of females' in both strains. Therefore, males' daily emergence was affected only by H_2O_2.

Table 3. Summary of the ANOVA and LSD statistically significant values obtained for the daily emergence parameter.

‡Factorial ANOVA								‡LSD
Strain		Treatment		Day		Strain-Treatment-Day		H_2O_2
(flr^3, OR(R))		(W, H_2O_2, DMSO, TOL)		(1-10)		interaction		vs. W
Females	n.s.	Females	n.s.	Females	$p = 0$	Females	n.s.	$p = 0.001$
Males	n.s.	Males	$p = 0.014$*	Males	$p = 0$	Males	n.s.	

‡Statistically significant differences when $p \leq 0.05$.*See the corresponding LSD value in the column on the right; n.s. = non-significant values.

Sexual proportion of P_0: In both strains all treatments yielded a sexual proportion that was statistically different from that of the W control, with lower medians in males than in females (Table 2 and Figure 3), but no differences between strains. Clearly, there is a biological component in males that makes them more susceptible than females, but it seems that the Cyp450s' different levels between the *flr*^{3} and OR(R) did not affect these results.

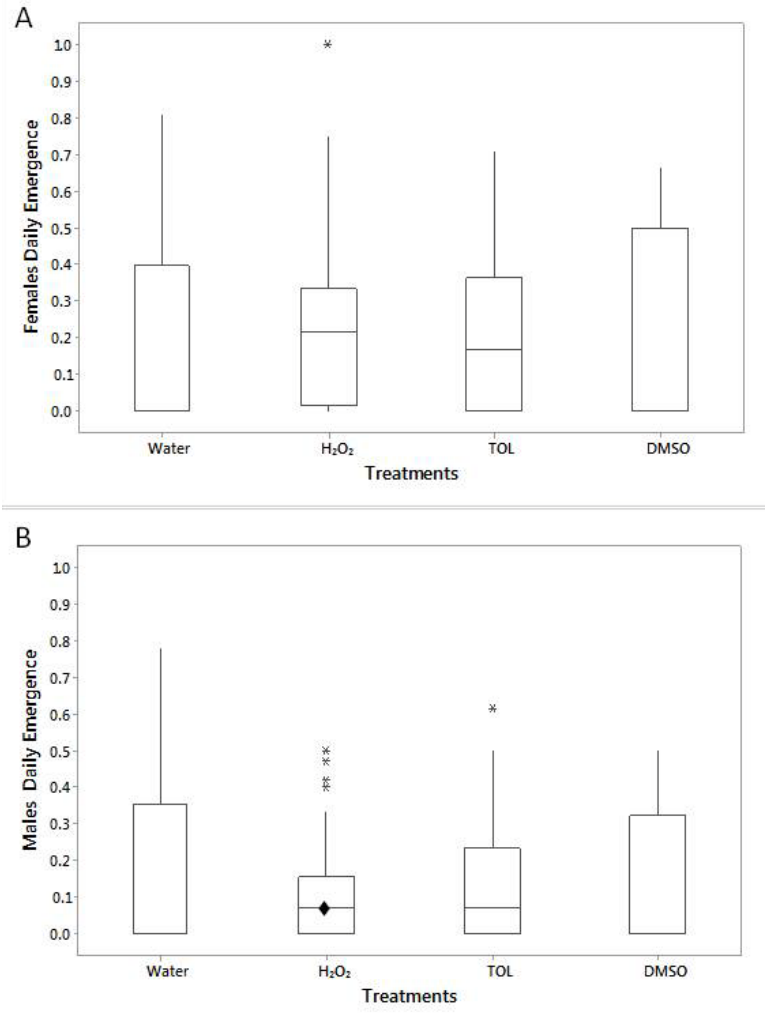

Figure 2. Medians and dispersions of (A) females and (B) males daily emergence per treatment (Water, H_2O_2, 20 mM; TOL, 10 mM; DMSO, 0.03%) and strain (*flr*^{3}, OR(O)). ♦LSD test statistically significant differences ($p < 0.05$).

Reproductive parameters

Total fecundity: As was expected, W produced the most homogeneous data of total fecundity, but they were not statistically different from H_2O_2's. TOL's data were the most dispersed and its median was similar to DMSO's. The analyses showed differences when comparing between the following treatments: H_2O_2 *vs*. DMSO, H_2O_2 *vs*. TOL, DMSO *vs*. W, and TOL *vs*. W (Table 2 and Figure 4), because total fecundity was lower in the DMSO and TOL treatments, remarkably with less dispersion in the first one. Then, these two compounds affected notably this parameter in the same manner to both strains.

Reproductive performance: The total number of hatched imagoes during 10 days did not show differences between strains, treatments, or interactions.

Fertility percentage: For the treatment-strain interaction ANOVA analyses, the fertility percentage was different ($p = 0.036$) with very low numbers in TOL *vs*. *flr*^{3}. When comparing between them we obtained LSD test differences between treatment-strain (Table 2 and Figure 5). Therefore, third instar larvae from the *flr*^{3} strain, with basal and inducible levels of Cyp450s, were more affected by TOL.

Discussion

H_2O_2

It is well-known that H_2O_2 produces the hydroxyl radical that has a very high oxidation potential that can decompose organic compounds in a very short time (Siu *et al*., 2009); it is also well documented that H_2O_2 is a free radical generator that can alter cell functions (Halliwell and Gutteridge, 2007) and it has been proved to exert toxic effects on *D. melanogaster* (Pomatto *et al*., 2017). On the other hand, it has been reported that it up-regulates developmental pathway, signaling and nucleobase metabolism genes (Landis *et al*., 2012). Here, the H_2O_2 altered the development parameters of both strains by delaying the total and daily emergence of imagoes from exposed third instar larvae. Furthermore, it affected the 1:1 sex proportion with a significant decrease in male individuals. Interestingly, Pomatto *et al*. (2017) demonstrated in *D. melanogaster* that female, but not male, adults adapt to H_2O_2 (10 and 100 μM) stress. Also, Pickering *et al*. (2013) demonstrated in *D. melanogaster* adults that pretreatment with 10–1000 μmol l^{-1} H_2O_2 yields a progressive

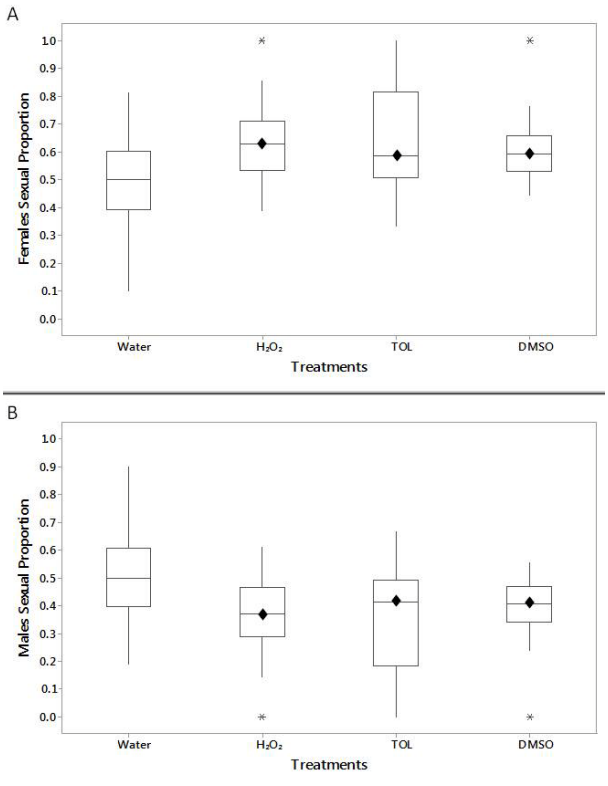

Figure 3. Medians and dispersions of sexual proportions of (A) females and (B) males per treatment (Water, H_2O_2, 20 mM; TOL, 10 mM; DMSO, 0.03%). ♦LSD test statistically significant differences ($p < 0.05$).

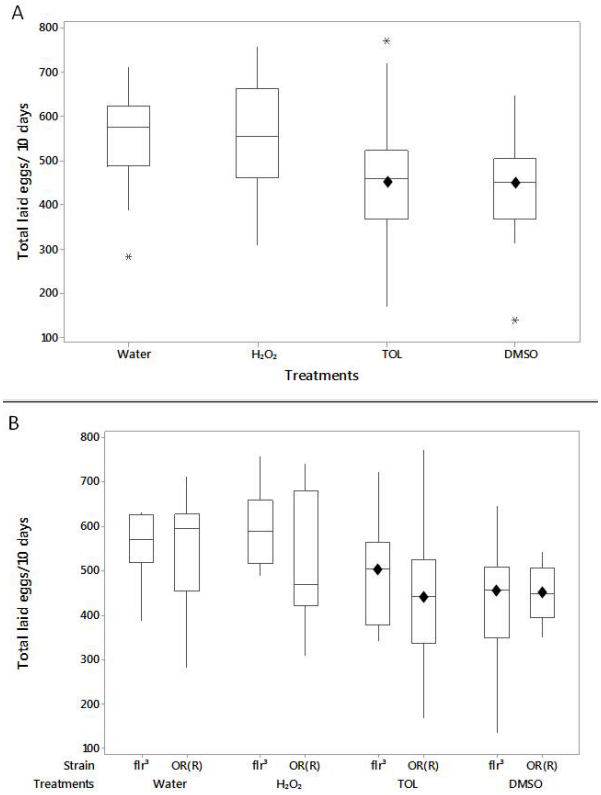

Figure 4. Medians and dispersions of (A) total fecundity (total laid eggs/10 days) per treatment (Water, H_2O_2, 20 mM; TOL, 10 mM; DMSO, 0.03%) and (B) total fecundity per treatment and strain (flr^3, OR(O)). ♦LSD test statistically significant differences ($p < 0.05$).

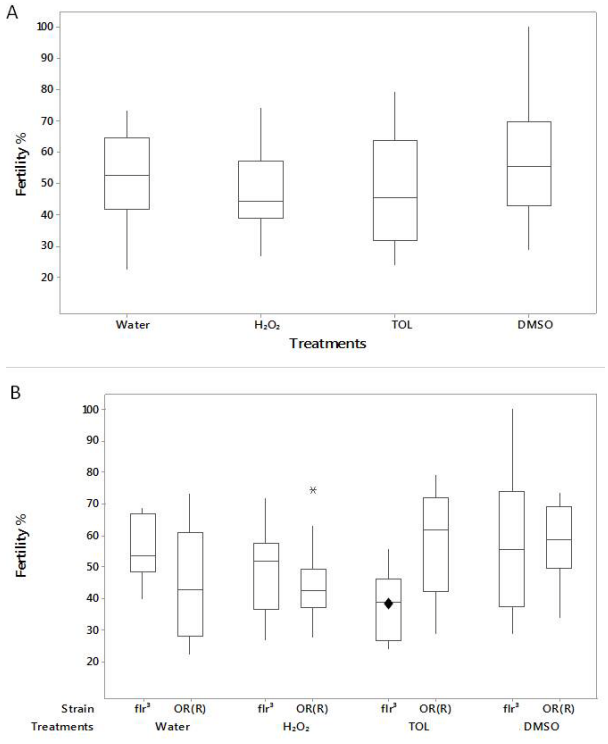

Figure 5. Medians and dispersions of (A) fertility percentage (No. flies in 10 days/No. of laid eggs in 10 days × 100) per treatment (Water, H_2O_2, 20 mM; TOL, 10 mM; DMSO, 0.03%) and (B) fertility percentage per treatment and strain (flr^3, OR(O)). ♦LSD test statistically significant differences ($p < 0.05$).

increase in the proteolytic capacity of females' lysates, but a decrease of proteolytic capacity from male fly lysates. We fed third instar larvae with a higher H_2O_2 concentration (20 mM), and we suggest that the difference in sex proportion, due to males' decrease in total and daily emergence, could be related to the less male's capacity to cope with oxidative stress as reported by Pomatto *et al*. (2017) and Pickering *et al*. (2013). So,

we infer that the observed effects in development parameters of treated larvae of both strains might be due to an oxidative stress and the different levels of Cyp450s between strains did not influences effects of H_2O_2. Surprisingly, H_2O_2 did not affect the reproductive parameters of adults obtained from treated larvae; it is well known that H_2O_2 treatment could induce the pro-survival program characterized by an alteration in insulin-like signaling, an increase in mitochondrial biogenesis and an increase in the de-acetylase activity of sirtuins (Stefanatos *et al*., 2012) or the clearances of damage tissue through apoptosis (Siu *et al*., 2009); then, putative cell defense mechanisms linked to this compound could explain the lack of detectable effect on the reproductive parameters only if damage in larvae's germ line cells could be repaired, or their damaged cells could be eliminated by apoptosis, during larval and metamorphosis stages.

TOL

We used TOL (10 mM) as a positive control, because Singh *et al.* (2009) reported in a strain with inducible Cyp450s, as the *flr*^{3} strain used here, that TOL (10-100 mM) affected the reproduction of *D. melanogaster,* by delaying emergence of imagoes up to 48 h, significantly decreasing the number of imagoes, and diminishing the reproductive performance in an inverse proportion to the concentration. They suggested that it was due to an alteration in development that could be the result of an increase in ROS that induced apoptosis (Singh *et al.*, 2009). Our experiments with both strains showed that exposure to TOL affected the development parameter: sexual proportion and the reproduction parameters: total fecundity and fertility percentage (Table 2), but contrary to Singh *et al*. (2009) it did not affect total emergence. Interestingly, TOL treatment was the only one where we got statistical differences in fertility percentage, between treatment-strain factors and between TOL and controls (LSD values in Table 2), confirming that alteration of fertility percentage by TOL occurred only in the strain with inducible Cyp450s. Because of the well described differences in Cyp450s expression levels between these strains (Saner *et al*., 1996; Vázquez-Gómez *et al*., 2010), we suggest that in the TOL treatment, the differences found in development parameter and reproductive parameters must be related to these different enzyme levels. Contrary to Kim *et al*. (2015), affirmations that TOL's metabolism is related only with GSTM1 or GSTT1 enzymes, our results contribute to assure the Cyp450s enzymes participation in TOL's metabolism (Kawamoto *et al*., 1995; Nakajima *et al*., 1997). We propose that the high levels of Cyp450s in the insecticide resistant OR(R) strain diminished cell damage, and TOL's biotransformation caused the differences observed when comparing its results with those of the *flr*^{3} strain.

DMSO

We used DMSO (0.3%) as a solvent control of TOL solution. Nazir *et al*. (2003) reported that a dietary concentration of 0.3% of DMSO is not harmful for the development of *D. melanogaster* and Singh *et al*. (2009) used in this model this concentration as a negative control. Also, Traut (1983) reported that a 2% solution does not induce aneuploidy in oocytes, making it a good solvent for mutagenicity screening in *Drosophila melanogaster*. Moreover, in the wing spot test we observed lower frequencies of mutant clones when DMSO was used as a solvent, presumably due to its scavenger properties (Dueñas-García *et al*., 2012). While DMSO is generally considered an antioxidant (Da Silva Duarte *et al*., 2004), under certain circumstances it causes oxidative stress (Sadowska-Bartosz *et al*., 2013). Surprisingly in this work, exposure to a DMSO concentration of 0.3% affected the 1:1 sexual proportion in both strains with a significant decrease in male individuals. This finding directly contradicts Singh *et al*. (2009) results and the observed decrease in total fecundity agrees with a cytotoxic effect reported by Nazir *et al.* (2003), although they used a higher concentration of 0.5%. Our results will be added to other adverse effects of DMSO demonstrated in human cells (Ruiz-Delgado *et al*., 2009; Shu *et al*., 2014) and other models (Choi *et al*., 2015; Herrid *et al*., 2016; Sadowska-Bartosz *et al*., 2013).

Conclusions

In conclusion, H_2O_2 significantly affected total emergence, daily emergence, and sexual proportion in both strains. TOL affected sexual proportion, total fecundity, and fertility percentage. Although TOL was dissolved in DMSO, the different Cyp450s levels between the two strains used, *flr*^{3} and OR(R), yielded

differences only for TOL treatment. This is important, because it supports the fact that the effects of this chemical on cells could depend on the expression levels of Cyp450s enzymes. Exposure to DMSO treatment affected in strains, sexual proportion, and total fecundity. We demonstrated that these three chemicals affect the studied parameters. Our results agree with the concern that chronic exposure to these compounds may affect human and animal development and reproductive processes (Pulver *et al.,* 2011; Misra *et al*., 2014).

Declaration of interest and acknowledgments: The authors report no declarations of interest. Also, they acknowledge the technical assistance of Ma. de Jesús Laura Castañeda-Partida. This work was supported by UNAM-Iztacala program PAPCA #34, 2011-2012.

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Thermotolerance of *Drosophila* hybrids: a new mode of adaptation in extreme climatic conditions.

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Abstract

Hybridization is not common in natural *Drosophila* populations. Trait expression and ecological performance of hybrids determine the consequences of hybridization such as degree and direction of gene flow or generation of phenotypic novelty. We investigated plastic effects on thermoresistance traits by growing parental species namely, *Drosophila jambulina* and *Drosophila punjabeinsis* and their hybrids, at four growth temperatures. Hybrids show increased variation in thermotolerance traits than their parental species. Hybrids show higher plasticity as well as cumulative fitness under variable environmental conditions. Acclimation effects were also significant in hybrids concluding adaptive potential. Results suggest that hybridization increases genetic variation that produces adaptation to new environments. We conclude that plasticity studies on hybrids and their progenitors are useful for testing basic predictions about evolution, as well as for understanding the evolutionary significance of hybrids. Key words: sibling species; *Drosophila jambulina*; *Drosophila punjabeinsis*; hybrid zone; genetic variation; fitness; acclimation

Introduction

Evolutionary significance of natural interspecific hybridization has gained much attention in recent years. In hybrids, trait expression and their ecological performance depends on the genetic control of the traits and the nature of genetic differences in their parental species. The outcome of natural hybridization varies in different taxonomic groups, but it has played a part in the evolution of large number of contemporary and