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<http://dx.doi.org/10.1289/EHP211>

Received: 23 March 2016

Revised: 13 June 2016

Accepted: 23 June 2016

Published: 22 July 2016

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***In Utero* Exposure to Benzo[a]Pyrene Increases Mutation Burden in the Soma and Sperm of Adult Mice**

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Acknowledgments:

Mutagenicity data, statistical analysis, and detailed methodology described in this paper are available in Supplementary Materials. Sequence data for *lacZ* mutants are archived in the NCBI sequence read archive. We acknowledge technical assistance from Marianela Rosales, John Gingerich, and Lynda Soper. We are thankful for comments received from Drs. Daniel

Desaulniers and Mike Wade. This work was supported by the Health Canada intramural funding. Stipend support to MJM and JOB was provided by the Natural Sciences and Engineering Research Council of Canada; to MJM, JOB and MAB by the Canadian Institute of Health Research (CIHR) Training Program in Reproduction, Early Development, and the Impact on Health; and to MAB by the National Sciences and Engineering Research Council.

Competing Financial Interests:

The authors declare they have no actual or potential competing financial interests.

ABSTRACT

Background: Mosaicism, the presence of genetically distinct cell populations within an organism, has emerged as an important contributor to disease. Mutational events occurring during embryonic development can cause mosaicism in any tissue, but the influence of environmental factors on levels of mosaicism is unclear.

Objectives: We investigated whether *in utero* exposure to the widespread environmental mutagen benzo[a]pyrene (BaP) impacts the burden and distribution of mutations in adult mice.

Methods: We used the MutaTMMouse transgenic rodent model to quantify and characterize mutations in the offspring of pregnant mice exposed to BaP during postconception days 7 through 16, covering the major period of organogenesis in mice. Next-generation DNA sequencing was then used to determine the spectrum of mutations induced in adult mice that were exposed to BaP during fetal development.

Results: Mutation frequency was significantly increased in the bone marrow, liver, brain, and sperm of F1 males. Developing embryos accumulated more mutations and exhibited higher proportions of mosaicism than exposed adults, particularly in the brain. Decreased sperm count and motility revealed additional negative impacts on reproductive function of F1 males.

Conclusion: We show that *in utero* exposure to environmental mutagens contributes to somatic and germline mosaicism, permanently impacting both the genetic health of the F1 and the population gene pool.

INTRODUCTION

Our understanding of human genetic disease is predicated on the idea that most mutations are inherited through the germline. However, mounting evidence suggests that disease-associated genetic changes also arise during embryonic development (Biesecker and Spinner 2013; Erickson 2010; Lupski 2013, 2015). These postzygotic events (which may include mutations, large-scale rearrangements, or aneuploidies) produce a varied distribution of altered genomes throughout the individual – a phenomenon known as mosaicism (De 2011). Any cell type in the body can accumulate such mutations, including stem cells or primordial germ cell precursors, inducing permanent changes in the individual or their offspring (Campbell et al. 2015; Cohen et al. 2014; Rahbari et al. 2015). Recent genome-scale studies have revealed unexpected levels of mosaicism in seemingly normal tissues (Campbell et al. 2015; Erickson 2010; Rahbari et al. 2015), and we may still be vastly underestimating the prevalence and health burden of low-level mosaicism (Campbell et al. 2014; Spinner and Conlin 2014).

Recent work by Rahbari et al. (2015) exemplifies the prevalence of mutations occurring early during development and their significant contribution to somatic and germline mosaicism in adults. They also provide compelling evidence that germline mutation rates are not constant throughout the lifetime of an organism, and that spontaneous mutation may be more likely during the expansion of male primordial germ cell precursors in embryogenesis than during other developmental stages or post-pubertal spermatogenesis. Although individual differences were observed in the prevalence of germline mosaicism, no studies since the pioneering work of Russell et al. (1992; 1999; 1988) have investigated whether environmental factors during development alters the induction of mosaic mutations in the germline. This previous work

demonstrated, using phenotypic markers in the specific locus test, that the perigametic interval was a significant source of spontaneous as well as chemical- or radiation-induced mosaic mutations. However, other stages of development, such as fetal growth, have remained uncharacterized with respect to the induction of mosaicism by environmental factors. Mutation assays in general measure both unique and clonally expanded mutations; however, the relative contribution of mosaicism to overall mutation burden is rarely considered in these assays. Moreover, the influence of mutagen exposure during critical developmental stages on the degree of tissue-wide mosaicism remains a significant gap in understanding in the field of genetic toxicology.

In utero exposure to toxicants can cause a range of deleterious health effects in adulthood [e.g., reproductive defects (Fowler et al. 2008; Mocarelli et al. 2011), increased cancer susceptibility (Autrup 1993), impaired cardiac function (Buscariollo et al. 2014), and neurodegenerative disease (Modgil et al. 2014)]. However, the extent to which *in utero* exposure to environmental chemicals contributes to adult disorders resulting from the induction and distribution of mutations in developing tissues is unknown (Ritz et al. 2011). We used a transgenic rodent model combined with next-generation sequencing to investigate the effects of *in utero* exposure to benzo[a]pyrene (BaP), a common environmental pollutant and human carcinogen produced from a variety of sources, on the burden and distribution of mutations in adults. This study presents the first evidence that fetal exposure of mice to a mutagenic chemical can directly result in an excess burden of mutations and increased mosaicism in both somatic tissues and germ cells of adult F1 mice.

METHODS

Animal Treatment

The use of animals in these experiments was approved by the Health Canada Ottawa Animal Care Committee. Animals used in this study were humanely treated with regard to the alleviation of suffering following the guidelines of the Canadian Council on Animal Care (http://www.ccac.ca/en/_standards/policies/policy-ethics_animal_investigation). Both male and female MutaTM Mouse mice were obtained from a colony maintained at Health Canada. Males were housed with up to four females and every morning females were checked for the presence of vaginal plug as indication of mating. Pregnant mice were dosed with 0, 10, 20, or 40 mg/kg/d BaP (Sigma-Aldrich Canada Ltd), dissolved in olive oil (at a volume of 0.15 mL for 30 g body weight) and administered on postconception days 7 through 16 by oral gavage (with postconception day 1 indicated by the presence of a vaginal plug). Each pregnant female was housed individually. Pups were weaned at three weeks of age and euthanized 10 weeks after birth in accordance with Health Canada's ethical guidelines and tissues collected. The bone marrow, brain, liver, testis, and cauda epididymis (one per mouse) were flash frozen in liquid nitrogen immediately following necropsy, and stored at -80 °C until DNA extraction was performed.

LacZ Transgene Mutation Assay

The LacZ transgenic rodent mutation assay was performed as previously described, in a manner consistent with OECD Test Guideline 488 (O'Brien et al. 2014; OECD 2011). DNA was isolated from tissues using phenol/chloroform extraction and packaged into lambda phage (Transpack packaging extract, Agilent Technologies). The packaged reporter constructs were

subsequently plated on a lawn of *Escherichia coli* (*galE*) grown on LB media, and positive selection for mutant plaques was performed using phenylgalactoside (Gossen et al. 1992). Mutant plaques were counted, and a subset of plaques were collected in MilliQ water (3 uL per plaque) for sequencing. The dose-response of mutant frequency was tested for significance with generalized linear modeling in R using a quasi-Poisson distribution (R Core Team 2014) as well as dose-response modeling using both the R-package PROAST and benchmark dose software (BMDS v. 2.6 from the US-EPA; <http://www.epa.gov/ncea/bmds/>).

Computer Assisted Sperm Analysis

Computer assisted sperm analysis (CASA) was performed on an IVOS instrument (Hamilton Thorne Research, USA) using sperm from one of the cauda epididymis taken from mice at the time of necropsy. Each cauda was minced into 2.5 mL of prewarmed M16 or M199 medium (Sigma, USA) for the 10 mg/kg/d BaP or the 20 and 40 mg/kg/d/ BaP, respectively. After 3 minutes at 37 °C in 5% CO₂, a 16 µL of a 1:4 dilution of sperm was added into the two wells of a 2X-CEL 80 µm deep chambered slide (Hamilton Thorne Research). At least 10 fields per chamber, automatically selected by the IVOS, were imaged with a 4x objective and analyzed with the IVOS Animal software v14. Setting for CASA analysis were: frame rate, 60Hz; 30 frames acquired/samples; minimum contrast, 40; minimum cell size, 3.

Statistical Analyses

The P-values for body weight, liver somatic index (LSI), testis somatic index (TSI), and sperm motility metrics were determined using an ANOVA followed by a Bonferroni post-hoc multiple comparison, relative to the control group. Dose-response data from the *lacZ* transgene

assay was analyzed in R using the glm function. The number of mutant plaques were compared to dose, setting log(total plaques) as the offset and using the quasi-Poisson distribution family to account for over-dispersion. The resulting p-values were corrected for multiple comparisons using the Bonferroni method. A likelihood ratio test was used to eliminate outliers between technical replicates (within animals), and subsequently between animals within dose groups. Dose response analysis for all tissues was done using all available models in BMDS v. 2.6, followed by selection of the best fit model using the Akaike information criterion (AIC).

Ion Proton Sequencing

Pooled plaques collected from the transgenic rodent assay were subjected to PCR following heating at 95 °C and centrifugation to remove *E. coli* cellular debris. PCR was performed in duplicate technical replicates for each sample using NEB Phusion DNA Polymerase according to the manufacturer's directions. PCR amplified DNA was purified with the QIAquick PCR purification kit, and then used to create a fragmented DNA library via ligation to P1 adapter and barcoded A adapter, via the NEBNext Ion Kit. The resulting libraries were pooled in equimolar quantities after quantification on the Agilent Tapestation D1000. These libraries were used in template preparation on the Ion Chef robot, and sequenced using a P1 chip on the Ion Proton instrument.

Computational Analyses

Mutations were called as previously described (Beal et al. 2015). Reads were aligned to the *lacZ* sequence from MutaTMMouse using bowtie2 with the “very sensitive local” option enabled. Pileups were created using samtools mpileup v 0.1.19 and mutations were called with a

customized R script (available online at <http://usegalaxy.org>). Based on the pileups, a proportion of each base call at each position of the *lacZ* gene was determined for each library (each of which constitutes a different sample of pooled plaques, performed in technical replicates). Putative mutations were filtered based on the criteria that they must be present above the pooled mutation calling threshold (1/# plaques sequenced) in both technical replicate DNA libraries, and the background rate of the mutation must be less than 2%. For calculating clonal expansion, the count of each mutant was determined by multiplying the percentage of reads containing each unique mutation by the number of plaques sequenced for that animal; since we observed high variability among low numbers of mutant counts, we applied a limit of detection/linear model to correct the counts (Beal et al. 2015). This method relies on the conservative assumption that any two reads derived from the same biological sample, and possessing an identical mutation within one tissue, are likely to be the product of clonal expansion rather than independent events. Clonally expanded mutants were then considered to be those with a corrected count greater than 1, while mutants with a corrected count of 1 were considered as singletons. Mutation spectra were generated using the counts for each unique mutation type.

RESULTS

Mutations in somatic tissues after *in utero* BaP exposure

To determine the genetic effects of transplacental exposure to an environmental mutagen, BaP was administered in olive oil at 0, 10, 20, or 40 mg/kg/day by oral gavage to pregnant MutaTMMouse females on postconception days 7-16, comprising the period of organogenesis in

mice (Mitiku and Baker 2007). These doses were chosen based on previous literature indicating that such an exposure affected the fertility of the F1 generation (MacKenzie and Angevine 1981). Neither litter size at birth nor body weight of the F1 generation (at 10 weeks of age) were significantly affected by BaP administered during pregnancy, indicating that BaP exposure in the dam did not cause embryo loss or significantly impact postnatal development.

We then measured mutations in three somatic tissues derived from each germ layer (ectoderm: brain; mesoderm: bone marrow; endoderm: liver) using the recoverable *lacZ* reporter transgene within the MutaTMMouse genome (Lambert et al. 2005). Transplacental BaP exposure induced dose-dependent increases in mutations in the somatic tissues of F1 males (Figure 1 and Supplementary Table S1). At the highest dose, mutant frequencies increased 16, 18, and 33-fold ($P < 0.0001$) above controls in bone marrow, brain, and liver, respectively. At the low dose, brain showed a 7-fold increase in mutant frequency ($P < 0.0001$), liver a 3-fold increase ($P = 0.04$), and bone marrow was unaffected ($P = 0.13$). Although BaP adduct levels are typically elevated in maternal tissues relative to fetal tissues (Lu et al. 1986), *in utero* exposed liver and brain had 3- and 19-fold higher numbers of mutants per mg/kg-bw than adult BaP-exposed tissues, respectively (Supplementary Figure S1, Supplementary Table S2). Conversely, mutagenic response in bone marrow did not differ between *in utero* and adult exposures. This is likely because all tissues are mitotically active during *in utero* development and therefore susceptible to mutation fixation; in contrast, adult liver and brain have very low mitotic indices. These results show that the placenta does not shelter the embryo from polycyclic aromatic hydrocarbons and that embryonic development is a highly susceptible window for the induction of somatic mosaicism.

Effects of *in utero* BaP exposure on reproductive health

Next, we assessed the effects of *in utero* exposure to BaP on the reproductive health of the offspring. Although no effects occurred at 10 mg/kg/d, we observed dose-related effects on all male reproductive parameters at the other doses. At 40 mg/kg/day, testis weight decreased by 77% (Figure 2A) and sperm concentration decreased >90% (Figure 2B). Computer assisted sperm analysis indicated that *in utero* BaP exposure significantly decreased all of the measured motility parameters (Table 1, Figure 2C). Furthermore, *in utero* exposure to 20 mg/kg/d BaP caused a significant increase (3-fold, $P < 0.0001$) in *lacZ* mutants in sperm (Figure 3A). At 40 mg/kg/d, so few sperm were present in the cauda epididymis that samples required pooling to obtain sufficient DNA for mutation analysis. These pooled samples had significantly higher mutant frequencies than lower doses and controls (Figure 3A; 16-fold, $P = 0.0003$), and the average mutant frequency (46.3×10^{-5}) was 4-fold higher than that seen in sperm of adult males exposed to 100 mg/kg/d ENU (O'Brien et al. 2015), the most potent germ cell mutagen known. This equates to 11-fold higher mutants per mg/kg-bw of BaP than sperm from exposed adults (O'Brien et al. 2016). Mutant frequencies in whole testes (Figure 3B) closely matched those in sperm.

Clonal expansion of mutations and the induction of mosaicism

We used next-generation sequencing to determine the mutation spectra induced by transplacental BaP exposure and assess clonal expansion in both somatic tissues and sperm. We characterized 233 and 648 unique *lacZ* mutations from controls and BaP-treated tissues, respectively. In all four tissues, BaP exposure increased the proportions of *lacZ* mutations that were clonally expanded (Figure 4, Supplementary Table S3). Interestingly, clonality correlates

with the adult replication rate of each tissue, with bone marrow and sperm (proxy for spermatogonial stem cells) showing the highest clonality and having higher adult proliferation rates (19% and 35% dividing cells, respectively; (Edwards and Klein 1961)). Conversely, brain and liver showed the lowest clonality, with adult proliferation rates of neurons being negligible (<0.1% dividing; (Edwards and Klein 1961)), and hepatic cells dividing very rarely (0.53% dividing; (Edwards and Klein 1961)). This suggests that mutations were induced in stem cells during embryonic development and that the chance of detecting cells carrying the same mutation is affected by the rate of stem cell division in that tissue during adulthood.

Analysis of the mutation spectra provided insights into the mechanism(s) of BaP mutagenesis *in utero* and whether these differ among tissues. As BaP mainly induces G→T transversions via mispairing of adducted DNA (Keohavong and Thilly 1992), increased proportions of G→T (as well as G→C transversions and deletions) were observed (Figure 1B). Mutation types were consistent among the three somatic tissues and with the mutation spectrum observed in the bone marrow of adult-exposed mice (Beal et al. 2015). The sperm spectrum was similar except that deletions were not increased (Figure 3C). Thus, the reactive metabolite of BaP (benzo[a]pyrene-7,8-dihydrodiol-9,10-epoxide) was likely active through a similar genotoxic mechanism in fetal tissues as in adults. The presence of the mutational signature of BaP also confirms that the increased proportion of *lacZ* mutations is caused by the reactivity of the chemical itself, and not through induction of genomic instability.

DISCUSSION

We demonstrate that BaP exposure during fetal development causes severe somatic and germ cell consequences in F1 animals, and that tissues originating from all three germ layers, as well as the germ cells, are highly mutagenized in adult offspring. Thus, *in utero* development is a critical window of risk for environmentally induced mutations that can lead to adverse health effects during adult life.

Historically, the discussion of somatic mutations has revolved around their role in tumorigenesis. In light of evidence that the number of lifetime stem cell divisions is a major contributor to cancer formation (Tomasetti and Vogelstein 2015), any first-hit mutations in critical genes taking place *in utero* would increase the likelihood for loss of heterozygosity in the affected locus. Because only three to six mutated driver genes are sufficient to initiate most cancers (Vogelstein et al. 2013), increased numbers of *de novo* mutations could accelerate cancer progression later in life. Furthermore, extrinsic risk factors such as environmental exposures may contribute 70 to 90% of lifetime cancer risk, suggesting – in the context of data presented in this study – that variation in developmental exposure to environmental mutagens plays a meaningful role in determining an individual's risk (Wu et al. 2016). However, because mutations arising during *in utero* development are often observed as widely distributed mosaic variants, they can also result in diseases other than cancer.

Mosaicism in the nervous system is associated with diseases including epilepsy, lissencephaly, hemimegalencephaly, early-onset Alzheimer's, autism spectrum disorders, and others (Poduri et al. 2013; Sanders et al. 2012; Yuen et al. 2015). Normal brains also possess distinct cell lineages with genetic variants that originate during development (Lodato et al.

2015). Here, the brains of mice exposed *in utero* showed a higher mutagenic response at lower doses of BaP than other tissues ($P < 0.01$) providing a novel mode of action for neurological effects induced by chemical exposures. We estimate that BaP-induced mutations increase by one standard deviation in the brain at 0.68 mg/kg-bw for a 10-day exposure (Supplementary Figure S2). This is a relatively small cumulative dose of 6.8 mg/kg-bw. In comparison, cancer induction in mice is estimated to occur at 0.8 mg/kg-bw over two years (a cumulative dose of 584 mg/kg-bw) (Moffat et al. 2015). Therefore, mutations in the developing brain are induced by lower cumulative doses of BaP than those required for adult cancer formation. Coupled with the observation that *in utero* BaP exposures as low as 0.3 mg/kg/d impaired neuronal activity in rats (McCallister et al. 2008), the developing brain demonstrates unprecedented sensitivity to BaP (Chepelev et al. 2015).

Developing germ cells differed in their response from developing somatic tissues in two major aspects: increased sensitivity to cell killing and mutation spectrum. The reduced sperm count in exposed offspring demonstrates increased sensitivity to BaP-induced apoptosis of primordial germ cell precursors (PGCs) via cellular toxicity, consistent with the framework proposed by Rube et al. (Rube et al. 2011). This is supported by a study in *Caenorhabditis elegans* that found somatic cells to be more resistant to DNA damage-induced apoptosis than germ cells (Gartner et al. 2000). Because PGCs were proliferating during the exposure window in this study, PGC toxicity likely reduced the pool of germline stem cells in the offspring, and, consequently, drastically reduced sperm count permanently. Furthermore, the BaP-induced mutation spectrum in sperm was significantly different from other tissues (Fisher's exact, Bonferroni adjusted, $P < 0.001$ compared to liver and brain, $P = 0.097$ compared to bone marrow),

showing fewer deletions. Germline stem cells are thought to be more effective at repairing DNA damage because the integrity of their genome is vital to the faithful replication of DNA for the organism's descendants (Rübe et al. 2011). It is therefore possible that deletions in sperm were under high selective pressure. These findings suggest that developing germ cells are more susceptible to environmental chemicals than adult spermatogonial stem cells, which may partially explain the higher mutation rate in primordial germ cells observed by Rahbari et al. (2015), and that the health effects of *in utero* mutagen exposure impact not only the exposed individual, but future generations as well.

Recent research has suggested that a large proportion of *de novo* mutations in humans are in fact the result of low-level somatic and/or germline mosaicism in the parents (Rahbari et al. 2015). In a similar manner, our study used clonal expansion of independent transgene mutations as a method to quantify mosaicism. Figure 4 shows the relative quantity for each recovered mutation, where the size of the bubble correlates with the number of cells which carried that mutation in a given tissue. The transgenic rodent *in vivo* mutation assay positively selects *lacZ* from cells carrying a mutant copy and enables their relative quantification with NGS; thus, even though the physical distribution of these mutations *in situ* remains unknown, we are effectively able to interrogate very large numbers of cells that could not be feasibly analyzed with single-cell sequencing based on current costs. Rahbari et al. (2015) suggested that many of these low-level mosaic mutations arose spontaneously in the parents during embryogenesis (particularly during expansion of primordial germ cell precursors). Although individual families in their study clearly exhibited different propensities to pass on such mosaic mutations, the underlying cause for the variation remains enigmatic. We propose that one contributor to variation in mutation

burden among individuals is differential exposure to environmental mutagens during development.

The doses used in this study are higher than the levels of BaP to which most individuals in the population would be exposed. The International Agency for Research on Cancer has concluded that dietary exposure to BaP may range as high as 17 $\mu\text{g}/\text{person}/\text{day}$, or 0.28 $\mu\text{g}/\text{kg}/\text{d}$ for a 60 kg human (IARC 2010). This equates to 1.9 $\mu\text{g}/\text{kg}/\text{d}$ in mice after an allometric interspecies correction (USEPA 2011), or 300-fold less than the lowest dose inducing mutations in the brains of developing mice. It should be noted that humans are exposed to complex mixtures of multiple chemicals, through both oral and inhalation routes. Occupational exposures or lifestyle factors may result in highly exposed individuals, and because the pharmacokinetics of these complex mixtures and their interactions with the placenta and fetus are not well known, we believe that further research in this area is warranted.

In summary, this study provides clear evidence that *in utero* exposure to a common environmental pollutant induces somatic mosaicism in tissues originating from all three germ layers and germline mosaicism in the F1 generation. Thus, *in utero* development represents a sensitive window for the genesis of mosaicism via environmental exposures to chemical mutagens, and the potential health consequences apply not only to the developing organisms, but also to their progeny, which can ultimately influence the genetic health of an entire species.

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Table 1. *In utero* exposure to BaP negatively impacts sperm quality in F1 males as measured by computer-assisted sperm analysis.

Parameter	Fraction of control values* [^]		
	10 mg/kg/d	20 mg/kg/d	40 mg/kg/d
Progressive Motility	1.02	0.36	0.02
Path Velocity (VAP)	1.03	0.77	0.19
Progressive Velocity (VSL)	1.04	0.85	0.20
Track Velocity (VCL)	1.01	0.70	0.18
Amplitude Lateral Head (ALH)	1.03	0.66	0.16
Beat Cross Frequency (BCF)	0.98	0.90	0.50
Straightness (STR)	1.00	1.07	0.64
Linearity (LIN)	1.01	1.21	0.76

*bold indicates statistical significance of $P < 0.05$ in ANOVA followed by a Bonferroni post-hoc multiple comparison.

[^] Non-normalized data is available in Supplementary Table S4.

FIGURE LEGENDS

Fig. 1. Male mice exposed transplacentally to BaP during organogenesis exhibited increased mutant frequencies in multiple somatic tissues. Dams were dosed at 0, 10, 20, or 40 mg/kg/d, and mutant frequencies (in 10 week old F1 males) were determined using the *lacZ* transgene mutation assay. (A) *In utero* exposure to BaP induced a significant dose-dependent increase in mutant frequencies in somatic tissues originating from distinct primary germ layers, including the bone marrow (mesoderm), brain (ectoderm), and liver (endoderm). Error bars represent 95% CI. * $P < 0.05$, *** $P < 0.0001$, generalized linear model with quasi-Poisson distribution. (B) Next-generation sequencing of the *lacZ* reporter gene from animals exposed *in utero* shows the well-characterized molecular signature of BaP exposure, visible in the pie charts as an increase in the proportion of G→T mutations. The number of unique *lacZ* mutations sequenced is noted beneath each pie chart.

Fig. 2. *In utero* exposure to BaP significantly decreases multiple reproductive parameters in F1 adult male mice. BaP caused a significant dose-dependent decrease in the testis weight (shown as percentage body weight, A), as well as a drastic decrease in sperm concentration (B) and percentage of motile sperm (C). Liver weight (D) was unaffected. * $P < 0.05$, ANOVA. Error bars represent 95% CI.

Fig. 3. Mutant frequencies were significantly increased in caudal sperm (A) and whole testes (B) of F1 males exposed *in utero* to 20 and 40 mg/kg/d BaP. Mutant frequencies in sperm at 40 mg/kg/d were measured by pooling several DNA samples because of extremely low sperm counts. Error bars represent 95% CI. ** $P < 0.001$, *** $P < 0.0001$, generalized linear model with quasi-Poisson distribution. (C) Next-generation sequencing of the *lacZ* reporter gene from caudal

sperm. BaP exposure (20 and 40 mg/kg/d combined) increased the proportion of G→T and G→C mutations but had no effect on deletions. The number of unique *lacZ* mutations sequenced is noted beneath each pie chart.

Fig 4. Clonal expansion of the mutations characterized in each somatic tissue and caudal sperm. The proportion of mutations that underwent clonal expansion is consistently higher in animals exposed to BaP *in utero*, illustrating the potential for increased mosaicism. As in somatic tissues, the proportion of clonally expanded mutations is higher in BaP-exposed sperm, highlighting the potential for increased germline mosaicism. Compared to exposure in adults (data from Beal et al., 2015 and O'Brien et al., 2016), *in utero* exposure results in a higher degree of clonal expansion. Each circle within a group corresponds to an independent mutation scored, and the area of each circle represents the number of times that mutation was observed per animal.

Figure 1.

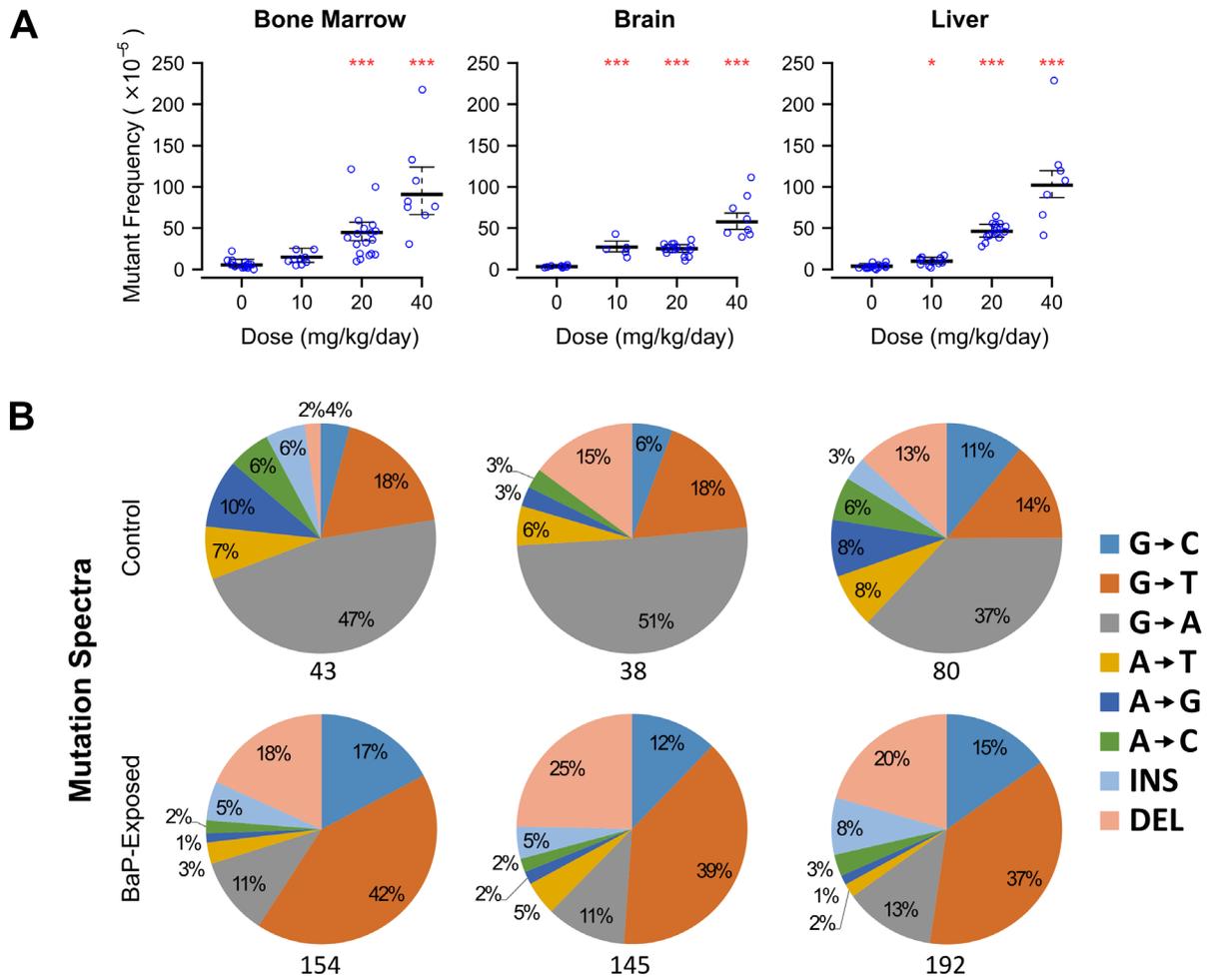


Figure 2.

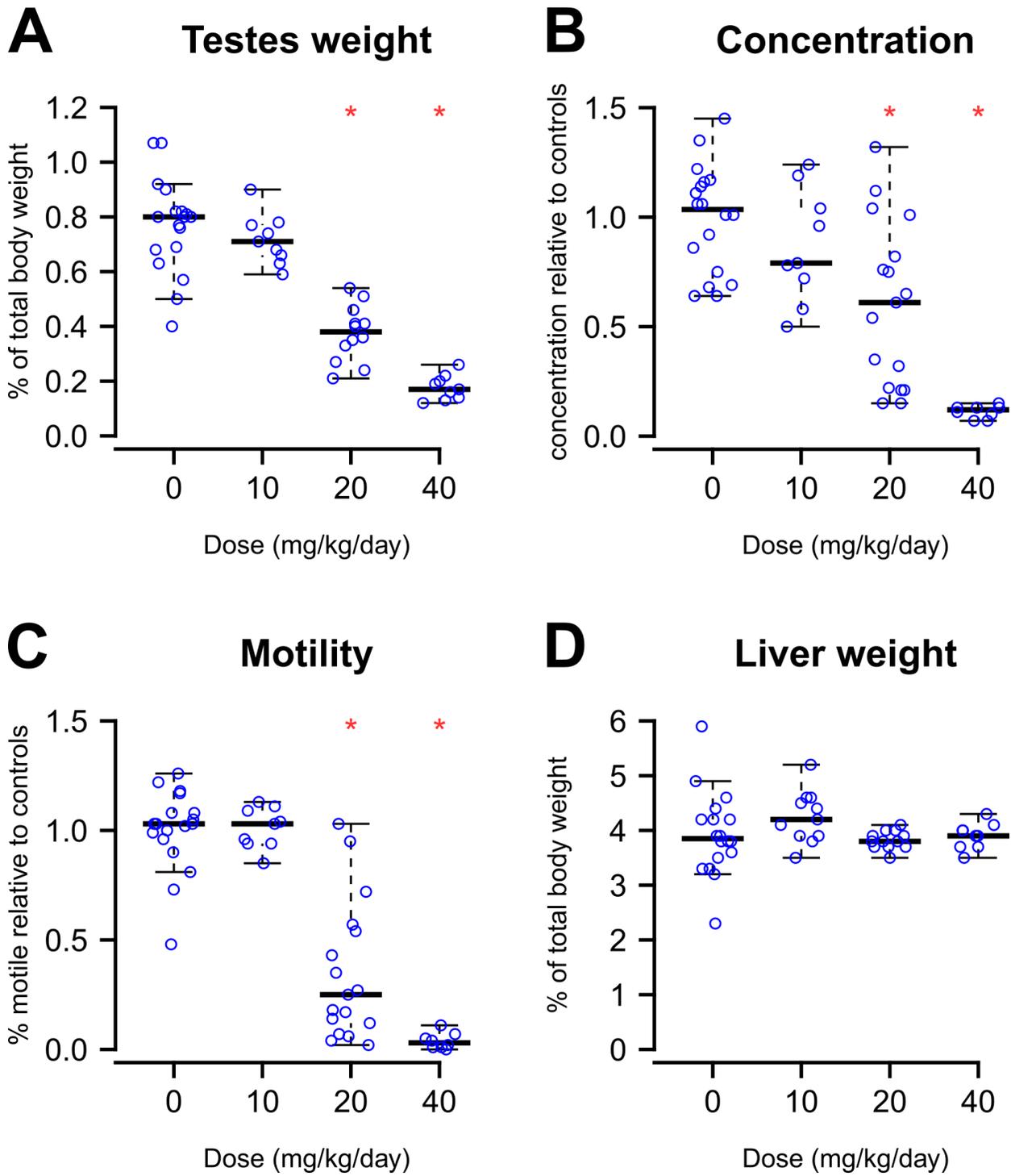


Figure 3.

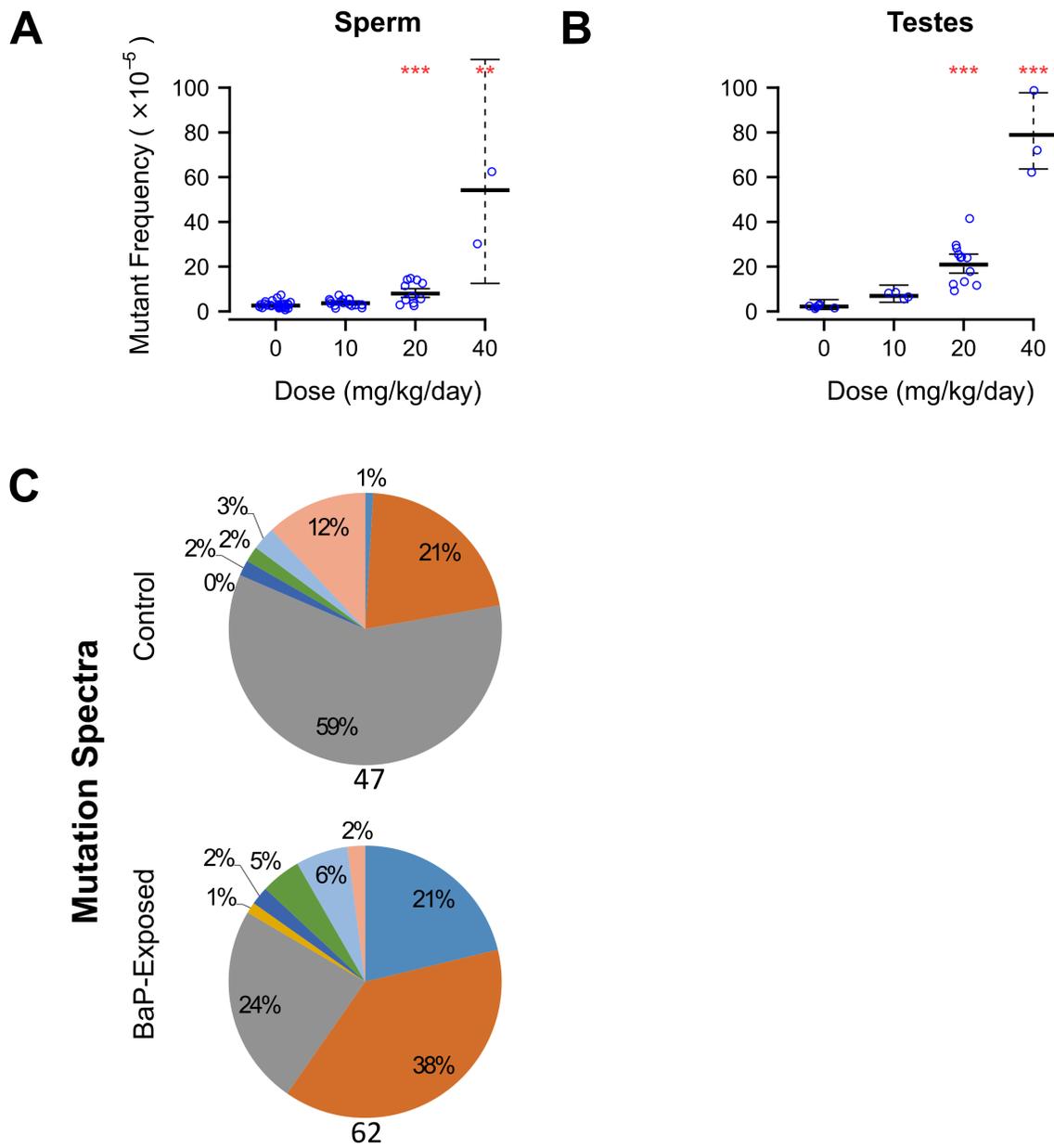


Figure 4.

