Supporting Information

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SI Materials and Methods

Animal Husbandry. Diets were administered 2 wk before mating, and dams remained on the diet throughout pregnancy and lactation. At weaning (21 d of age), all offspring were placed on the control (CTL) diet (Fig. 1). The animal rooms were maintained at 70–72 °F temperature, 40–50% humidity, and on a 12-h light/12-h dark cycle with lights on at 0700 hours and lights off at 1900 hours CST. All animals were fed and provided water on an ad libitum basis. To minimize any background bisphenol A (BPA) exposure, the mice were housed in polypropylene (ASRC colony) or polystyrene (Bond Life Sciences Center colony) cages (7.5" width × 11.75" length × 5.0" height) (Allentown) and provided glass water bottles, with water that had been subjected to a triple carbon filter filtration and shown to be free of BPA contamination. Approximately 1 wk before parturition, the females were placed in individual cages. Once their offspring were born and weaned, select females were paired with another breeder male to provide data over multiple parities. BPA (lot AOHOK CAS: 80-05-7) was obtained from the National Institute of Environmental and Health Sciences, ethinyl estradiol (EE) was purchased from Sigma Chemical (catalog number E4876), and genistein (G) (catalog number G-6055; lot number CH-148) was obtained from LC Laboratories.

Offspring Coat Color Analyses. These classifications were performed by two independent observers with interobserver agreement of 0.9. In total, 2,824 offspring were analyzed with 530 CTL, 426 low-dose BPA, 149 middle-dose BPA, 520 upper-dose BPA, 407 BPA-plus-G, 299 G, and 493 EE pups. To determine whether the classifications performed by each individual equated to quantifiable differences in coat color assignment, a sampling of the later images of mice on the control diet were further analyzed by a digital densitometric method. Although all of the litters were photographed, background and lighting conditions were incompatible with digital analysis of the early litters. The digital assessments were performed solely to determine whether mice that had previously been classified by the two observers as Y1–Y2, Y3, or Y4 conformed to these designations (1). Those that were classified as Y0 (all brown) or Y5 (all yellow) were included as negative controls, but the absence of either yellow or brown fur patterns in these mice prevented them from being included in the statistical analysis. To obtain consistent images, A^{vy}/a offspring were placed in a pipette-tip box (4" length × 5" width × 2.5" height) lined with light blue construction paper (Staples). To minimize glare and shadows, images were obtained under fluorescent lighting with a Sony α330 camera (Sony) placed 18" distance from the mouse. Images were downloaded into the GIMP 2 program (www.gimp.org/) to unify the background and any external colored anatomical features, such as the eyes and ears, that did not contribute to the coat color. The "masked" images were downloaded into the Layer Pilot program (http://layer-pilot.software.informer.com/), which permits the viewer to select regions of "yellow or brown" and yield a single flattened image. These densitometric colored images were analyzed by Adobe Photoshop C5.5 Extended Version (Adobe Systems) so that, with use of the magic wand tool at a tolerance of 30, each section of brown and yellow could be selected separately and measured by using a filter provided by the Fovea Pro, version 3 (Reindeer Graphics) and Adobe Photoshop (Adobe Systems) programs. The text file generated from this document was imported into Microsoft Excel (Microsoft Office 2007) to determine the actual percentage of yellow to brown for each mouse. Examples of flattened images for a Y2 (brown with slight yellow mottling) and Y4 (yellow with slight brown mottling) analyzed by this method are illustrated in Fig. S1.

PCR Analysis to Confirm Genotype Status of Presumptive A^{vy}/a Breeder Males. DNA was isolated from either the tail or testes by using the Qiagen DNeasy kit (Qiagen), and its concentration was measured on an Epoch Microplate Spectrophotometer (BioTek). DNA (20 ng) was PCR amplified by using the A^{vy} forward primer sequence, 5′-AATTTTCAGCCCTATCTTAA-3′, and the reverse primer sequence, 5′-GAGTTTAGCACATACCTTCT-3′. Positive control primers against *Actb* were used to verify that the DNA was intact (2). To confirm the sampling of breeder males that had failed to produce any *a*/*a* (nonagouti) offspring were heterozygous, densitometric analysis of the A^{vy} and *Actb* amplicons was performed by using the Fovea Pro, version 3 (Reindeer Graphics) and Adobe Photoshop (Adobe Systems). After controlling for background, the ratio of these values for each suspect male was compared with those obtained for proven A^{vy}/a males. Nonagouti (*a*/*a*) males were also analyzed as negative controls.

Statistical Analyses. ***Coat color.*** To provide sufficient statistical power to allow comparisons between individual A^{vy} coat color groups and their responses to the seven diets, offspring coat color patterns were broken down into four groups: black (*a*/*a*), brown Y0–Y2 (A^{vy}/a), mottled Y3 (A^{vy}/a), and yellow Y4–Y5 (A^{vy}/a) (Fig. 1). The PROC GLIMMIX procedure was then applied to the model above to allow the analysis of both multinomial data, e.g., on the three groups of A^{vy}/a coat colors, and binomial data when comparing two A^{vy}/a coat colors. When the analysis was based on more than two coat color groups, i.e., multinomial, a cumulative logit link (SAS statement: Link = cLogit) and a multinomial distribution were used (SAS statement: dist = multinomial). When the analysis was based on two offspring coat colors, i.e., binomial, a logit link (SAS statement: Link = Logit) and a binomial distribution were used (SAS statement: dist = binary). The binomial analyses included comparison of brown (Y0–Y2) versus yellow (Y4–Y5), and black (*a*/*a*) to all agouti A^{vy}/a offspring (Y0–Y2, Y3, and Y4–Y5).

Sex ratio. Additional PROC GLIMMIX in SAS analyses were performed to test whether sex ratio (fraction males) was influenced by maternal diet. In this case, the model contained the effect of diet, and the denominator of F was either dam ID or litter effects within diet. A logit link and a binary distribution were applied.

Parity effects. PROC GLIMMIX in SAS was used to determine whether parity had any effect on color pattern (brown, Y0–Y2, versus yellow, Y4–Y5, and nonagouti, *a*/*a*, versus agouti, A^{vy}/a). Because not all treatment groups had more than three parities, wherever possible, these analyses included the first three parities. The middle-dose BPA did not have sufficient brown to yellow A^{vy}/a pups in the third parity to permit these analyses, and both the low and middle doses of BPA did not have sufficient nonagouti (*a*/*a*) to agouti (A^{vy}/a) in the third parity. The linear statistical model included the effects of maternal diet and parity and the interaction of diet by parity. Dam ID or litter effect within diet was the denominator of F to test diet, and the residual mean square was the denominator of F for effects of parity and interaction of diet by parity. If the analysis above was a multinomial distribution, e.g., all three A^{vy}/a coat color groups, pairwise differences were determined by using the ESTIMATE statement in SAS. If the distribution was binary, the pairwise difference used

the LSMEANS statement in SAS (SAS, version 9.2, software; SAS Institute).

Pups born. An analysis of covariance was performed to test if the number of pups born changed between the diets and across parities. The general linear model (GLM) procedure was used, and the model contained the effect of parity and diet, with parity serving as the covariate.

Densitometric analysis of coat color. A one-way ANOVA was performed to test the difference in mottled yellow to mottled brown coat color patterns in the Y1–Y2, Y3, and Y4 control mice whose images had been recorded by digital photography.

χ^2 analysis. For completeness and for consistency with earlier studies (3–5), differences in coat colors across diets was also performed for pups in the Y0–Y2 and Y4–Y5 groups ($n = 1264$). This analysis was achieved by using a 7×2 row-by-column PROC FREQ χ^2 analysis in SAS with the row number corresponding to the seven diets used in the study (Table S2). It should be noted that, in these analyses, the unit was the pup rather than the litter.

Densitometric analysis of the A^{vy} to ACTB amplicon ratio. A one-way ANOVA with the GLM function of SAS and post hoc analysis was performed by using Tukey's test to determine whether there was any difference in this ratio between questionable heterozygous A^{vy} males ($n = 14$), known heterozygous A^{vy} males ($n = 12$), and a/a (black, nonagouti) males ($n = 10$).

1. Ounpraseuth S, et al. (2009) A method to quantify mouse coat-color proportions. *PLoS One* 4(4):e5414.
2. Schroder AL, Pelch KE, Nagel SC (2009) Estrogen modulates expression of putative housekeeping genes in the mouse uterus. *Endocrine* 35(2):211–219.
3. Anderson OS, et al. (2012) Epigenetic responses following maternal dietary exposure to physiologically relevant levels of bisphenol A. *Environ Mol Mutagen* 53(5):334–342.
4. Dolinoy DC, Huang D, Jirtle RL (2007) Maternal nutrient supplementation counteracts bisphenol A-induced DNA hypomethylation in early development. *Proc Natl Acad Sci USA* 104(32):13056–13061.
5. Dolinoy DC, Weidman JR, Waterland RA, Jirtle RL (2006) Maternal genistein alters coat color and protects Avy mouse offspring from obesity by modifying the fetal epigenome. *Environ Health Perspect* 114(4):567–572.

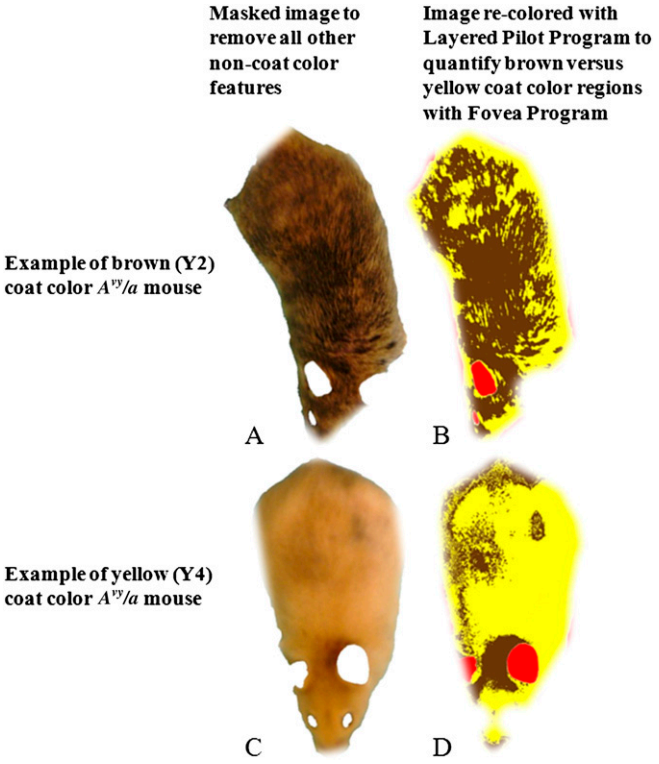

Fig. S1. Examples of densitometric analysis of coat color for brown (*A* and *B*) and yellow (*C* and *D*) coat color A^{vy}/a offspring mice.

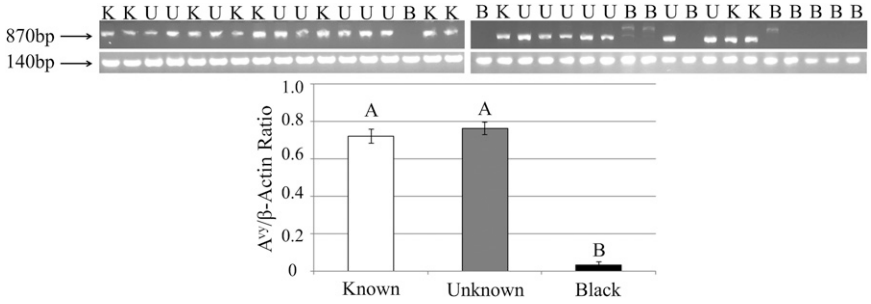

Fig. S2. Gel electrophoresis images of A^{vy} (*Upper*) and *Actb* (*Lower*; internal control) genotyping. B, Black (*a*/*a*, nonagouti) coat color males (negative control); K, known heterozygous males; U, unknown/questionable males. After controlling for the background color of the gel pictures, densitometric analysis showed no difference between known heterozygous males (ratio range, 0.52–0.89) and questionable heterozygous males (ratio range, 0.52–0.94). There was a significant difference between black coat color (*a*/*a*) (ratio range, 0–0.12) versus known and questionable heterozygous (A^{vy}/*a*) males ($P < 0.0001$). As indicated on the Mutant Mouse Regional Resource Center website (www.mmrrc.org/strains/375/ctr_protocol.pdf), in some instances a nonspecific higher doublet can be observed in *a*/*a*, nonagouti mice, as shown in 3 out of the 10 black (*a*/*a*) mice tested. Data are represented as mean ± SEM, and different superscripts represent significant differences ($P < 0.0001$) among groups. $n = 12$ known heterozygous males, 14 questionable heterozygous males, and 10 black (*a*/*a*) males.

Table S1. Effects of maternal diet endocrine disruption on litter size and total number of male and female offspring

Maternal diet	Litter size (mean ± SEM)	Total no. of male offspring	Total no. of female offspring	Fraction male pups	No. of male biased litters/total no. of litters
Control	6.4 ± 0.3^{a}	303	227	$0.57^{a,*}$	43/84
Low-dose BPA	$6.5 \pm 0.4^{a,b}$	218	208	$0.51^{a,b}$	29/67
Middle-dose BPA	5.5 ± 0.6^{a}	76	73	$0.48^{a,b}$	14/27
Upper-dose BPA	$6.8 \pm 0.4^{a,b}$	267	253	$0.51^{a,b}$	36/76
Upper-dose BPA + G	$6.6 \pm 0.4^{a,b}$	214	193	$0.53^{a,b}$	30/61
G	$6.5 \pm 0.5^{a,b}$	137	162	0.46^{b}	16/46
EE	7.5 ± 0.4^{b}	271	222	$0.55^{a,*}$	38/65

Values with different superscripts (^{a,b}) differ from each other ($P < 0.05$).
*These values differ from the expected 1:1 ratio.

Table S2. χ^2 analysis with only brown (Y0–Y2) and yellow (Y4–Y5) A^{vy}/a offspring to examine for differences in overall expression patterns based on maternal diet

Maternal diet*	Brown (Y0–Y2)	Yellow (Y4–Y5)	Total frequency percent
Control	104	118	222
	8.2	9.3	17.5
	46.8	53.1	
	18.1	17.1	
Low-dose BPA	75	121	196
	5.9	9.6	15.5
	38.3	61.7	
	13.1	17.5	
Middle-dose BPA	30	44	74
	2.4	3.5	5.9
	40.5	59.5	
	5.2	6.4	
Upper-dose BPA	94	101	195
	7.4	8.0	15.4
	48.2	51.8	
	16.4	14.6	
Upper-dose BPA + G	108	110	218
	8.5	8.7	17.2
	49.5	50.5	
	18.8	15.9	
G	47	77	124
	3.7	6.1	9.8
	37.9	62.1	
	8.2	11.2	
EE	116	119	235
	9.2	9.4	18.6
	49.4	50.6	
	20.2	17.2	
Total no. of pups column	574	690	1,264
Percent	45.4	54.6	100

χ^2 statistics: df = 6; value, 11.3434; probability, 0.0783.
*For each diet group, the first row is frequency or number, the second row is the percent for each category, the third row is the row percent going across, and the fourth row is the column percent.

Table S3. Comparison between diets for percentage of brown (Y0–Y2) to yellow (Y4–Y5) offspring

Maternal diet 1	Maternal diet 2	*P* value
CTL	Low-dose BPA	0.19
CTL	Middle-dose BPA	0.38
CTL	Upper-dose BPA	0.92
CTL	Upper-dose BPA + G	0.84
CTL	G	0.19
CTL	EE	0.77
Low-dose BPA	Middle-dose BPA	0.87
Low-dose BPA	Upper-dose BPA	0.24
Low-dose BPA	Upper-dose BPA + G	0.14
Low-dose BPA	G	0.85
Low-dose BPA	EE	0.11
Middle-dose BPA	Upper-dose BPA	0.43
Middle-dose BPA	Upper-dose BPA + G	0.31
Middle-dose BPA	G	0.76
Middle-dose BPA	EE	0.27
Upper-dose BPA	Upper-dose BPA + G	0.77
Upper-dose BPA	G	0.22
Upper-dose BPA	EE	0.70
Upper-dose BPA + G	G	0.14
Upper-dose BPA + G	EE	0.94
G	EE	0.11

Table S4. Comparison of brown (Y0–Y2) versus yellow (Y4–Y5) coat color A^{vy}/a offspring across parity

Diet (total litters born in each parity)	Brown (Y0–Y2) coat color offspring in individual parities, %	Parity comparison		Estimated difference, mean ± SEM	*P* value
CTL					
Parity 1 = 37	45.7	1	2	−0.33 ± 0.37	0.37
Parity 2 = 19	53.9	1	3	−0.93 ± 0.68	0.17
Parity 3 = 8	68.1	2	3	−0.60 ± 0.69	0.39
Low-dose BPA					
Parity 1 = 21	46.6	1	2	0.50 ± 0.43	0.24
Parity 2 = 13	34.5	1	3	−0.22 ± 0.51	0.67
Parity 3 = 10	52.0	2	3	−0.72 ± 0.54	0.18
Middle-dose BPA	N/A	N/A	N/A	N/A	N/A
Upper-dose BPA					
Parity 1 = 35	50.0	1	2	0.65 ± 0.40	0.1
Parity 2 = 21	34.4	1	3	−0.81 ± 0.49	0.1
Parity 3 = 10	69.3	**2**	**3**	**−1.46 ± 0.51**	**0.004**
Upper-dose BPA +G					
Parity 1 = 26	50.1	1	2	−0.33 ± 0.38	0.38
Parity 2 = 15	58.4	1	3	0.51 ± 0.41	0.22
Parity 3 = 9	37.6	**2**	**3**	**0.84 ± 0.42**	**0.05**
G					
Parity 1 = 15	32.4	1	2	−0.37 ± 0.53	0.48
Parity 2 = 11	41.0	1	3	−0.24 ± 0.54	0.65
Parity 3 = 9	37.9	2	3	0.13 ± 0.58	0.82

N/A, not applicable. The effects of parity on offspring coat color in the middle dose BPA group could not be analyzed, as there was insufficient number of offspring born in later parities for this group. Bold numbers are significantly different with the indicated *P* value.

Table S5. Comparison of *a/a* versus A^{vy}/a offspring across parities

Diet (*n* = no. of litters)	*a/a* offspring at individual parities, %	Parity comparison		Estimated difference, mean ± SEM	*P* value
CTL					
Parity 1 (37)	41.8	1	2	−0.21 ± 0.25	0.37
Parity 2 (19)	46.9	1	3	−0.11 ± 0.35	0.75
Parity 3 (8)	44.3	2	3	0.10 ± 0.37	0.78
Low-dose BPA	N/A	N/A	N/A	N/A	N/A
Middle-dose BPA	N/A	N/A	N/A	N/A	N/A
Upper-dose BPA					
Parity 1 (35)	54.3	1	2	0.11 ± 0.23	0.62
Parity 2 (21)	51.2	1	3	0.29 ± 0.28	0.30
Parity 3 (10)	46.7	2	3	0.18 ± 0.29	0.54
Upper-dose BPA +G					
Parity 1 (26)	37.3*	1	2	0.25 ± 0.28	0.38
Parity 2 (15)	31.8*	1	3	0.95 ± 0.38	**0.01**
Parity 3 (9)	18.6*	2	3	0.70 ± 0.38	0.06
G					
Parity 1 (15)	56.8	1	2	0.33 ± 0.34	0.33
Parity 2 (11)	47.0	1	3	0.68 ± 0.37	0.07
Parity 3 (9)	40.4	2	3	0.35 ± 0.41	0.40
EE					
Parity 1 (35)	43.0	1	2	0.47 ± 0.26	0.07
Parity 2 (17)	32.0*	1	3	0.29 ± 0.31	0.34
Parity 3 (9)	35.9	2	3	−0.18 ± 0.34	0.60

N/A, not applicable. The effects of parity on offspring coat color in the low- and middle-dose BPA groups could not be analyzed, as there was insufficient number of *a/a* compared with A^{vy}/a offspring born in later parities. Bold numbers are significantly different with the indicated *P* value.
*Indicates parities that differed from the expected 1:1 ratio of *a/a* to A^{vy}/a mice.

Table S6. Number of litters and pups in previous published studies that examined the effects of developmental exposure to BPA and G in A^{vy}/a mice

Ref(s).	Maternal diet (no. of litters)	Offspring born	A^{vy}/a offspring analyzed
1	AIN control (n = 15)	NR	52
	Genistein diet (n = 12)	NR	44
2	AIN control (n = 16)	120	60
	50 mg BPA/kg fw (n = 17)	124	73
	50 mg BPA/kg fw BPA + methyl diet (n = 14)	95	54
	BPA + G (250 mg/kg fw) (n = 13)	81	39
3	AIN control (n = 11)	86	39
	50 ng BPA/kg fw (n = 14)	107	48
	50 μg BPA/kg fw (n = 9)	67	32
	50 mg BPA/kg fw (n = 13)	91	45

NR, not reported.

1. Dolinoy DC, Weidman JR, Waterland RA, Jirtle RL (2006) Maternal genistein alters coat color and protects Avy mouse offspring from obesity by modifying the fetal epigenome. *Environ Health Perspect* 114(4):567–572.
2. Dolinoy DC, Huang D, Jirtle RL (2007) Maternal nutrient supplementation counteracts bisphenol A-induced DNA hypomethylation in early development. *Proc Natl Acad Sci USA* 104 (32):13056–13061.
3. Anderson OS, et al. (2012) Epigenetic responses following maternal dietary exposure to physiologically relevant levels of bisphenol A. *Environ Mol Mutagen* 53(5):334–342.