Report

Current Biology

Replacement Bisphenols Adversely Affect Mouse Gametogenesis with Consequences for Subsequent Generations

Highlights

- Replacement bisphenols are structural BPA variants with similar biological effects
- Common bisphenols are germline toxicants that induce meiotic effects in both sexes
- Genotoxic bisphenol exposure effects may persist for several generations in males
- Environmental contaminants can undermine science by affecting data and conclusions

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In Brief

Horan et al. report changes in meiotic data in mice coinciding with physical damage to polysulfone cages. LCMS analyses implicate replacement bisphenols. Subsequent controlled experiments demonstrate that, like BPA, common replacement bisphenols induce meiotic effects in both sexes that, in males, may persist for several generations.

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Replacement Bisphenols Adversely Affect Mouse Gametogenesis with Consequences for Subsequent Generations

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SUMMARY

20 years ago, accidental bisphenol A (BPA) exposure caused a sudden increase in chromosomally abnormal eggs from our control mice [1]. Subsequent rodent studies demonstrated developmental effects of exposure with repercussions on adult health and fertility (e.g., [2–9]; reviewed in [10–17]). Studies in monkeys, humans, fish, and worms suggest BPA effects extend across species (e.g., [18–30]; reviewed in [31–33]). Widespread use has resulted in ubiquitous environmental contamination and human BPA exposure. Consumer concern resulted in "BPA-free" products produced using structurally similar bisphenols that are now detectable environmental and human contaminants (e.g., [34–41]). We report here studies initiated by meiotic changes mirroring our previous BPA experience and implicating exposure to BPS (a common BPA replacement) from damaged polysulfone cages. Like with BPA [1, 2, 5], our data show that exposure to common replacement bisphenols induces germline effects in both sexes that may affect multiple generations. These findings add to growing evidence of the biological risks posed by this class of chemicals. Rapid production of structural variants of BPA and other EDCs circumvents efforts to eliminate dangerous chemicals, exacerbates the regulatory burden of safety assessment, and increases environmental contamination. Our experience suggests that these environmental contaminants pose a risk not only to reproductive health but also to the integrity of the research environment. EDCs, like endogenous hormones, can affect diverse processes. The sensitivity of the germline allows us to detect effects that, although not immediately apparent in other systems, may induce variability that undermines experimental reproducibility and impedes scientific advancement.

Results and Discussion

In the course of meiotic studies in male and female mice, we observed variation in meiotic recombination (measured by the number of MLH1 foci in pachytene stage meiocytes), with levels in some controls reaching values characteristic of BPA-exposed animals [2, 5]. Although the change in pooled data was subtle, variation among litters was striking (Figure 1). Given our previous experience with BPA leaching from polycarbonate cages and water bottles [1], damaged materials were an obvious suspect. When white residue was evident on the surface of some polysulfone cages in our facility (Figure 2A), we suspected that exposure to chemicals leaching from the damaged polymer was eliciting meiotic effects.

An Unexpected Contaminant

Polysulfone is comprised of BPA and diphenyl sulfone (Figure 2B); thus, we suspected that these were the contaminants of interest. Liquid chromatography-tandem mass spectrometry (LC-MS)/MS analysis of a methanol extraction of damaged cages, however, demonstrated the presence of both BPA and BPS (Figures 2C–2F). Because polymeric aromatic ethers, like their monomeric counterparts, cannot undergo nucleophilic substitution to generate an unsubstituted aromatic ring at the reaction site, degradation results in the formation of a phenolic group. Therefore, damaged polysulfone is, in fact, more likely to generate BPS than diphenyl sulfone is (Figure 2B). Unfortunately, high signal levels in both control and solvent blanks made it impossible to determine if diphenyl sulfone was a significant contaminant.

Replacement bisphenols have rapidly emerged in consumer products, and studies of them are limited. However, plastics containing them can leach estrogenic chemicals [43, 44], and exposure has been reported to induce adverse effects similar to BPA (e.g., [45–52]; reviewed in [53]). Our findings suggest that, although newer polymers like polysulfone are more resistant to chemical damage than polycarbonate is, damage can occur in the course of normal use and may result in the release of contaminants that are not constituent components of the polymer.

Bisphenol Analogs Elicit Meiotic Effects

To eliminate contamination, all caging materials in the facility were replaced, new breeding stocks were purchased, and studies were conducted to confirm that control values in both

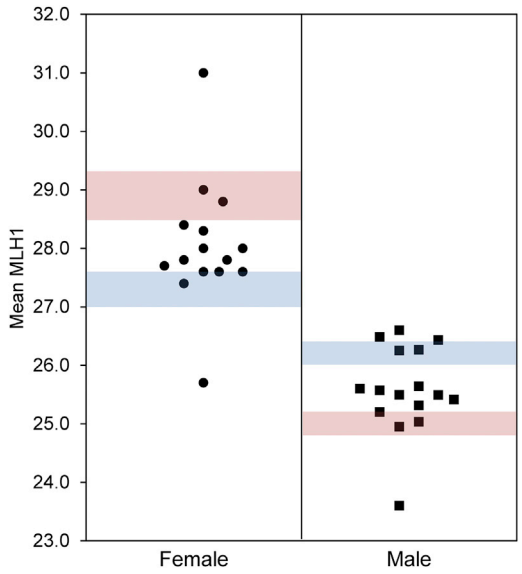

Figure 1. Variation in Control Data Suggests Environmental Contamination

Data from 15 litters (1–2 fetuses each) of C57BL/6 females (circles) and 16 litters (2–4 adults each) of 129S1/SvimJ males (squares) showing variation in mean MLH1 counts in control animals analyzed during a 6-month period. Shaded bars denote historical laboratory means ± SEM for control (blue) and exposed (pink) animals, showing an exposure-induced increase in females and decrease in males as reported previously [2, 5, 42].

sexes had returned to expected levels. To verify that the contaminant bisphenols elicit meiotic effects, we designed experimental studies.

Our previous studies in mice suggest that a brief, appropriately timed exposure to BPA can impact the entire germ cell population in both sexes, although the timing and mechanisms differ. In females, all oocytes enter meiosis in the fetal ovary, and *in utero* exposure coinciding with meiotic onset increases levels of meiotic recombination [2, 18]. The subtle changes induced are compatible with continued oocyte survival but increase the frequency of aneuploid eggs and embryos produced by the adult female [2]. In contrast, in males, BPA and other estrogenic exposures also can affect the entire germline—not by epigenetically modifying cells entering meiosis, but rather germline stem cells. Neonatal exposure coinciding with the establishment of the spermatogonial stem cell (SSC) population of the testis causes a permanent reduction in recombination levels in all descendant spermatocytes [5, 42].

While rebuilding our colony and confirming that contamination had been eliminated, we initiated studies to assess the effects of the putative contaminants BPS and diphenyl sulfone using timed pregnant females purchased from the Jackson Laboratory. Oral doses of 20 ng/g BPA (positive control), BPS, diphenyl sulfone (Figure 3A), or placebo (vehicle-only) control were administered at 14 and 15 days post coitum (dpc) to coincide with the time of meiotic entry in the fetal ovary. 20 ng/g is below the US EPA tolerable daily intake level for BPA (50 ng/g/day) and thus is a low dose with human relevance. By comparison with unexposed female fetuses, BPA and BPS exposure induced a significant increase in mean MLH1 counts (27.1 ± 0.5, 29.2 ± 0.3, and 29.3 ± 0.4, respectively; post hoc $p < 0.01$; Figure 3B). Diphenyl sulfone also elicited an increase (28.6 ± 0.4) but was not significant due to the limited sample size. Our previous studies in both mice and monkeys demonstrated similarly increased levels of meiotic recombination in developing oocytes as a result of maternal BPA exposure [2, 18].

In males, we assessed the effects of neonatal exposure to the putative contaminants, BPS and diphenyl sulfone, and two other common replacement bisphenols, BPF and BPAF (Figure 3A). Males were given daily oral doses of 20 ng/g BPA, BPS, diphenyl sulfone, BPF, BPAF, or placebo from 1–8 days postpartum (dpp) and meiotic analyses were conducted on 6-week-old adults. As shown in Figure 3C, all bisphenols induced significant meiotic effects. By comparison with controls (26.0 ± 0.1), mean MLH1 counts in exposed males were significantly reduced, with diphenyl sulfone eliciting the strongest effect: 25.2 ± 0.1, 25.3 ± 0.1, 24.8 ± 0.1, 25.1 ± 0.1, and 25.0 ± 0.1 for BPA, BPS, diphenyl sulfone, BPF, and BPAF, respectively (Figure 3C; post hoc $p < 0.01$).

Low recombination rates are deleterious because spermatocytes with homologs that fail to undergo recombination face certain death due to the actions of a robust spindle assembly checkpoint mechanism that causes arrest and demise of cells with unpartnered chromosomes at metaphase I [5, 54, 55]. As predicted on the basis of previous studies [5, 42], reduced recombination levels in bisphenol-exposed males resulted in an increase in the frequency of spermatocytes with at least one synaptonemal complex lacking an MLH1 focus (i.e., MLH1 null SCs; Figure S1).

Although "BPA free" is a valuable marketing tool, and most consumers interpret this label as an indication of a safer product, our findings add to growing evidence from studies in *C. elegans* [56], zebrafish [46, 49, 52, 57–59], mice [47, 50, 51, 60–62], and rats [63–65], as well as human *in vitro* studies [25, 45, 48, 66], that replacement bisphenols have the potential to induce adverse effects similar to those reported for BPA. Meiosis is both a sensitive indicator of environmental contamination and, because recombination directly affects the amount of genetic diversity in a population, an evolutionary driver. Thus, exposures that influence recombination are cause for concern. Importantly, meiotic effects of bisphenol exposure are clearly not limited to mice. Remarkably similar effects of BPA and replacement bisphenols have been reported in *C. elegans*, although subtle mechanistic differences among bisphenols are evident [56]. While understanding the mechanism of action of individual chemicals is important, our data suggest that bisphenols as a class should be considered germline toxicants.

Exposure Effects Persist in Males for Several Generations

Meiotic recombination is quantitative, making it a powerful means of tracing exposure effects across generations. Our previous studies suggest that meiotic effects induced by neonatal estrogenic exposure in male mice are transmitted to offspring, and exposure effects intensify with successive generations of exposure [42]. Thus, inadvertent exposure of our animals provided an opportunity to determine if and for how long exposure effects persisted after the elimination of environmental bisphenol contamination. Three 129S1/SvimJ males from the exposed colony served as founders (F0) for an analysis of four successive

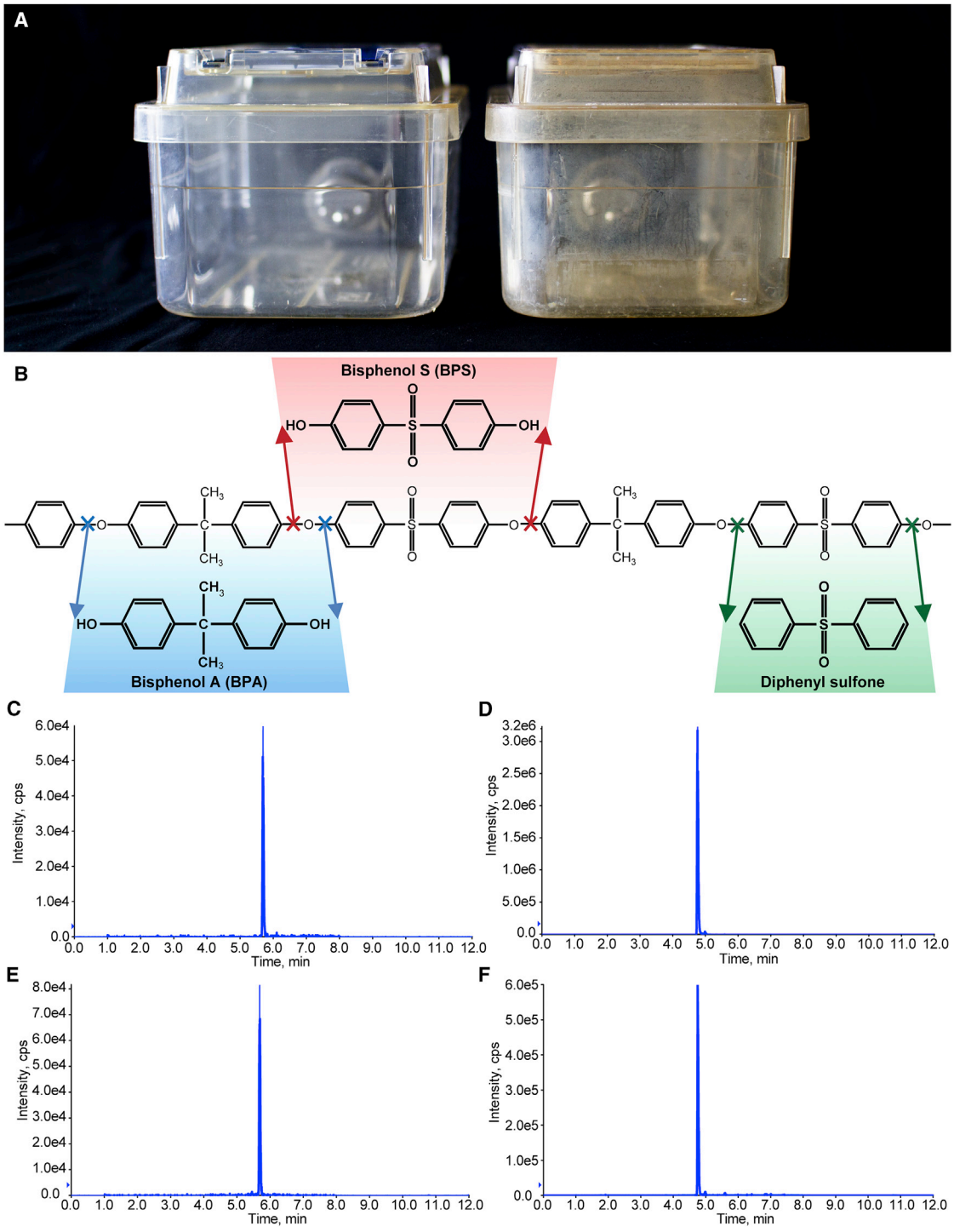

Figure 2. BPA and BPS Are Released from Damaged Polysulfone

(A) Comparison of an undamaged polysulfone cage (left) and cage with white residue indicative of damage (right).

(B) Structure of the BPA-diphenyl sulfone dimer that comprises polysulfone. Arrows denote cleavage sites that would result in the release of BPA (blue), BPS (red), and diphenyl sulfone (green).

(C–F) Extracted ion chromatogram results showing BPA and BPS standards at 10 ng/mL (C and D, respectively) and results from the analysis of BPA and BPS in white residue scraped from a damaged cage (E and F, respectively). The concentration of BPS detected in the damaged cage was greater than BPA (note differences in y axis).

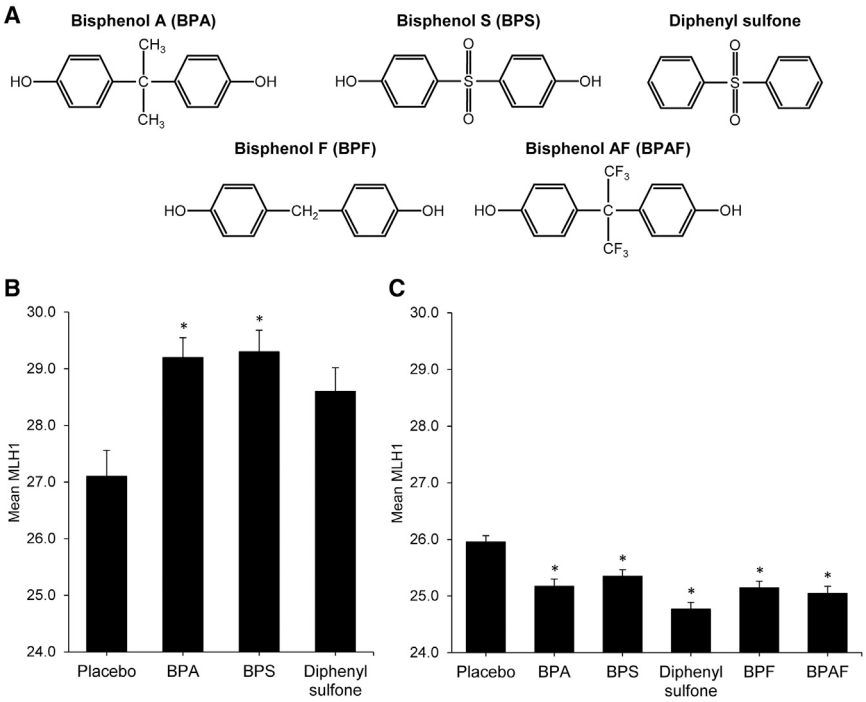

Figure 3. Bisphenol Analog Exposure Elicits Male and Female Meiotic Effects

(A) Chemical structures of BPA and four replacement bisphenols.

(B) Mean MLH1 counts ± SEM for females treated 14–15 dpc with placebo or 20 ng/g BPA, BPS, or diphenyl sulfone. Groups represent 37, 100, 88, and 56 cells for 3 placebo, 7 BPA, 6 BPS, and 6 diphenyl sulfone females, respectively.

(C) Mean MLH1 counts ± SEM for males treated from 1–8 dpp with placebo or 20 ng/g BPA, BPS, diphenyl sulfone, BPF, or BPAF. Groups represent 420, 270, 330, 300, 385, and 270 for 14 placebo, 9 BPA, 11 BPS, 10 diphenyl sulfone, 13 BPF, and 9 BPAF males, respectively. Groups were compared by one-way ANOVA ($F = 4.1$, $p < 0.01$ for females; $F = 11.4$, $p < 0.0001$ for males). Significant differences were determined by Tukey-Kramer post-hoc test (asterisk denotes $p < 0.01$ post hoc comparison with the placebo).

See also Figure S1.

generations of unexposed male descendants. F0s were born and weaned in contaminated cages but transferred as adults to new cages with sibling females to produce F1 males. On average, 10 males from at least 3 litters were produced each generation for each family. Male progeny from new 129S1/SvimJ breeding stock served as unexposed controls. Our analysis of over 120 male progeny provided evidence both of the variability of the exposure effect on our animals and that exposure effects spanned several generations.

As shown in Figure 4, families 1 and 2 exhibited similar trends, with a significant reduction in recombination levels by comparison with controls for the first three generations (F0–F2; assessed by one-way ANOVA; Figure 4). In contrast, the MLH1 mean of the founder for family 3 (26.3 ± 0.4) was in the control range, and subsequent generations of offspring did not deviate significantly. The variation among founder males is consistent with the inter-litter variation that characterized the exposure effect (Figure 1) but effectively reduced our generational study to the analysis of two families.

Recombination levels in founders from both families 1 and 2 were low by comparison with controls (24.0 ± 0.3, 24.3 ± 0.4, and 26.0 ± 0.0, respectively; post hoc $p < 0.01$; Figure 4). In both families, F1 males showed an increase in mean MLH1 levels (24.9 ± 0.2 and 25.2 ± 0.2, respectively) by comparison with their fathers, although the difference did not reach significance. By the F2 generation, mean MLH1 values reached an intermediate level (25.6 ± 0.1 in both families) that was significantly different (post hoc $p < 0.05$) from both the F0 and the new colony mean, providing evidence of a transgenerational effect. However, in the F3 generation, mean values for both families (25.7 ± 0.1 and 26.0 ± 0.1, respectively) were not significantly different from the new colony, and this return to expected control values was evident in the F4 generation (Figure 4).

Although we cannot pinpoint the onset of the accidental exposure in our 129S1/SvimJ colony, evidence of exposure effects in some families but not others suggests that the duration of exposure was limited to a single generation. Indeed, one founder male exhibited no evidence of exposure. Thus, while our data suggest eradication of male germline effects after several generations, they do not allow us to draw conclusions about a scenario with greater human relevance—i.e., the resolution of effects following multiple generations of exposure. This is an important consideration in view of our recent finding that the testis phenotype is exacerbated by successive generations of exposure [42].

Brave New World

DuPont's 20th century slogan "better living through chemistry" has been borne out. Remarkable technical advances allow us to synthesize molecules and create subtle variations in them. Innovation, however, has outpaced our ability to understand the implications of the release of rapidly generated families of structurally similar chemicals into our environment. Our data add to and extend the growing concern about the harmful reproductive effects of one such family, the bisphenols. Although most data derive from rodent studies, given the developmental and reproductive similarities, concerns almost certainly extend to humans. Importantly, bisphenols are not the only chemical family with an ever-increasing array of diverse members; other prominent environmental contaminant families include the parabens, perfluorinated compounds (PFCs), phthalates, flame retardants, and quaternary ammonium compounds.

The ability to rapidly enhance the properties of a chemical has tremendous potential for treating cancer, enhancing medical and structural materials, and controlling dangerous infectious agents. Importantly, this technology has paved the way for "green chemistry," a healthier future achieved by engineering chemicals to ensure against hazardous effects (e.g., [67]). Currently, however, regulatory agencies charged with assessing chemical safety cannot keep pace with the introduction of new chemicals. Further, as replacement bisphenols illustrate, it is

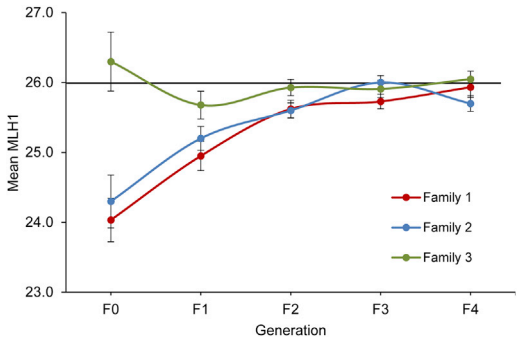

Figure 4. Effects of Inadvertent Exposure on Male Meiotic Recombination Rate Persist for Several Generations

Black line denotes MLH1 mean for new colony 129S1/SvimJ males (26 ± 0.0 foci per cell, n = 1848 cells from 63 males from 22 litters). Colored lines show MLH1 mean ± SEM for three different founder males (F0) from the exposed colony and four subsequent generations of unexposed male offspring (F1–F4). 25–30 pachytene cells were analyzed per male. F1 groups consisted of 4–6 males per family, F2 of 11–14 males per family, F3 of 12–16 males per family, and F4 of 8–12 males per family. Means for each generation were compared to the new colony mean using one-way ANOVA ($F = 13.4$, $p < 0.0001$ for family 1; $F = 12.0$, $p < 0.0001$ for family 2; and $F = 1.6$, $p = 0.2$ for family 3), and significant differences between groups were assessed using a Tukey-Kramer post hoc. For both families 1 and 2, the F0, F1, and F2 generations had significantly lower mean MLH1 values by comparison with the new colony (post hoc $p < 0.05$); the F2, F3, and F4 generations had significantly higher mean MLH1 values by comparison with the F0 (post hoc $p < 0.05$).

easier and more cost effective under current chemical regulations to replace a chemical of concern with structural analogs rather than determine the attributes that make it hazardous.

The environmental exposure underlying this study is the third such inadvertent environmental contamination encountered in the course of studies in our laboratory [1, 68]. The sensitivity of the germ cell endpoints we study has made it possible to rapidly detect the effects of these environmental contaminants, but identifying and eliminating them has impeded our research. Because we study environmental effects, we are vigilant about controlling the animal environment and testing contact materials. Thus, repeated inadvertent contamination in the course of our studies is an indicator of the sheer number of contaminants and their ubiquitous presence in daily life. This represents a hazard not only to human health, but also to the ability of scientists to conduct sound and meaningful studies. For example, initial data suggest that inadvertent contamination may have compromised the CLARITY-BPA project sponsored by the US Food and Drug Administration (FDA) and the National Toxicology Program (NTP) [69, 70]. CLARITY-BPA is a multi-investigator initiative conducted under federal oversight and designed to comprehensively test the effects of BPA exposure. Thus, because findings from this initiative will inform regulatory decisions regarding BPA in the United States, evidence of possible contamination of control animals in the CLARITY-BPA project is disturbing. As our data demonstrate, common endocrine-disrupting chanels (EDCs) that are prevalent environmental contaminants have the potential to introduce significant variability in research studies. The NIH considers rigor and reproducibility "the cornerstones of science advancement." Thus, compromised studies in both our laboratory and the CLARITY-BPA project suggest that, by interfering with reproducibility of results, environmental contamination can undermine scientific interpretation.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

- KEY RESOURCES TABLE
- CONTACT FOR REAGENT AND RESOURCE SHARING
- EXPERIMENTAL MODEL AND SUBJECT DETAILS
- METHOD DETAILS
  - Detection of contaminants in damaged cages
  - Breeding paradigm for recovery analysis
  - Treatment solutions
  - Meiocyte preparations and immunostaining
  - MLH1 analysis
- QUANTIFICATION AND STATISTICAL ANALYSIS
- DATA AND SOFTWARE AVAILABILITY

SUPPLEMENTAL INFORMATION

Supplemental Information includes one figure and can be found with this article online at https://doi.org/10.1016/j.cub.2018.06.070.

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AUTHOR CONTRIBUTIONS

Conceptualization and Methodology, P.A.H. and T.S.H.; Investigation, T.S.H., H.P., C.L., R.G., S.M., M.C.G., and C.V.S.; Formal Analysis, T.S.H., H.P., C.L., S.M., R.G., and P.A.H.; Visualization, T.S.H., C.L., and S.M; Resources, P.A.H. and R.G.; Funding Acquisition, P.A.H.; Writing—Original Draft, Review, and Editing, T.S.H., P.A.H., H.P., and R.G.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Purified Mouse Anti-Human MLH-1	BD PharMingen	Cat#550838; RRID: AB_2297859
Rabbit polyclonal to SCP3	Abcam	Cat#ab15093; RRID: AB_301639
SCP3/SYCP3 Antibody	Novus Biologicals	Cat#NB300-232; RRID: AB_2087193
Alexa Fluor 488 AffiniPure Donkey Anti-Mouse IgG (H+L)	Jackson Immunoresearch Laboratories	Cat#715-545-150; RRID: AB_2340846
Cy3 AffiniPure Donkey Anti-Rabbit IgG (H+L)	Jackson Immunoresearch Laboratories	Cat#711-165-152; RRID: AB_2307443
Rhodamine (TRITC) AffiniPure Donkey Anti-Rabbit IgG (H+L)	Jackson Immunoresearch Laboratories	Cat#711-025-152; RRID: AB_2340588
Chemicals, Peptides, and Recombinant Proteins		
4,4'-Sulfonyldiphenol	Santa Cruz Biotechnology	Cat#sc-238983
Bis(4-Hydroxyphenyl)Methane 98%	Sigma-Aldrich	Cat#B47006-1G
4,4′-(Hexafluoroisopropylidene)diphenol 97%	Sigma-Aldrich	Cat#257591-25G
Diphenyl sulfone, 97%	Sigma-Aldrich	Cat#P35359-100G
Bisphenol A	Sigma-Aldrich	Cat#80-05-7
Tocopherol Stripped Corn Oil	MP Biomedicals	Cat#901415
ProLong Gold Antifade reagent with DAPI	Invitrogen	Cat#P36931
Bovine Serum Albumin (BSA)	Fischer Scientific	Cat#BP9700100
Normal Donkey Serum, ChromPure, NovoProtein	Jackson ImmunoResearch	Cat#017-000-121
Experimental Models: Organisms/Strains		
129S1/SvimJ mice (MGI:5658424)	The Jackson Laboratory	JAX: 002448
C57BL/6J mice (MGI:5656552)	The Jackson Laboratory	JAX: 000664
Software and Algorithms		
GenASIs Case Data Manager	Spectral-Imaging	http://www.spectral-imaging.com/products-technologies/data-management
GenASIs MetScan	Spectral-Imaging	http://www.spectral-imaging.com/products-technologies/scan-analysis
Axiovision LE	Zeiss	https://www.zeiss.com/microscopy/us/products/microscope-software/axiovision.html; RRID: SCR_002677
AB Sciex Analyst 1.6	Sciex	https://sciex.com/products/software/analyst-software; RRID: SCR_015785

CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Patricia A. Hunt (pathunt@wsu.edu).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Breeding stocks of adult inbred 129S1/SvimJ and C57BL/6J mice (The Jackson Laboratory) were mated in brother/sister breeding pairs with mating beginning at 6-weeks of age (sexual maturity). Pups resulting from these matings were weaned at 20 days post-partum (dpp) and housed in polysulfone cages (Allentown) separated by sex, with no more than 5 mice per cage. Cages were kept on ventilated racks (Allentown, Jag 75 micro isolator model) on a 14-hr light/10-hr dark cycle, in a climate controlled, specific pathogen-free facility, monitored quarterly by sentinel mice. Cages contained Sanichip 7090A bedding (Harlan Laboratories) and a nestlet (Ancare) for enrichment. Drinking water in polysulfone bottles and irradiated food (Envigo Teklad 2920) were autoclaved and

provided *ad libitum*. Littermates of the same sex were randomly assigned to experimental groups. All adult mice were killed using inhaled CO_2 until cessation of breathing was observed, followed by secondary internal cervical dislocation. Fetal mice were euthanized using decapitation as specified by the Washington State University Institutional Care and Use Committee. All protocols were approved and followed the National Institute of Health standards established by the Guide for Care and Use of Laboratory Animals (The National Academies Press). Washington State University is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care.

METHOD DETAILS

Detection of contaminants in damaged cages

Cage extractions were obtained by sequentially rinsing five mouse cages with 100 mL of absolute methanol. The resultant methanol extraction was analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) using an Agilent LC1260 (Agilent, Santa Cruz, CA)- AB Sciex 5500 Triple Quadrupole MS (AB Sciex, Foster City, CA) at the University of California San Francisco. Samples from cage filters and scrapings were extracted with methanol, evaporation, and reconstitution in 10% methanol for injection into the LC-MS/MS. One mL aliquots of methanol were included as a negative control. Extracts were injected into an Agilent Extend-C18 (2.1 × 100 mm, 1.8 μm) column, maintained at 50°C. Chromatographic separation of the analytes was achieved by gradient elution using water with 0.05% ammonium acetate (pH 7.8) as mobile phase A and methanol with 0.05% ammonium acetate (pH 7.8) as mobile phase B. The elution gradient employed was- 0– 0.5 min = 30%B; 1 min = 75%B; 4 min = 100%B; 4– 6 min = 100%B; and 6.01– 12 min = 30% B. The limit of detection (LOD) was 0.2 ng/mL for BPA and 0.01 ng/mL for BPS. Data analysis was done using AB Sciex Analyst 1.6 software package. Identification and confirmation of each analyte in the sample was based on its retention time and the peak area ratio between its two transitions. A signal/noise (S/N) ration of > 3 was used to define qualitative signals.

Breeding paradigm for recovery analysis

Three males born and weaned in contaminated cages served as F0 founders of three families. At 6-weeks of age, founder males were placed in new, undamaged cages and paired with two female siblings to produce second-generation (F1) offspring. Each founder produced 2-4 litters, and each litter was weaned into new, undamaged cages. At 6-weeks of age, one male from one litter of each family was paired with two sister females to produce the third-generation (F2). Each male produced 3-4 litters. This pattern was repeated to produce fourth-generation (F3) and fifth-generation (F4) offspring for each family. In total, there were 3 F0, 15 F1, 39 F2, 41 F3, and 31 F4 129S1/SvimJ males from 2, 9, 10, 12, and 12 litters, respectively. Adult males of all generations were killed between 6–12-weeks of age as specified above and their testes surgically collected in phosphate-buffered saline (PBS: 136.9 mM NaCl, 53.7 mM KCl, 29.4mM KH_2PO_4, 129.6 mM Na_2HPO_4, pH 7.4).

Treatment solutions

For intentional exposure studies, new breeding stocks of 129S1/SvimJ and C57BL/6 mice were obtained from The Jackson Laboratory to generate offspring for analysis. To verify the elimination of contamination, we analyzed meiotic recombination levels in replacement animals born in our facility. Levels in 129S1/SvimJ males appeared normal, but slightly high mean recombination levels were observed in C57BL/6 females. When levels remained high after several months, we resorted to the use of timed pregnant females purchased from The Jackson Laboratory to experimentally assess the effects of exposure to the putative bisphenol contaminants. Females arrived on 13 days post coitum (dpc) and were treated 14-15 dpc with 20 ng/g BPA, BPS or diphenyl sulfone, or placebo (equal volume ethanol/corn oil vehicle). All chemicals were dissolved in 100% ethanol and diluted in tocopherol-stripped corn oil to a 2.5% (v/v) ethanol solution and administered orally by pipette as previously stated for the male mice. Female dams were given a 20 ng/g body weight dose as determined via electronic scale daily. Dams were killed on 17.5 dpc, and fetuses were collected in phosphate-buffered saline (PBS). Female sample size consisted of 3 placebo, 7 BPA, 6 BPS, and 6 diphenyl sulfone C57BL/6 females from 2, 3, 2, and 3 litters, respectively.

Male 129S1/SvimJ mice were treated from 1-8 dpp with 20 ng/g of either diphenyl sulfone, bisphenol A (BPA), bisphenol S (BPS), bisphenol F (BPF), bisphenol AF (BPAF), or placebo (an equal volume ethanol/corn oil vehicle). Treatment stock solutions were made by dissolving powder solid chemicals in 100% ethanol and reduced to treatment dosages by serial dilutions. All treatment solutions were made containing 1% (v/v) ethanol in tocopherol-stripped corn oil (MP Biomedicals), such that solution concentrations were 20 ng/μl of solution. Mice were dosed with one μL treatment solution per gram of body weight, with pup weights estimated as average weight for age and strain. This produced a final daily dose of 20 ng/g which was chosen for several reasons: First, it is below the US EPA tolerable intake level for BPA (50 ng/g/day), and thus represents a low dose with human relevance; second, a 20 ng/g dose of BPA elicits meiotic effects in male mice [5]; and third, using the same dose for all chemicals makes it possible to compare their relative potency. Adult males were killed at 6-weeks of age as specified above and their testes were surgically collected in PBS. Male samples consisted of 14 placebo, 9 BPA, 11 BPS, 10 diphenyl sulfone, 13 BPF, and 9 BPAF 129S1/SvimJ males from 5, 3, 3, 3, 3, and 4 litters, respectively.

Meiocyte preparations and immunostaining

Meiocyte preparations were made according to the method developed by Peters and colleagues [71]. Testes were cleaned in PBS and incubated in hypotonic solution (30mM tris, pH 8.2-8.4; 50 mM sucrose; 17 mM sodium citrate; and 5 mM EDTA, pH 8.2) for

20 min. Several seminiferous tubules from each testis were separated and macerated in 500 mM sucrose. Cell suspensions were spread on slides coated in paraformaldehyde (PFA: 1% paraformaldehyde, 50 μL Triton X-100, pH 9.2). Ovaries were cleaned in PBS and incubated in hypotonic solution for 12 min. Both ovaries from each fetus were macerated together in 500 mM sucrose and the cell suspension was spread on slides coated in PFA. Slides were incubated in a humid chamber for 2 hr. and washed with 0.4% Photo-flo 200 solution (Kodak Professional). Immunofluorescence staining of slides was performed as described previously [5, 42]. Slides were stained with MLH1 primary antibody (BD PharMingen, 550838, at 1:60) overnight followed by SYCP3 primary antibody (Novus, NB300-232, at 1:200) for 2 hr., and counterstained with Alexa Fluor 488-conjugated AffiniPure Donkey Anti-Mouse (AFDAM) secondary antibody (Jackson Immunoresearch Laboratories, 715-545-150, at 1:75) and either 2 hr. in Cy3-conjugated AffiniPure Donkey Anti-Rabbit (CDAR) secondary antibody for spermatocytes (Jackson Immunoresearch Laboratories, 711-165-152, at 1:1500) or 1 hr. in Rhodamine-conjugated AffiniPure Donkey Anti-rabbit (RDAR) secondary antibody for oocytes (Jackson Immunoresearch Laboratories, 711-025-152, at 1:200). All staining was done in 1X ADB (10X stock consisted of 10 mL normal donkey serum, 3.0 g BSA, 50 μL Triton X-100, and 90 mL PBS that was then sterile filtered with a 45 μm filter), and slides were incubated at 37°C.

MLH1 analysis

Spermatocytes were imaged using the GenASI Scan & Analysis platform with an Olympus BX61 microscope. Oocytes were imaged using a Zeiss Axio Imager epifluorescence microscope. MLH1-FITC, SYCP3-TRITC, and DAPI were imaged sequentially, and brightness adjusted using either GenASI MetScan or Axiovision software. The number of MLH1 foci in composite MLH1/SYCP3 images was counted by two independent observers who were blinded with regard to exposure group.

QUANTIFICATION AND STATISTICAL ANALYSIS

Twenty-five to thirty pachytene spermatocytes were scored per animal, and minor counting discrepancies were resolved. No minimum or maximum number of oocytes was analyzed per animal. Cells with major scoring discrepancies, poor staining, or synaptic defects were excluded from analysis. In addition, cells with greater than two MLH1 null SCs were excluded to eliminate potential bias due to poor immunostaining.

Average MLH1 counts were determined for each animal and pooled averages were determined for each treatment group (n = number of cells). Male sample sizes comprised of: 3 F0 males (90 cells), 15 F1s (441 cells), 39 F2s (1167 cells), 41 F3s (1218 cells), 31 F4s (928 cells), and 91 new colony (2675 cells) for persistence of inadvertent exposure in the "old colony"; 14 placebo (420 cells), 9 BPA (270 cells), 11 BPS (330 cells), 10 diphenyl sulfone (300 cells), 13 BPF (385 cells), and 9 BPAF (270 cells). Female sample sizes consisted of 3 placebo (37 cells), 7 BPA (100 cells), 6 BPS (88 cells), and 6 diphenyl sulfone (56 cells). Differences in mean MLH1 foci counts and MLH1 null frequencies among exposure groups or generations were analyzed by one-way ANOVA. For statistically significant differences ($p < 0.05$), a Tukey-Kramer post hoc test was performed to infer which groups differed. All statistical analysis was performed using Microsoft Excel.

DATA AND SOFTWARE AVAILABILITY

Data are available on request. Please contact the Lead Contact.