Supplementary Material

Identification of Drosophila based endpoints for the assessment and understanding of xenobiotic mediated male reproductive adversities

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Consists of supplementary figures S1-S3, Tables S1-S3 and additional methodological details.

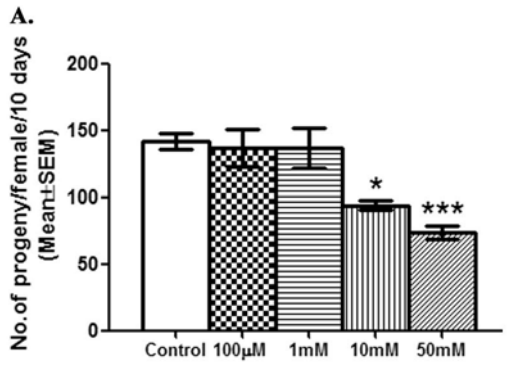

Fig. S1. Reduced fertility of males exposed to DBP for 72h. Panel (A) represents the number of progeny produced over a period of 10 days by females mated to males exposed to various concentrations of DBP at the adult stage (within 24 h of their emergence) for 72 h. We observed significant reduction in the number of progeny produced by females mated to males exposed 10 mM (*$p<0.05$) or 50 mM (***$p<0.001$) DBP for 72 h. All experiments were repeated three times (N=15-20 males/mates per replicate/group).

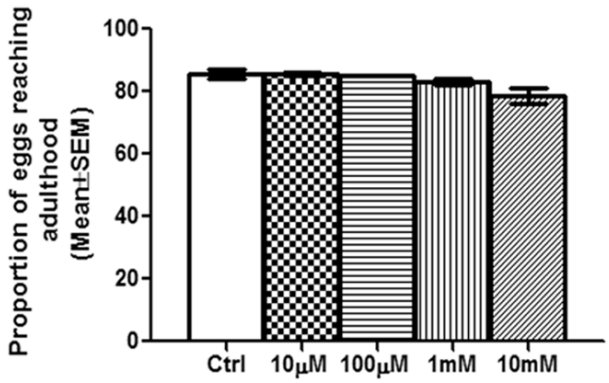

Fig. S2. Proportion of eggs reaching adulthood on control and DBP food. To determine, if exposure to DBP causes developmental lethality, 100 eggs in triplicate were placed on food containing DBP at concentrations ranging from 10 μM to 10 mM or food without test chemical (control). Similar proportions of eggs reached the adult stage in both control and DBP foods ($p=0.06$ based on one-way ANOVA). In addition, we did not observe any gross morphological, reproductive tract abnormalities, in flies exposed to DBP during their development, when compared to controls.

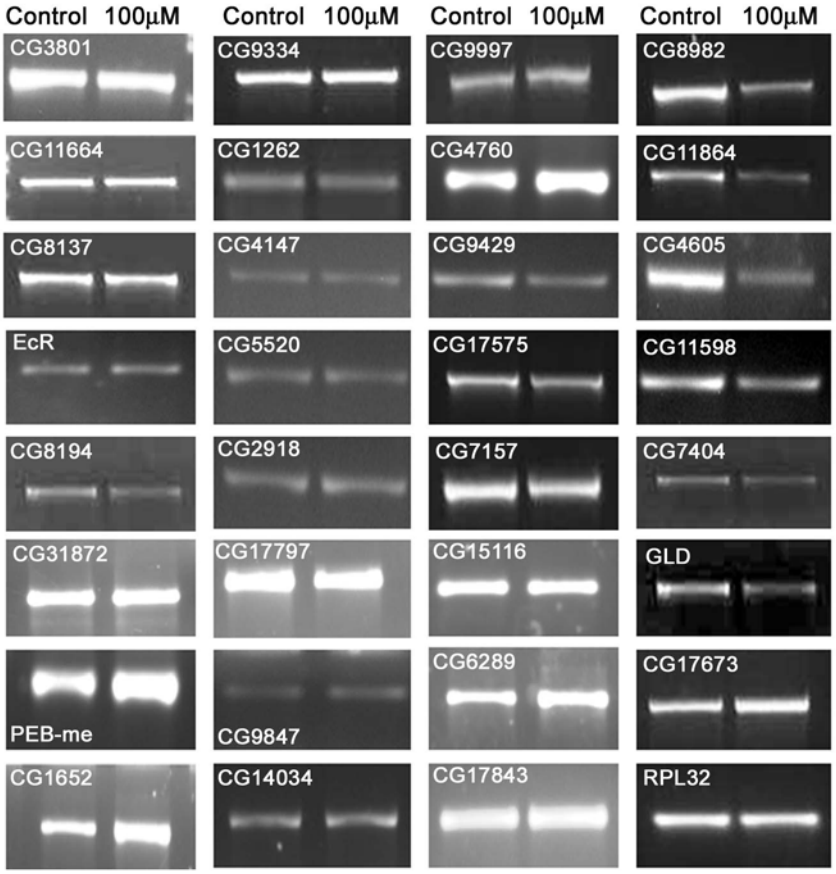

Fig S3. Modulation of a few genes expressed in the reproductive tract or encoding seminal proteins in males exposed to DBP during development. To determine the consequence of exposure to DBP at the gene expression level, we analyzed the transcript levels of 31 genes (represented in panels) in control (control lane) or exposed males (100 µM DBP). We used RPL32 (RPL 32 panel) as an internal control for the quality as well as quantity of the template. Of the four columns, the last vertical column represents the panels of the candidate genes, whose transcript levels were significantly up or down in response to chemical exposure, according to densitometry.

Table S1 GC-MS/MS parameters for estimation of DBP and MBP in Drosophila

Compound	Retention time (min)	Quantitative transition	Qualitative transitions
DBP	12.09	149 → 65@ 30eV	149 → 93@ 20 eV
			223 → 149@ 10 eV
MBP	9.33	223 → 75@ 10eV	149 → 121@ 10 eV
			223 → 75@ 10 eV

Table S2. Genes and their predicted gene ontology categories included in the present study (Schmittgen and Livak 2008):

S.No	Predicted gene ontology categories	Genes
1.	Protease inhibitor	Acp76A (CG3801), CG8137, CG9334, CG6289, Acp62F (CG1262)
2.	Lipase activity	CG17097, CG18284, CG11598, CG14034, CG31872
3.	Peptides and post mating	Acp36DE (CG7157), Sex peptide (CG17673), Acp32CD (CG4605), Ovulin (CG8982), Est-6 (CG6917), GLD (CG1152), PEB-me (CG2668)
4.	Lectins/ Ca^{2+} binding	Acp29AB (CG17797), CG1652, CG1656, CG7304, CG9429
5.	Protease	CG11864, CG9997, CG11664
6.	Hsp cognates	CG4147, CG2918, CG5520
7.	FAD linked sulfahydryl oxidase	CG17843
8.	Isomerase activity	CG6988, CG9847
9.	Ribonuclease	CG8194
10.	Cysteine rich secretory protein	CRISP (CG17575)
11.	Ligand activated DNA binding	Estrogen related receptor (CG7404, ERR) and Ecdysone receptor (EcR)
12.	Boule (Drosophila homolog of vertebrate DAZ ; Eberhart *et al.* 1996)	CG4760

Table S3. Primers used for Real time PCR

RPL32	Forward CCAGTCGGATCGATATGCTAA Reverse CCGATGTTGGGCATCAGATA
CG8982	Forward ATACTGTGAACCGCAGACTTT Reverse GTTGGCTTCCTGAAACTGATTG
CG11864	Forward CGTTCACTTGGGCTATAGGAATAA Reverse GTGAAGGAGCTCGTGGATAATG
CG10586	Forward GGCTTATCAAACCCGTGTTATTG Reverse CCGCCGCAGATGAAATTATTG
CG17843	Forward GTTGAGCTCGAAGCAGAGTATAA Reverse ACGTATCGCCTTGCCTTAAA
CG4605	Forward CCACGCTACATCTGCTGATAAT Reverse GAGTCTTTGGAGTCGCTCTTG
ERR	Forward CACAGCGCATGGAAAGAATATC Reverse GAACTCTGATCATCCAGCAGAA
GLD	Forward CCCACCTCTATCCTACGCTATT Reverse CCGTCATCTGGGCAATCATAAA
CG11598	Forward GCTCACACGAGATGGGTATTT Reverse GACATTCCGTGCTGAAAGAGA
CG17673	Forward TTGGTTCTCGTTTGCGTACT Reverse GCACCACTTATCACGAGGATT

Supplementary methods

Estimation of DBP and MBP in DBP exposed flies

Flies (3 days old) were homogenised in 3 ml of milli-Q water using bead beater. The pH of the homogenate was adjusted to 6.0-6.5 using 900 µL of 1M ammonium acetate (pH 7.0). To the homogenate 110 µl of β-glucuronidase (Sigma, USA) was added and the mixture was hydrolyzed at 37°C for 3 h. After enzymatic hydrolysis, 0.7 g of anhydrous sodium sulphate was added. Considering the pKa of mono phthalate ester (pKa 3.4-3.6), 1.2 ml of 2 M acetic acid was added to adjust the pH to 4.0. Extraction was carried out using 5.0 ml of ethyl acetate and vortexed for 15 min. The organic phase was separated by centrifugation at 6000 rpm. This extraction procedure was repeated thrice and nitrogen stream was used to remove the solvent (ethyl acetate) from the pooled extract. Subsequently, 60 µl of pyridine and 90 µl of BSTFA + 1 % TMCS were added to this pooled extract and mixed over thermo mixer for 35 min at 65 °C. Finally the extract was diluted with 200µl of hexane and 1.0 µl was used for the GC-MS/MS analysis. The oven temperature was programmed as follows: initial temperature was 100°C and then increased to 200°C at a rate of 15°C per min and maintained for 5 min and then increased further to 280°C at a rate of 10°C finally hold for 5.0 min. Transfer line and ion source temperatures were set at 290°C and 220°C respectively. Estimations were repeated with a minimum of two replicates per group (100 flies/replicate for control or exposed groups).

Conditions applied for Real time PCR

1 cycle of pre incubation (50ºC for 2 min and 95ºC for 10 min) followed by 40 amplification cycles (95ºC for 15 seconds, and 60ºC for 1 min) and melting curve detection (95ºC for 5 sec, 65ºC for 1 min). Experiments were preformed in duplicate for each sample for nine genes (please see Table S3 for primer sequences). The gene expression data were analyzed using

the comparative $2^{-\Delta\Delta}$CT (Schmittgen and Livak 2008) considering RPL32 as the internal control. All results are given as Mean±Standard Error of the mean.

Sample Preparation and Western blot Analysis

Typically, protein equivalents of two pairs of male accessory glands or five female reproductive tracts (in 2X SDS sample buffer 0.125 M Tris pH 6.8, 10% glycerol, 2% SDS, 5% 2-Mercaptoethanol, 1 mg bromophenol blue) formed the samples. Samples were separated on 13% SDS-polyacrylamide gel, and western blotting was performed using GFP specific primary antibody (anti-GFP generated in Rabbit, diluted 1:1500 in 1X TBST, Cell signaling technology, USA). and Peroxidase–Affinipure Goat anti-rabit secondary antibody (at a dilution of 1:3000, in 1X TBST, Jackson, USA). The blots were developed with chemiluminiscent reagent following manufacturer's protocol (Femto; Thermo-scientific, USA), and patterns were documented using Versa-doc Imaging system (Bio-Rad, USA). The protein quantities in terms of intensities of signals in samples from control/exposed males and their mates were semi-quantitatively assessed using volume analysis algorithm of Quantity One software (Bio-Rad, USA).

X-gal staining

Briefly, the dissected tissues were fixed in 2.5% glutaraldehyde for 30 min, and washed twice with 1X PBS. The wash buffer was replaced by pre warmed (65ºC for atleast 15 min) staining buffer (150 mM NaCl, 1 mM $MgCl_2$, 3 mM $K_4[Fe(CN)_6]$, 3 mM $K_3[Fe(CN)_6]$ in 1X PBS), and then by prewarmed staining solution containing 1/30 volume of 8% X-gal in DMSO. Staining reactions were allowed to develop for 2 h at 37°C. Tissues were washed thrice with 1X PBS and were subsequently placed on slides in a drop of physiological saline. The

coverslips were placed, sealed with nail polish, and images were captured through an inverted microscope (Nikon, Japan) at a total magnification of 100X.

Eberhart, C. G., Maines, J. Z., and Wasserman, S. A. (1996). Meiotic cell cycle requirement for a fly homologue of human Deleted in Azoospermia. *Nature* **381**, 783-5.

Schmittgen, T. D., and Livak, K. J. (2008). Analyzing real-time PCR data by the comparative C(T) method. *Nature protocols* **3**, 1101-8.