High Intake of Dietary Sugar Enhances Bisphenol A (BPA) Disruption and Reveals Ribosome-Mediated Pathways of Toxicity

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ABSTRACT Bisphenol A (BPA) is an organic compound to which human populations are ubiquitously exposed. Epidemiological data suggest BPA exposure might be associated with higher rates of diabetes and reproductive anomalies. Health concerns also include transgenerational consequences, but these mechanisms are crudely defined. Similarly, little is known about synergistic interactions between BPA and other substances. Here we show that acute and chronic exposure to BPA causes genome-wide modulation of several functionally coherent genetic pathways in the fruit fly *Drosophila melanogaster*. In particular, BPA exposure causes massive down-regulation of testis-specific genes and upregulation of ribosome-associated genes widely expressed across tissues. In addition, it causes the modulation of transposable elements that are specific to the ribosomal DNA loci, suggesting that nucleolar stress might contribute to BPA toxicity. The upregulation of ribosome-associated genes and the impairment of testis-specific gene expression are significantly enhanced upon BPA exposure with a high-sugar diet. Our results suggest that BPA and dietary sugar might functionally interact, with consequences to regulatory programs in both reproductive and somatic tissues.

EXPOSURE to environmental toxins is widespread. It is well documented, for instance, that human populations are commonly exposed to bisphenol A (BPA), bis(2-ethylhexyl) phthalate (DEHP), dioxins, and other organic molecules produced at industrial scales. Toxins might act by modulating epigenetic pathways to change the expression of genes and normal cellular homeostasis and could result in transgenerational consequences. The fast pace at which new compounds are invented and become ubiquitous in the environment poses a challenge to safety assessment. This challenge emerges not only from the large number of compounds to evaluate, but also from the high dimensionality of toxic responses across tissue types and developmental stages. This is further aggravated by the potential for unexpected synergism among toxins in mixtures. Sensitivity might also be strongly dependent on the specific epigenomic features and genetic makeup of an individual (Du *et al.* 2004).

Genomic analyses have uncovered levels of human genetic variation not surmised a few decades ago (*e.g.*, Sudmant *et al.* 2010). Cost-effective epigenetic and physiological models are needed for (i) addressing tissue-specific responses to toxins, (ii) gaining insights into mechanisms of epigenetic toxicity, and (iii) evaluating the manifestation of these responses across naturally occurring genotypes. In particular, cost-effective models with massive publicly available genetic information (Zwarts *et al.* 2011; King *et al.* 2012a,b; Mackay *et al.* 2012) might allow for genome-wide evaluation of toxic responses and assessment of coexposure outcomes in conjunction with the evaluation of unique individual responses.

BPA is one of the most abundantly produced chemicals worldwide, reaching an annual volume >4 million tons (Burridge 2003). Multiple studies find consistently high levels of BPA in human subjects, presumably due to continued exposure (vom Saal *et al.* 2007). Indeed, BPA can be detected in the urine of >90% of the American population (Calafat *et al.* 2005). Exposures occur not only in isolation, but also in mixtures with other toxins. Layered on top of exposures to novel combinations of toxins is the observation

that the human diet has changed substantially in the last few centuries; sugar in particular has increased dramatically in the western diet. Mouse studies suggest that BPA disrupts insulin production and glucose homeostasis (Alonso-Magdalena *et al.* 2006) and epidemiological studies (Lang *et al.* 2008; vom Saal and Myers 2008) found that increased BPA levels in human urine were associated with significantly increased risks of diabetes and heart disease. However, mechanisms for understanding BPA's contribution to diabetes are not well defined and raise the question of whether the relationship between BPA and diabetes is causal. Furthermore, epidemiological data have been inconclusive (Magliano and Lyons 2013). The relationship between BPA and diabetes is further confounded because BPA is often co-ingested with high sugar. Ingestion of drinks and food packaged in vessels lined with BPA appears to be a major source of exposure. BPA and high sugar might individually have long-term effects on disease susceptibility, but might also display synergistic interactions with each other. Altogether, poorly defined mechanisms not only hamper the development of bottom–up causal models of toxic BPA action but also prevent the optimal identification of high-risk individuals.

We investigated BPA toxicity under typical and high-sugar diets, using a genetically tractable *Drosophila melanogaster* model. Here we report significant interactions between BPA and sugar intake. We observed genome-wide modulation in both somatic and germline tissues of flies raised on a typical diet or on a high-sugar diet, with and without coexposure to BPA. Mode of exposure resulted in distinct outcomes: short-term acute BPA exposure prominently affected the expression of gut-specific genes, while chronic exposure impaired the activity of genes expressed in testis. Interestingly, combined exposure to BPA and high sugar activated the transcription of several genes that code for ribosomal proteins. These results reveal novel pathways contributing to BPA toxicity and highlight potential synergism among substances that are ubiquitous and often co-ingested in the diet.

Materials and Methods

Drosophila strains and treatments

The *y[1]*; *bw[1]*; *e[4]*; *ci[1] ey[R]* strain was maintained in vials containing the same standard diet in use at the Bloomington *Drosophila* Stock Center at Indiana University without light malt extract (http://flystocks.bio.indiana.edu/Fly_Work/media-recipes/bloomfood.htm). The base recipe for the control diet contains 150 mM of sucrose. For the treatments, the standard diet was prepared, and after the food cooled down to 60°, 3.7 g/liter of powdered BPA and/or 102.7 g/liter of powdered sucrose and/or 0.8% (v/v) of DEHP were added to the food and homogenized with a mixer. Hence, the treatments contain 450 mM of sucrose (high sugar), 16 mM of BPA, and/or 2 mM of DEHP. The

food was poured in vials and kept at room temperature overnight to dry. For acute exposure, four replicas per treatment were set up with 30 flies (2-day-old males) and exposed for 48 hr (supporting information, Figure S1). For chronic exposure, four replicas per treatment were set up with 15 virgin females and 10 males. Two genotypes were used for chronic exposure [*Yohio* and *Ycongo* (Lemos *et al.* 2008)]. Vials were kept and newly emerged adults, which developed in the diets containing the different compounds, were collected and aged for 2 days with continued exposure to the same rearing condition in which they developed (Figure S2). All flies were grown at 25°, with ~65% of relative humidity, and a 14-hr light/10-hr dark cycle. All flies used for gene expression analyses were flash-frozen in liquid nitrogen and stored at −80°.

Gene expression analysis

An ~18,000-feature array spotted primarily with PCR products designed for single exons was used. This gene set was supplemented with PCR products designed for Y-linked genes, *Drosophila* testis ESTs, and transposable elements (Lemos *et al.* 2008). Designs for microarray data collection are shown in Figure S3 and Figure S4 for acute and chronic exposures, respectively. Total RNA was extracted from whole flies, using TRIzol (Life Technologies). The synthesis of cDNA probes and hybridization conditions were carried out using 3DNA protocols and reagents (Genisphere). Microarray slides were scanned with an Axon 400B scanner (Axon Instruments) and the data extracted with GenePix Pro 6.0 software. We used stringent quality-control criteria to ensure reliability of foreground intensity reads for both Cy3 and Cy5 channels (Lemos *et al.* 2008). This procedure screens out poor-quality arrays. It ensures that the resulting data are minimally sensitive to the specifics of normalization. Foreground fluorescence of dye intensities was normalized by the Loess method in Bioconductor/Limma (Smyth and Speed 2003). We used the normexp method for background correction and the loess and Aquantile methods for within- and between-array normalization, respectively (Smyth and Speed 2003). The significance of variation in gene expression was assessed with the Bayesian analysis of gene expression levels (BAGEL) (Townsend and Hartl 2002). Gene expression estimates from duplicate spots were averaged previous to the computation of statistical significance of differential expression. Results were checked for consistency, using Linear Models in Limma. False discovery rates were empirically estimated by permutation of the data set. Specifically, we have used permutation of columns (*i.e.*, slides) rather than rows (*i.e.*, genes) because the former is more conservative. The microarray gene expression data reported here can be obtained at the Gene Expression Omnibus database under accession nos. GSE55655 and GSE55670. For quantitative PCR analyses, four biological replicates of each sample were used to make cDNA, using the QuantiTect Reverse-Transcription Kit (QIAGEN, Valencia, CA), and real-time qPCR analyses were carried out with the

Fast Sybr Green Master Mix (Applied Biosystems, Foster City, CA), using 7900HT Fast Real-Time PCR (Applied Biosystems). The expression level of the R1 and R2 genes was analyzed with the software REST (Pfaffl *et al.* 2002).

Metrics of tissue perturbation

Tissue specificity of differentially expressed (DE) genes was assessed using the FlyAtlas (Chintapalli *et al.* 2007; Robinson *et al.* 2013). We filtered the data to include only a nonredundant set of adult tissues: brain, eye, adult thoracicoabdominal ganglion (tag), adult salivary gland (sg), testis, accessory glands (acc), crop, midgut, tubule, hindgut, heart, and adult fat body. Genes with <200 expression counts were conservatively set to zero. We calculated a tissue prevalence index [TP(i)] for tissue i in a set of n tissues as:

$$TP(i) = \frac{\text{No. of DE genes with evidence of expression in tissue}(i)}{\text{Total no. of DE genes}}.$$

TP(i) of 1 means that all genes in the set of differential expressed genes show evidence of expression in the focal tissue (Branco *et al.* 2013). The tissue salience ratio [TSr(i)] is calculated for a set of DE genes as the ratio between DE genes that display relative abundance in tissue i above and below the average expression measured in the whole organism:

$$TSr(i) = \frac{\text{No. of DE genes with expression in tissue}(i) \text{ above whole organism mean}}{\text{No. of DE genes with expression in tissue}(i) \text{ below whole organism mean}}.$$

The ratio allows us to evaluate the relative impact of a perturbation on genes that are highly expressed relative to genes that are lowly expressed in a particular tissue. Significance was calculated with standard Fisher's exact tests on contingency tables with counts of genes displaying differential expression. Analyses of enrichment in gene ontology categories were performed with standard methods as previously implemented (Branco *et al.* 2013) and confirmed with the FlyMine platform (Lyne *et al.* 2007).

Results

Rapid expression responses and potentiation of BPA toxicity by coexposure with ubiquitous compounds

We investigated genome-wide expression responses of individuals treated with BPA (16 mM), high sugar (450 mM), and DEHP (2 mM) as well as their combination (BPA, high sugar, and DEHP) (Figure S1, Figure S3). The concentration of sucrose used for the high-sugar diet (450 mM) was three times the typical amount used in the base diet (150 mM), but below the concentration used in chronic high-sugar diets designed to induce insulin resistance and diabetic phenotypes in flies (1 M) (Baker and Thummel 2007; Musselman *et al.* 2011; Na *et al.* 2013). For each

toxin, the amount used in the mixture (BPA, high sugar, and DEHP combined) was identical to that used in the single exposures. To evaluate the effects of a short-term exposure, flies were transferred from regular food to the exposure food for 48 hr (Figure S1). Hereafter, 48-hr exposure is called acute exposure. Acute exposure did not increase mortality rates of adults in any treatment, which suggests that the treatments were not overly toxic. Nevertheless, acute adult exposure elicited rapid expression responses (Figure 1, A and B). A high-sugar diet led to a mild modulation of expression with 126 genes differentially regulated [76 genes were upregulated and 50 genes were downregulated; Bayesian posterior probability (BPP) > 0.97]. DEHP showed modulation of comparable magnitude with 96 genes differentially expressed (58 genes were upregulated and 38 genes were downregulated; BPP > 0.97). BPA treatment elicited the highest response with 237 genes disrupted upon exposure (141 genes were upregulated and 96 genes were downregulated; BPP > 0.97). The joint exposure of all three compounds resulted in the differential gene regulation of the same magnitude as that of BPA alone, with 219 differentially expressed genes (120 genes were upregulated and 98 genes were downregulated; BPP > 0.97). A substantial fraction of the upregulated (30%) and downregulated (41%) genes were unique to the mixture (Figure 1, A and B).

Acute exposure might elicit differential responses across organs due to variation in both distribution and concentration in different tissues. Specifically, nutrient absorption occurs primarily in the insect midgut (Ohlstein and Spradling 2006; Buchon *et al.* 2013), which might be expected to respond most rapidly to oral toxin intake. To address the issue we used two metrics, TP(i) and TSr(i), both of which combine our expression data sets with data available in the FlyAtlas database (Chintapalli *et al.* 2007; Robinson *et al.* 2013), a unique resource with tissue expression data collected across several dissected tissues. We observed that neither treatment with high sugar nor that with DEHP showed an overtly tissue-specific disruption in either sets of upregulated (Figure S5) or downregulated (Figure 2A) genes. On the other hand, genes knocked down upon BPA exposure were highly expressed in the midgut [Table S1; for BPP > 0.98, midgut salience ratio of 1.7 (BPA) *vs.* 0.45 (genomic background), 0.91 (high sugar), and 0.80 (DEHP), $P < 2.2e\text{-}16$, Fisher's exact test]. Gene ontology analysis indicated that these genes are associated with proteolysis (GO:0006508; 19 genes, $P < 3.03e\text{-}11$) and lipid metabolism (GO:0044238; 4 genes, $P < 0.015$). Furthermore, analysis of the BPA-containing mixture indicated that the downregulation of midgut-expressed genes was substantially enhanced with coexposure to high sugar and DEHP (Figure 2A). Indeed, more than half of the genes knocked down with this mixture were expressed specifically in the midgut (Figure 2B, Table S1; for BPP > 0.98, midgut salience ratio of mixture is 6.22). This result suggests that toxic effects of BPA in the gut can be substantially heightened through interaction with other compounds in a mixture. TSr(i)'s reinforced the conclusion
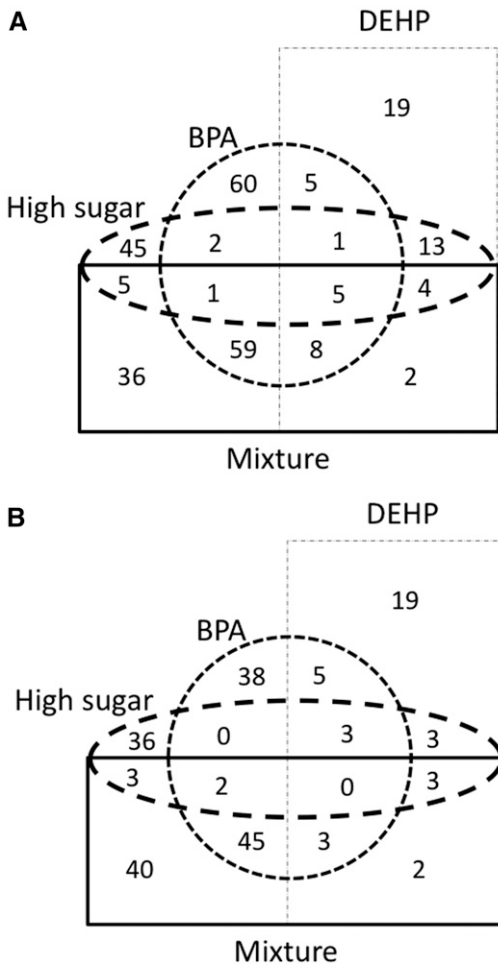
Figure 1 Acute exposures to BPA mixtures perturb gene expression in *Drosophila*. Upregulation (A) and downregulation (B) of gene expression in each acute treatment relative to the control diet (BPP > 0.97). The combined exposure (BPA, high sugar, and DEHP) reinforces individual effects and also results in the differential expression of a new set of genes.

that BPA and the BPA-containing mixture differ significantly in their gene regulatory consequences and suggest synergistic interactions (Figure 2, A and B; Table S1). Gene ontology analysis of genes differentially expressed due to the mixture confirmed the previous results, indicating significant enrichments for genes involved in protein degradation (GO:0006508; 23 genes, $P < 5.83e$-14) and metabolic processes (GO:0008152; 36 genes, $P < 3.12e$-4). Altogether, we conclude that acute exposure in adults caused rapid transcription responses and that common exposure sources might have synergistic effects when combined with BPA, even when their individual ability to disturb gene expression is comparatively mild.

Chronic exposure reveals sugar-mediated potentiation of BPA toxicity

To further address the specific interaction between BPA and sugar, we investigated the consequences of chronic BPA exposure during development (Figure S2). Flies were raised
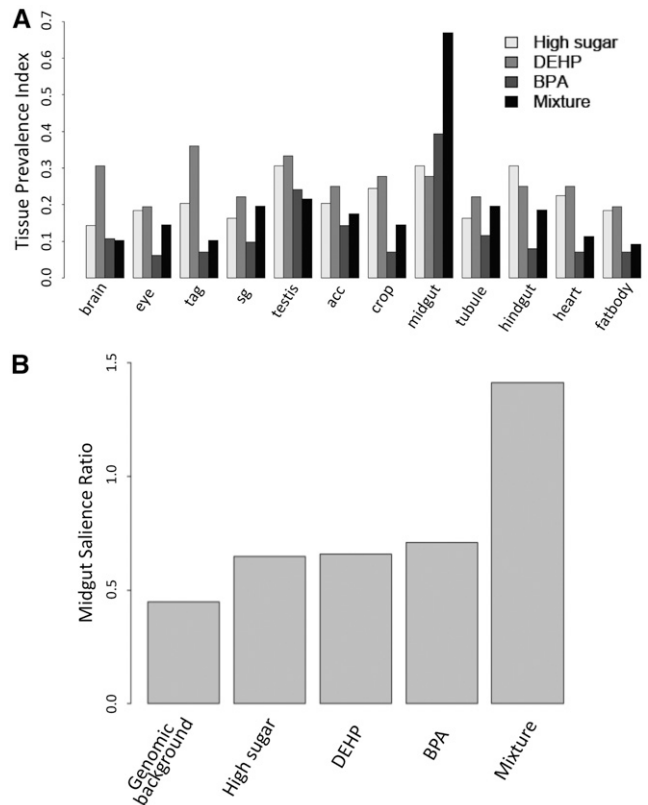

Figure 2 Acute exposures to BPA mixtures perturb the expression of midgut-specific genes. (A) Tissue prevalence indexes [TP(*i*)] for the set of downregulated genes (BPP > 0.95) across 12 tissues (see *Materials and Methods*). Data indicate that the acute treatment perturbs gene expression in the midgut ($P < 0.001$; Fisher's exact test for enrichment in the midgut). (B) Tissue salience ratio [TSr(*i*)] for the midgut indicates a significantly stronger gene knockdown with the mixture ($P < 0.001$, Fisher's exact test).

in (i) BPA, (ii) high sugar, and (iii) the combination of BPA and high sugar. As before, we used flies developed in a BPA-free control food with typical amounts of sugar (150 mM) as a reference. Flies that developed from eggs to adults on these treatment diets were considered chronically exposed. Male flies that emerged from these treatments were collected and used for genome-wide gene expression analysis (Figure S4). Noteworthy, we observed that exposure to the mixture of BPA and high sugar led to an evident delay in development. Relative to controls, the treatments with BPA or high sugar revealed the differential expression of 192 and 220 genes (BPP > 0.97), respectively. Only 48 differentially expressed genes were shared between the two conditions. On the other hand, flies chronically exposed to the BPA/high-sugar mixture showed a larger and unique disruption of gene transcription (Figure 3, A and B): 322 genes were affected by the BPA/high-sugar combination. About 45% and 36% of the genes up- and downregulated in the BPA/high-sugar mixture were also observed in the single exposures (Figure 3, A and B). Finally, we observed significant agreement between fold-change estimates for the effect of the BPA/high-sugar mixture in the two genotypes assayed

(Spearman's rank correlation $\rho = 0.41$, $P = 8.42e\text{-}05$; genes with BPP > 0.95 between control and treatment diet in both data sets). Altogether, the data point to unique and shared expression responses to chronic BPA, high sugar, and their combination. As observed for acute exposure, the combined exposure suggests that BPA and sugar generate a distinct gene regulatory response. Interaction in the combined exposure might happen either through the independent disruption of functionally related pathways or through the enhancement of the disruption of a single pathway targeted by each individual component in the mixture.

BPA exposure knocks down the expression of testis-expressed genes

BPA has been implicated in the disruption of a variety of reproductive traits in humans and model organisms (Allard and Colaiacovo 2010; Lawson *et al.* 2011; Hunt *et al.* 2012). To address the issue in *Drosophila*, we investigated the consequence of the BPA/sugar interaction on gene expression levels in the testis. In agreement with expectations, chronic BPA exposure showed a statistically significant increase in the prevalence of testis-expressed genes in the set of candidates that were downregulated (Figure 4A; $P < 0.001$, Fisher's exact test). Moreover, 24 of the 76 downregulated genes (BPP > 0.97) were expressed exclusively in the testis (32% testis-specific representation *vs.* 19% for the whole set of genes analyzed; $P < 0.01$, Fisher's exact test). On the other hand, testis-specific gene expression was not disrupted upon development in a high-sugar diet without BPA (19 genes of 86; 22% testis-specific representation). Furthermore, and in agreement with our observations concerning interactions between BPA and sugar, the BPA-mediated impairment of testis-expressed genes was enhanced upon coexposure with a high-sugar diet. Accordingly, BPA co-exposure with high sugar caused the downregulation of 47 testis-specific genes, which accounts for 37% of all genes downregulated. This is a statistically significant enrichment in testis-specific genes ($P < 0.001$, Fisher's exact test) and represents the disruption of nearly twice as many testis-specific genes as with BPA alone. Measurements of tissue salience further underscored the pattern. They showed that the set of genes that is downregulated with the BPA/high-sugar diet displays a substantial increase in the number of genes highly expressed in testis (Figure 4B and Table S2; $P = 1.05e\text{-}13$, Fisher's exact test). These results are in agreement with the observation that BPA impairs germ-line tissues (Allard and Colaiacovo 2010; Lawson *et al.* 2011; Hunt *et al.* 2012) and suggest that excess sugar might contribute to these toxic effects.

Finally, data for acute BPA exposure suggested that the 48-hr exposure elicited similar, albeit much subtler, signatures of testis gene downregulation. Accordingly, we observed a slight but statistically significant increase in the testis salience ratios of genes downregulated with BPA exposure (Table S3; $P < 0.05$, Fisher's exact test), which cannot be observed with either sugar or DEHP alone. Interestingly, acute exposure to the mixture led to a much
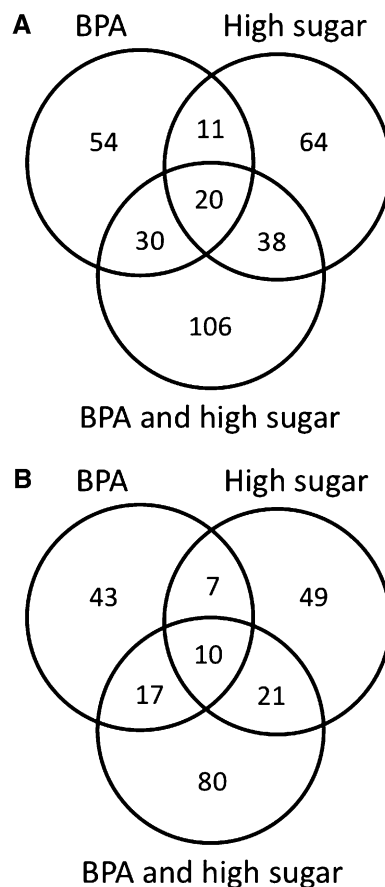

Figure 3 Chronic exposure to BPA perturbs gene expression in *Drosophila*. Upregulation (A) and downregulation (B) of gene expression in each acute treatment relative to the control diet (BPP > 0.97). The mixture (BPA and high sugar) reinforces individual effects and modulates the transcription of a new set of genes.

diminished testis salience possibly because of the stronger biases toward downregulation of midgut genes (*cf.* Table S1 and Table S3). These observations suggest that BPA might be sufficient to negatively affect testis gene expression within 48 hr of adult exposure. However, acute BPA disruption in the testis might depend on absorption rates that are modified when mixtures affect gene expression in gut tissues.

Upregulation of genes associated with energy metabolism during chronic BPA exposure

Excess sugar intake is expected to cause upregulation of energy metabolism. In agreement with this expectation, we observed that flies raised on a high-sugar diet displayed upregulation of pathways associated with energy metabolism. This included highly significant enrichments in genes associated with oxidative phosphorylation (GO:0006119; $P = 6e\text{-}07$), ATP synthesis-coupled electron transport (GO:0042773; $P = 2e\text{-}05$), and having electron carrier activity (GO:0009055; $P = 0.02$). This pattern was corroborated with the Kyoto Encyclopedia of Genes and Genomes (KEGG) database annotation, which pointed to significant
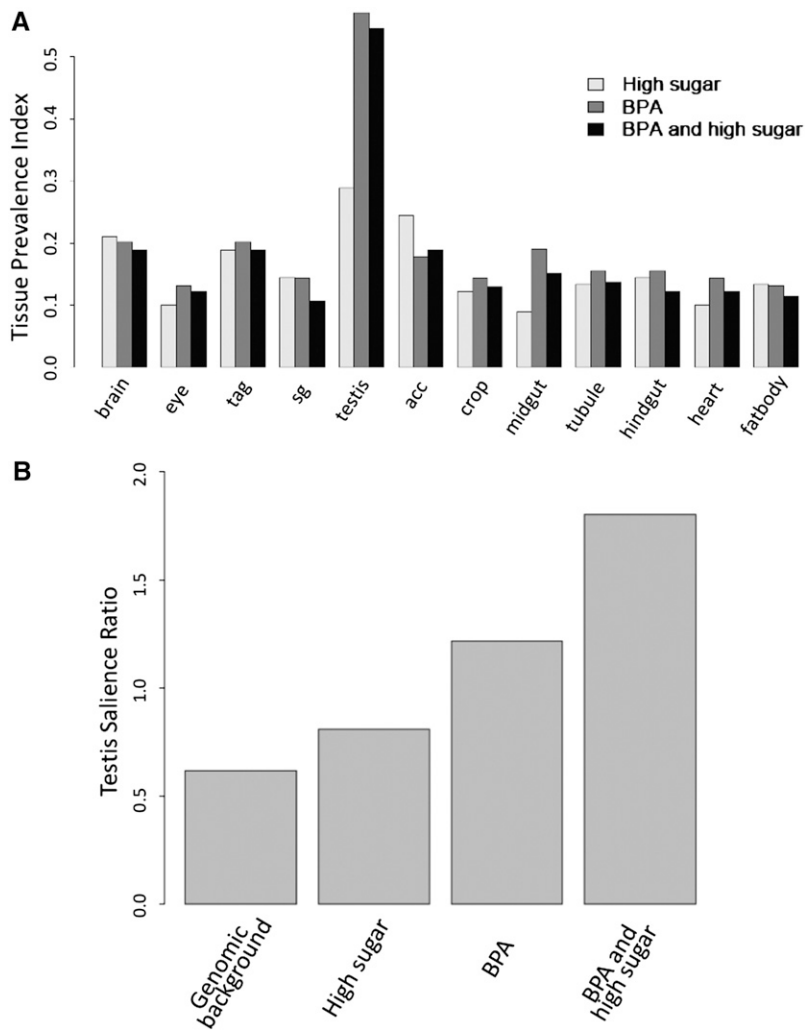
Figure 4 Chronic exposure to BPA perturbs the expression of testis-specific genes. (A) Tissue prevalence indexes [TP(i)] for the set of downregulated genes across 12 tissues (see *Materials and Methods*) indicate that the chronic treatment perturbs gene expression in the testis ($P <$ 0.001; Fisher's exact test for enrichment in the testis). (B) Tissue salience ratio [TSr(i)] for the testis indicates a significantly stronger testis gene knockdown with the mixture ($P <$ 0.001, Fisher's exact test).

enrichment in oxidative phosphorylation (dme00190; $P =$ 4e-08) and ubiquinone biosynthesis (dme130; $P =$ 0.01). Furthermore, we observed 30 genes whose protein products are localized in the mitochondria (GO:0005739; $P =$ 0.001), with 20 of those localized specifically to the mitochondrial inner membrane (GO:0005743; $P =$ 5e-05). We observed that a number of genes responsive to high sugar encode proteins that are localized to the nucleus, although this is not a set displaying statistically significant enrichment. This set includes candidates such as *P53, His4r, Trr, SRm160* (a splicing and mRNA-processing enzyme with a Piwi domain), *Hrb87F* (a ribonuclear protein), and *RpS3* (ribosomal protein S3).

Gene upregulation due to BPA exposure alone also showed significant statistical enrichment for targets involved in energy metabolism, with candidates localizing to the mitochondrial electron transport chain (GO:0005746; $P =$ 0.03). This observation was also supported by the KEGG metabolic pathway database, which identified eight candidates involved in oxidative phosphorylation (dme00190; $P =$ 0.06) and included genes such as the cytochrome C oxidase subunit Vb. The tissue prevalence index did not single out any individual tissue in the sets of genes upregulated by BPA, high sugar, and the BPA/high sugar combination (Figure S6). Notwithstanding, the TSr(i) suggested biases in the upregulation of genes that are typically highly expressed in the midgut and hindgut (Table S4). Collectively these data suggest that energy metabolism and mitochondria-associated processes might be commonly disrupted by both excess sugar and BPA toxicity, with potentially widespread consequences for gene expression across many tissues.

BPA-mediated disruption of pathways associated with the ribosome

While intriguing, BPA-mediated upregulation of mitochondria-associated genes is a fraction of all genes upregulated upon chronic BPA exposure. Noteworthy, the set of all genes upregulated by BPA exposure (BPP $>$ 0.97) also showed significant enrichments for candidates involved in translation (GO:0006412; $P =$ 0.002), with 23 proteins localizing to the ribosomes (GO:0005840, $P =$ 2e-09) and displaying enrichment for the large ribosomal subunit (15 genes, GO:0015934; $P =$ 7e-07). Furthermore, the set of upregulated

genes overlapped significantly with the set of genes observed in two studies designed specifically to identify ribosomal loci. Accordingly, we observed 22 genes in common ($P = 9e$-12) with Marygold *et al.* (2007) and 13 genes in common ($P = 9e$-10) with Alonso and Santarem (2006).

Furthermore, the addition of a high amount of dietary sugar appeared to increase BPA's disruption by further straining ribosome-related pathways. This pattern was stronger for cytoplasmic ribosomes (Figure 5; $P < 0.001$, Fisher's exact test), but disruption of a few mitochondrial ribosome genes was also verified (Figure S7). In the BPA/high-sugar treatment, we observed ribosomal genes that were not differentially expressed under BPA exposure with low sugar. We also observed larger fold changes in the common set of upregulated genes. Indeed, 77% of genes differentially expressed in the cytoplasmic ribosome were more induced in the BPA/high-sugar treatment than in the BPA treatment ($P < 0.01$; Figure 5). Specifically, BPA exposure under a high-sugar diet disrupted 32 genes associated with the ribonucleoprotein complex (GO:0030529; $P = 6e$-04), with 26 proteins localizing to the ribosomes ($P = 2e$-08) and displaying enrichment for components of both large (16 genes, GO:0015934; $P = 4e$-07) and small (10 genes, GO:0022627; $P = 2e$-07) ribosomal subunits. At a lenient BPP > 0.95 threshold, more than a third of the cytoplasmic ribosomal genes showed evidence of upregulation while none showed evidence of downregulation upon any combination of BPA/high sugar exposure (Figure 5, A and B). Collectively, the data indicate that a high-sugar diet might have unexpected and possibly synergistic modulation of BPA-mediated toxicity, even when its independent contribution to the disruption of specific pathways is mild.

Finally, further investigation of the acute exposure data sets revealed that the 48-hr treatments elicited similar, albeit much milder signatures of upregulation in ribosomal-related pathways. Accordingly, the set of upregulated genes in the acute exposure to the mixture (BPP > 0.95) showed significant enrichment in ribosome-related pathways, with 12 candidates that code for proteins belonging to the ribosomal subunits (GO:0044391; $P = 0.002$); 6 candidates from the small ribosomal subunit (RpS14a, RpS20, RpS21, RpS24, RpS26, and RpS27A; GO:0022627; $P = 0.009$), 5 candidates from the large ribosomal subunit (RpL4, RpL11, RpL24, RpL28, and RpL29), and 1 member of the mitochondrial ribosome complex (mRpL52).

Chronic exposure to BPA/high sugar shows signs of nucleolar stress

Expression of genes associated with ribosomes and the assembly of functional ribosomal subunits are tightly correlated with cellular activity and growth. Genes associated with ribosomes are highly expressed to ensure normal and continuous protein translation rates in the cell. These outstanding biosynthetic processes of transcription and translation emerge from the nucleolus, an intranuclear organelle responsible for assembling the ribosomal proteins translated in the cytoplasm and the locally transcribed ribosomal RNA (rRNA) to generate functional ribosomal subunits. Cell activity is partially regulated by the nucleolus, and consequences of its dysfunction are revealed in biological processes such as senescence, cell cycle regulation, and stress sensing (Boisvert *et al.* 2007; Nemeth and Langst 2011). Our gene expression data showed that BPA disrupts the expression of protein-coding genes necessary for ribosome synthesis, which suggests that the nucleolus might be affected. In *Drosophila*, the transposable elements R1 and R2 are specialized to insert into and reside exclusively in the 28s rDNA subunit. We addressed the expression pattern of R1 and R2 using quantitative PCR. Our results revealed that BPA alone does not affect the expression of R1 and R2, whereas sucrose slightly reduces the transcription levels of both transposon elements (Figure 6). On the other hand, the combined exposure to BPA and high sugar significantly increased the expression of R1 and R2, with a more than twofold change in the case of R1 [Figure 6; $P < 0.05$ as calculated with the REST software (Pfaffl *et al.* 2002)]. These observations corroborate genome-wide expression data, which showed that chronic exposure to the mixture of BPA and high sugar led to the disruption of genes associated with ribosomes. Altogether, the data indicated that BPA toxicity might be mediated through ribosomal pathways and that the disruption is enhanced when BPA is combined with a high-sugar diet.

Discussion

We investigated the extent of acute and chronic BPA toxicity and its interaction with a high-sugar diet. We observed that BPA exposure knocked down the expression of testis-specific genes and found that coexposure to a high-sugar diet significantly aggravated the impact of BPA. Surprisingly, BPA exposure substantially disrupted elements of the translational machinery, with significant disruptions to the expression of genes that code for both the small and large components of the ribosome. This signature was significantly more pronounced when BPA was combined with a high-sugar diet. The patterns were robust and observed under acute short-term exposures through oral ingestion as well as under chronic long-term exposure through development. Furthermore, BPA treatments impaired the expression of catabolic genes that are highly expressed in the midgut and might be independent of modulation of hormonal pathways. The analyses also showed that gene expression responses to BPA in the midgut were again enhanced by combination with a high-sugar diet. This suggests that these compounds might interact and act synergistically to perturb the physiology of gut tissues. Finally, our observations point to the disproportionate impact of BPA in the germline. Collectively, the data suggest that ribosome-mediated processes might modulate the sensitivity and consequences of BPA ingestion.
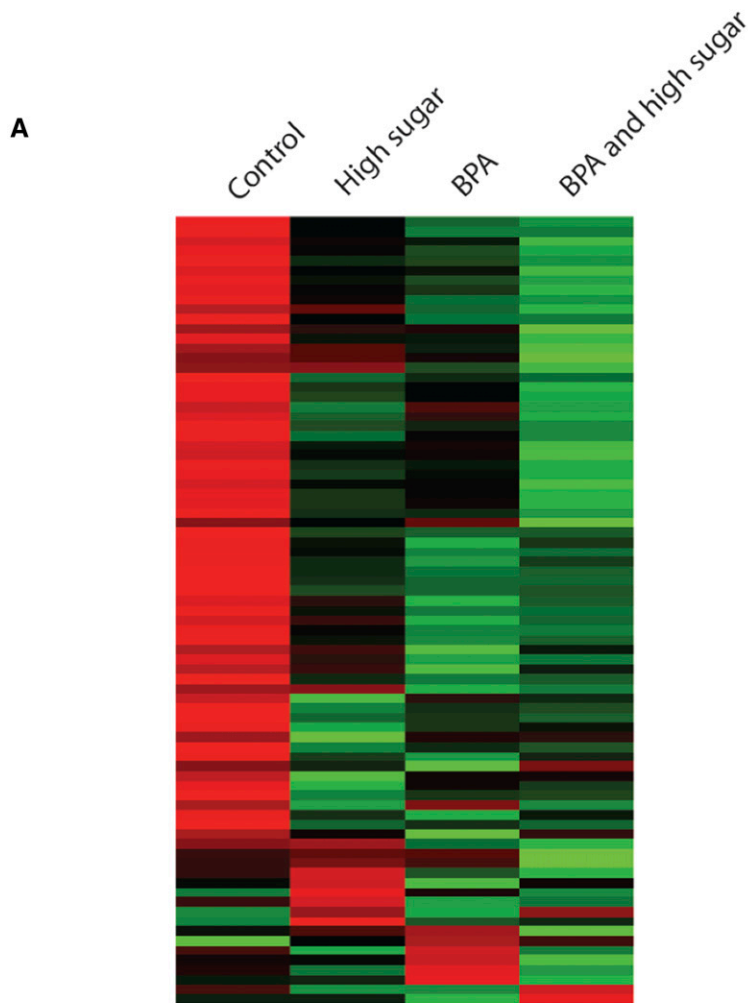
A

B

Cytoplasmic Ribosome	High sugar	BPA	BPA and high sugar
Up regulation	9	18	26
Down regulation	0	0	0

Figure 5 Chronic exposure to BPA and high sugar modulates the expression of genes encoding the protein components of the cytoplasmic ribosome. (A) Heat map shows global perturbation to the expression of 81 genes encoding components of the large and small subunits of the cytoplasmic ribosome. The scale color was normalized for each gene with green and red denoting high and low transcript abundance, respectively. (B) Up- and down-regulation in the subset of genes differentially expressed (BPP > 0.95). The association is statistically significant ($P <$ 0.0001, Fisher's exact test). No statistically significant association was observed for genes encoding components of the mitochondrial ribosome (Figure S7).

DNA methylation-independent pathways of BPA toxicity

Several studies using animal models and humans have indicated that BPA exposure might produce a broad range of adverse effects, including cancer (Weber Lozada and Keri 2011; Pupo *et al.* 2012), disruption of meiosis and other reproductive anomalies (Allard and Colaiacovo 2010; Hunt *et al.* 2012), impairment of genes that control brain development (Itoh *et al.* 2012), and behavior (Kundakovic *et al.* 2013). In some cases, the phenotypes are accompanied by disrupted expression of DNA methyltransferase 1 and 3A, two enzymes responsible for the incorporation of methyl marks on the DNA (Doherty *et al.* 2010; Itoh *et al.* 2012; Kundakovic *et al.* 2013). Alternative pathways for BPA modulation of the epigenome have been postulated; indeed, despite evidence showing that BPA might affect DNA methylation, it is unclear whether this is a direct outcome

of BPA action or a correlated change in the epigenome. *Drosophila* is an especially useful model system in this regard. This is because (i) the organism is amenable for high-throughput genome biology and large-scale screens, (ii) whole-body analysis might be an ideal first step in physiological dissection of tissue-specific outcomes, and (iii) DNA methylation is normally either absent or occurring at extremely low levels (Lyko *et al.* 2000; Phalke *et al.* 2009; Gou *et al.* 2010; Schaefer and Lyko 2010). The latter allows DNA methylation confounders to be excluded and might provide insights into alternative pathways of epigenetic disruption triggered by BPA exposure. In *Drosophila*, nucleolar status might be probed through the expression of two transposable elements (R1 and R2) that localize exclusively to the rDNA array. These specialized elements recognize DNA sequences that are specific to the 28s rDNA. The R1 and R2 elements do not occur in any other locus of the *Drosophila*
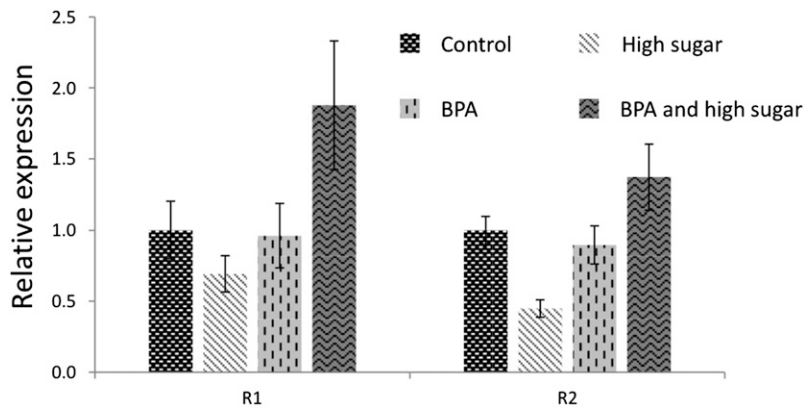
Figure 6 The combination of BPA and high sugar activates the expression of R1 and R2 elements. These transposon elements are located specifically in the rDNA array. Quantitative PCR shows that high sugar slightly decreased the expression of R1 and R2 while BPA alone had no effect on R1 and R2 transcription. However, flies chronically exposed to a diet containing BPA and high sugar showed induction of R1 and R2 expression ($P < 0.01$). Bars represent 95% confidence intervals for the mean.

genome. To address the hypothesis that a BPA/high-sugar diet might affect nucleolar attributes, we assayed R1 and R2 expression upon treatment with BPA, sugar, and the combined exposure. We observed a significant induction of the R1 and R2 retrotransposons in flies exposed to the mixture containing BPA and high sugar. In contrast, the high-sugar diet caused downregulation of the expression of these elements, whereas BPA alone only mildly induced transcription of the R1 and R2 elements. These results raise the prospect that nucleolar stress might be relevant in BPA-mediated toxicity.

Widespread consequences of disrupting the ribosomal machinery

Maintenance of chromosome integrity during cellular division is critical for cell viability. Distinct types of microtubules organize spindle position to establish accurate chromosome segregation and maintain cell orientation. Defects in spindle formation might result in cytokinesis errors, chromosome missegregation, and genome instability (Nousiainen *et al.* 2006). Remarkably, recent studies showed that ribosomal proteins such as RpS3, RpS3a, RpS4, RpL11, and RpL12 are associated with the mitotic spindle (Sauer *et al.* 2005; Torres *et al.* 2011). These proteins are typically studied as part of ribosomes, but post-translational modifications, including phosphorylation, dephosphorylation, and sumoylation, might modulate these proteins to activities outside of their main protein translation functions (Kim *et al.* 2005, 2006, 2009; Jang *et al.* 2011). For instance, studies on the ribosomal protein S3 (RpS3) suggest that it is associated with spindle formation (Jang *et al.* 2012a,b); RpS3-depleted cells displayed abnormal spindle shape, defects in chromosome movement, and mitotic arrest during metaphase.

Among genes upregulated by coexposure of BPA and high sugar, several suggest cross talk between the cytoskeleton and spindle machinery. Specifically, 33 genes were associated with cytoskeleton organization (GO:0007010; $P = 1.32e$-4). This set contains 15 ribosomal proteins, but also includes additional candidates that contributed to enrichment in the categories of "Cytoskeleton Organization" and "Mitotic Spindle Elongation" (GO:00000022; $P = 2.07e$-10).

These observations were corroborated by the Reactome database, which pointed to enrichment in "Eukaryotic Translation Elongation" (24 candidates, $P = 1.24e$-15), "Eukaryotic Translation Initiation" (27 candidates, $P = 3.43e$-15), and "Processing of Capped Intron-Containing pre-mRNA" (32 candidates, $P = 3.73e$-12). Genes belonging to these categories and perturbed by BPA include the tumor suppressor gene Pten, β-tubulin at 56D, tsunagi, actin-related protein 2/3 complex subunit 4, eukaryotic translation elongation factor 2b, eukaryotic initiation factor 5B, stubarista/ribosomal protein SA, and small ribonucleoprotein particle protein SmD2.

The effects of BPA exposure on the impairment of chromosome synapsis and double-strand DNA break repair have previously been observed during *Caenorhabditis elegans* oogenesis (Allard and Colaiacovo 2010), and similar observations were noted in rhesus monkey females (Hunt *et al.* 2012). Hunt *et al.* (2012) further observed abnormal centromere associations, although the mechanisms have remained unclear. One interpretation is that the spindle apparatus is directly disrupted by BPA toxicity. However, the possibility of cross talk between *bona fide* pathways involved in spindle formation and ribosomal protein function provides an intriguing alternate route of disruption. For instance, a proteomic analysis of the human spindle identified 50 ribosomal proteins, a surprisingly high number that suggests that these proteins might interact with the spindle machinery (Sauer *et al.* 2005). One hypothesis is that microtubules and proteins of the spindle not only play a role in generating force for chromosome segregation and organization, but also serve as tracks for the intracellular transport of the ribosomes (Sauer *et al.* 2005). On the other hand, the enrichment of ribosomal proteins in the spindle might reflect a fundamental connection with the nucleolus. Intriguingly, the candidates identified by Sauer *et al.* (2005) include nucleolin and fibrillarin, two proteins that specifically target the nucleolus and are required for its formation (Ochs *et al.* 1985; Ma *et al.* 2007), as well as several other proteins that also localize to this organelle (*e.g.*, nucleolar RNA helicases and small nucleolar ribonucleoproteins). Detailed investigation suggested that some proteins might play dual roles, forming associations with the microtubules during mitosis

as well as localizing to the nucleolus during interphase (Zatsepina *et al.* 1999). Altogether, these observations further raise the possibility that nucleolar stress might be one consequence of combined BPA/sugar toxicity.

Collectively, our observations point to synergistic toxic interactions between BPA and dietary sugar and indicate significant perturbations in the expression of ribosome-associated proteins and nucleolar stress. Our analyses show that the interaction between BPA and sugar results in heightened disruption of ribosome-associated genes and heightened activation of nucleolus-specific transposable elements R1 and R2. The data suggest DNA methylation-independent mechanisms through which BPA exerts its toxic effects and raise the question of how persistent the insult might be upon its removal and between generations.

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GENETICS

High Intake of Dietary Sugar Enhances Bisphenol A (BPA) Disruption and Reveals Ribosome-Mediated Pathways of Toxicity

Alan T. Branco and Bernardo Lemos

Figure S1

30 adult male flies were collected, and transfered to vials containing control food (which contains 150 mM of sucrose), food with BPA, high sugar (450 mM of sucrose), DEHP, or the mixture (BPA + DEHP + 450 mM of sucrose).
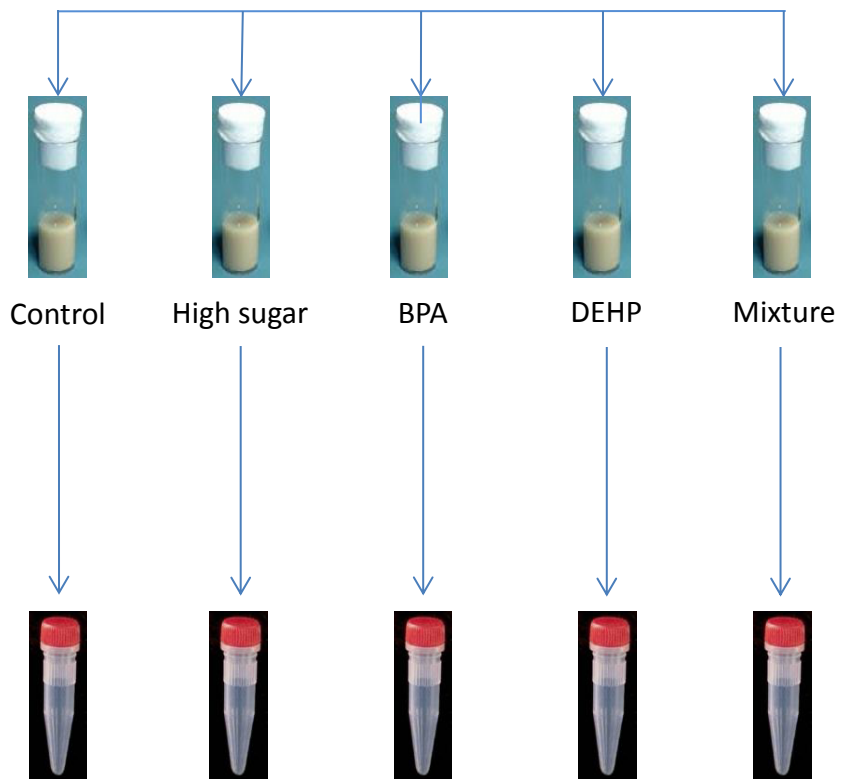
After 48 hours the flies were flash frozen in liquid nitrogen.

Figure S1. Cartoon with experimental design for acute exposure. We transferred adult male flies from the typical control laboratory diet to an identical diet in which BPA, DEHP, high sugar, or the mixture had been added. For the acute exposure flies were allowed to feed on the treatment diet for 48 hours. These flies were used to evaluate the effects of acute exposure on genome-wide gene expression. Four biological replicates were set up per treatment. Thirty males were exposed per biological replicate.

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Figure S2

10 males and 15 female flies mated for 5 days in control food (which contains 150 mM of sucrose), food containing BPA, high sugar (450 mM of sucrose), or BPA and high sugar mixed together.

Control | High sugar | BPA | BPA and High sugar

New male flies emerged from the food containing the compounds. They matured for 48hs in the same food they were raised.

Control | High sugar | BPA | BPA and High sugar

After 48 hours the flies were flash frozen in liquid nitrogen.
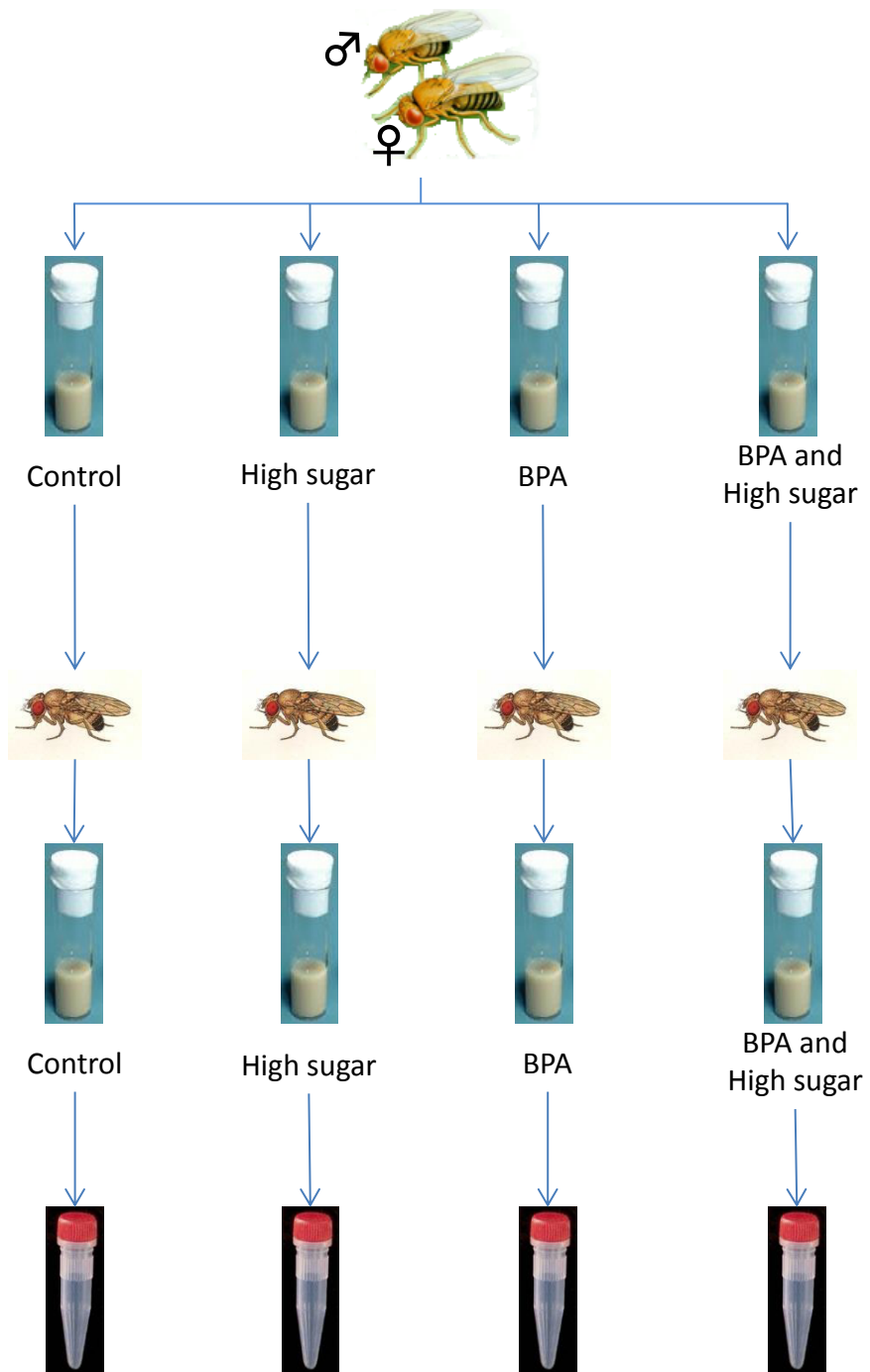
Figure S2. Cartoon with experimental design for chronic exposure. To study the chronic effect of BPA and high sugar, female flies laid eggs on the typical control laboratory diet or on an identical diet in which BPA, high sugar, or the mixture had been added. Flies were exposed to the treatment during development (from eggs to adults) and maintained in the treatment diet for 48hs upon emergence. These flies were used to evaluate genome-wide gene expression responses under chronic exposure. Four biological replicates were set up per treatment. Each treatment was further replicated in two genotypes.

Figure S3
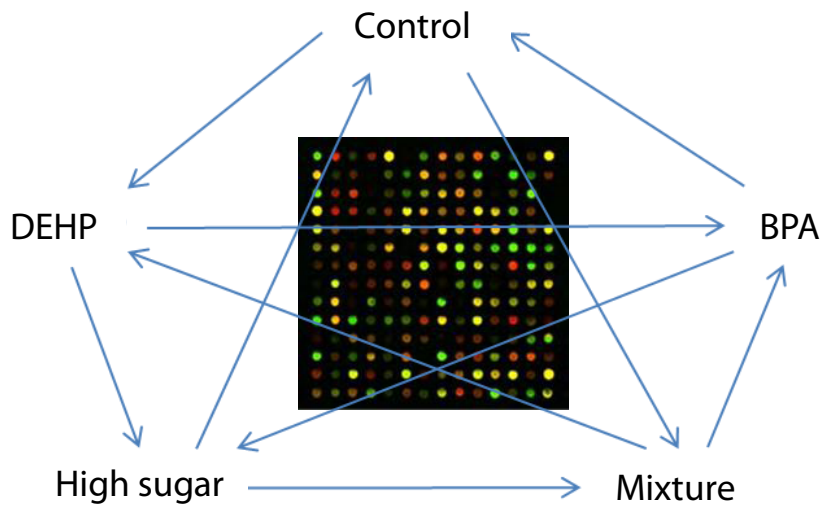
Figure S3. Microarray design for acute exposure. Dual-channel microarrays were used to study genome-wide gene expression. Two biological replicates were collected per treatment, with balanced dye swaps. Arrows represent the contrasts between 2 samples (1 microarray slide), and point from samples labeled with Cy5 to samples labeled with Cy3. In total, the expression signal of each treatment was collected from 4 distinct microarray slides.

Figure S4
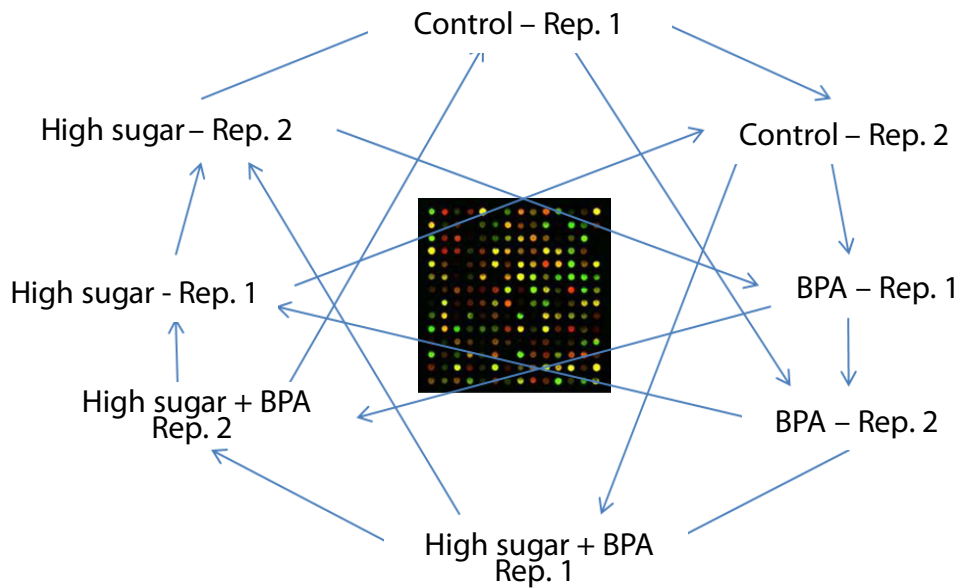
Figure S4. Microarray design for chronic exposure. Dual-channel microarrays were used for the genome-wide gene expression analysis. Each treatment was evaluated with two genotypes (rep 1 and rep 2). Each genotype replicate was evaluated twice (2 biological replicates per genotype and diet combination) with dye swaps. Arrows represent the contrasts between 2 samples (1 microarray slide), and point from samples labeled with Cy5 to samples labeled with Cy3. In total, the expression signal of each treatment was collected from 8 distinct microarray slides.

Figure S5

Figure S5. Tissue prevalence of genes up-regulated upon acute treatment with BPA, DEHP, and high sugar. Tissue prevalence indexes were calculated with the set of genes up-regulated in each treatment (BPP > 0.97). Tissue expression data are from the FlyAtlas database.
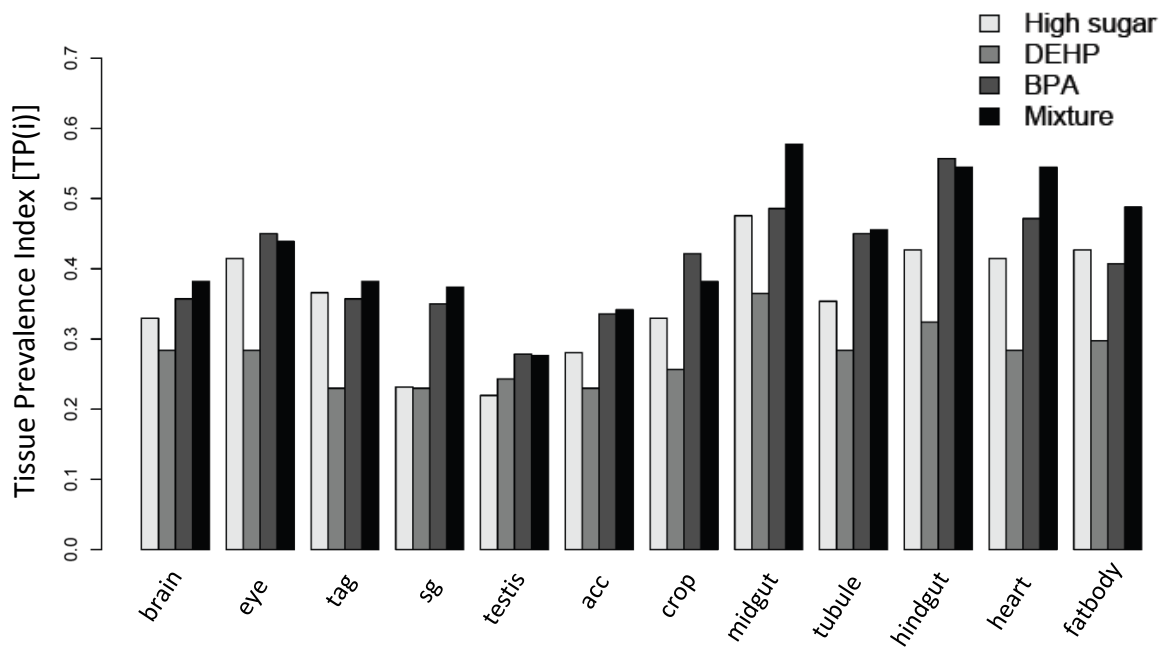
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Figure S6

Figure S6. Tissue prevalence of genes up-regulated upon chronic exposure to BPA and high sugar. Tissue prevalence indexes were calculated with the set of genes up-regulated in each treatment (BPP > 0.97). Tissue expression data are from the FlyAtlas database.

Figure S7

A

B

Mitochondrial Ribosome	High sugar	BPA	BPA and High sugar
Up regulation	4	4	2
Down regulation	1	1	0
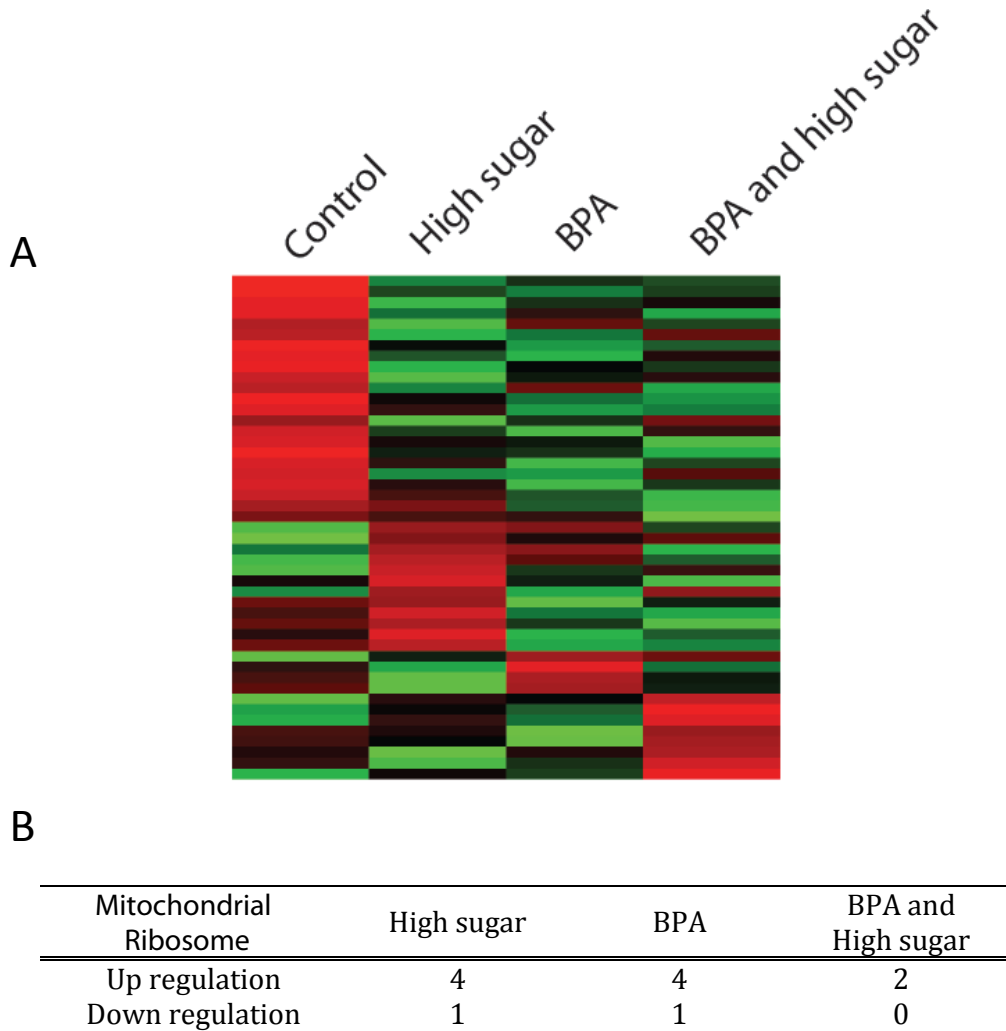
Figure S7. Expression profile of mitochondrial ribosome genes upon chronic exposure to BPA and high sugar. (A) Heat map shows the expression profile of 47 mitochondrial ribosome genes after chronic exposure to high sugar, BPA, and the mixture (BPA and high sugar). The scale color was normalized for each gene with green and red denoting high and low transcript abundance, respectively. (B) Number of mitochondrial ribosome genes significantly up-regulated (BPP > 0.95) in response to each treatment.

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Table S1. Midgut salience ratios of genes differentially regulated in response to acute exposure. Data is shown for genes differentially expressed in each of 4 treatments (High sugar, DEHP, BPA, and the mixture). Background refers to the complete set of genes that passed the quality control steps and entered the analysis of differential expression. "Up" and "Down" identifies gene sets expressed in the midgut and at a level that is above or below the whole organism average, respectivelly.

Midgut	Background	High sugar	DEHP	BPA	Mixture
Dataset of **down-regulated** genes (BPP > 0.95)					
Up	1594	30	22	64	86
Down	3545	46	33	90	61
Ratio	0.45	0.65	0.66	0.71	1.41
P-value = 1.006e-11, Fisher's exact test					
Dataset of **down-regulated** genes (BPP > 0.98)					
Up	1594	11	8	39	56
Down	3545	12	10	23	9
Ratio	0.45	0.91	0.80	1.70	6.22
P-value < 2.2e-16, Fisher's exact test					
Dataset of **up-regulated** genes (BPP > 0.95)					
Up	1594	45	47	93	84
Down	3545	77	44	95	72
Ratio	0.45	0.58	1.07	0.98	1.17
P-value < 0.001, Fisher's exact test					
Dataset of **up-regulated** genes (BPP > 0.98)					
Up	1594	13	14	32	28
Down	3545	22	20	40	30
Ratio	0.45	0.59	0.70	0.80	0.93
P-value < 0.002, Fisher's exact test					

Table S2. Testis salience ratios of genes down-regulated in response to chronic exposure. (A) Dataset of down-regulated genes with BPP > 0.95. (B) Dataset of down-regulated genes with BPP > 0.97. Data is shown for genes differentially expressed in each of 3 treatments (BPA, high sugar, and the mixture). Background refers to the complete set of genes that passed the quality control steps and entered the analysis of differential expression. "Up" and "Down" identifies gene sets expressed in the testis and at a level that is above or below the whole organism average, respectivelly.

A

TESTIS	Background	BPA	High sugar	Mixture
Up	1733	82	60	125
Down	2722	66	73	70
Ratio	0.64	1.24	0.82	1.79

P-value = 1.05e-13, Fisher's exact test

B

TESTIS	Background	BPA	High sugar	Mixture
Up	1733	41	30	72
Down	2722	26	38	35
Ratio	0.64	1.58	0.79	2.06

P-value = 2.55e-10, Fisher's exact test

Table S3. Testis salience ratios of genes differentially regulated in response to acute exposure. Data is shown for genes differentially expressed in each of 4 treatments (High sugar, DEHP, BPA, and the mixture). Background refers to the complete set of genes that passed the quality control steps and entered the analysis of differential expression. "Up" and "Down" identifies gene sets expressed in the testis at a level that is above or below the whole organism average, respectivelly.

TESTIS	Background	High sugar	DEHP	BPA	Mixture
Dataset of **down-regulated** genes (BPP > 0.95)					
Up	1985	29	23	66	36
Down	3554	53	41	85	98
Ratio	0.55	0.55	0.56	0.78	0.37

P-value = 0.06, Fisher's exact test

Dataset of **down-regulated** genes (BPP > 0.98)					
Up	1985	8	8	22	5
Down	3554	16	14	35	53
Ratio	0.55	0.50	0.57	0.63	0.09

P-value = 0.0001, Fisher's exact test

Dataset of **up-regulated** genes (BPP > 0.95)					
Up	1985	20	19	25	12
Down	3554	106	72	167	146
Ratio	0.55	0.19	0.26	0.15	0.08

P-value = 2.20e-16, Fisher's exact test

Dataset of **up-regulated** genes (BPP > 0.98)					
Up	1985	5	6	9	5
Down	3554	31	26	67	55
Ratio	0.55	0.16	0.23	0.13	0.09

P-value = 1.21e-11 Fisher's exact test

Table S4. Tissue salience ratio of genes up-regulated in response to chronic exposures. (A) Dataset of up-regulated genes with BPP > 0.95. (B) Dataset of up-regulated genes with BPP > 0.99. CNS stands for central nervous system. Data is shown for genes differentially expressed in each of 3 treatments (BPA, high sugar, and the mixture). Background refers to the complete set of genes that passed the quality control steps and entered the analysis of differential expression. "Up" and "Down" identifies gene sets expressed in the tissue at a level that is above or below the whole organism average, respectivelly.

A

MIDGUT	Background	BPA	High sugar	Mixture
Up	1158	68	65	102
Down	2971	87	123	138
Ratio	0.39	0.78	0.52	0.74

P-value = 1.342e-08, Fisher's exact test

HINDGUT	Background	BPA	High sugar	Mixture
Up	1415	73	96	133
Down	2633	76	91	104
Ratio	0.54	0.96	1.05	1.28

P-value = 1.151e-14, Fisher's exact test

CNS	Background	BPA	High sugar	Mixture
Up	1704	62	75	88
Down	2481	101	115	148
Ratio	0.69	0.61	0.65	0.60

P-value = 0.6754, Fisher's exact test

TESTIS	Background	BPA	High sugar	Mixture
Up	1733	42	37	39
Down	2722	136	168	221
Ratio	0.64	0.31	0.22	0.18

P-value < 2.2e-16, Fisher's exact test

B

MIDGUT	Background	BPA	High sugar	Mixture
Up	1158	13	10	24
Down	2971	13	26	36
Ratio	0.39	1.0	0.38	0.67

P-value = 0.020, Fisher's exact test

HINDGUT	Background	BPA	High sugar	Mixture
Up	1415	20	18	33
Down	2633	7	20	27
Ratio	0.54	2.9	0.90	1.22

P-value = 1.80e-06, Fisher's exact test

CNS	Background	BPA	High sugar	Mixture
Up	1704	10	13	23
Down	2481	19	29	43
Ratio	0.69	0.53	0.45	0.53

P-value = 0.415, Fisher's exact test

TESTIS	Background	BPA	High sugar	Mixture
Up	1733	5	6	3
Down	2722	28	38	61
Ratio	0.64	0.19	0.16	0.05

P-value = 3.459e-13, Fisher's exact test

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