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Research Article

Bisphenol AF and Bisphenol F Induce Similar Feminizing Effects in Chicken Embryo Testis as Bisphenol A

Anna Mentor [*,†] Mimmi Wänn,[*] Björn Brunström,[*,†] Maria Jönsson,[*,†] and Anna Mattsson [*,†,1]

[*]Department of Environmental Toxicology, Uppsala University, SE-752 36 Uppsala, Sweden and [†]Centre for Reproductive Biology in Uppsala (CRU), Uppsala, Sweden

[1]To whom correspondence should be addressed at E-mail: anna.mattsson@ebc.uu.se.

ABSTRACT

The plastic component bisphenol A (BPA) impairs reproductive organ development in various experimental animal species. In birds, effects are similar to those caused by other xenoestrogens. Because of its endocrine disrupting activity, BPA is being substituted with other bisphenols in many applications. Using the chicken embryo model, we explored whether the BPA alternatives bisphenol AF (BPAF), bisphenol F (BPF), and bisphenol S (BPS) can induce effects on reproductive organ development similar to those induced by BPA. Embryos were exposed *in ovo* from embryonic day 4 (E4) to vehicle, BPAF at 2.1, 21, 210, and 520 nmol/g egg, or to BPA, BPF, or BPS at 210 nmol/g egg and were dissected on embryonic day 19. Similar to BPA, BPAF and BPF induced testis feminization, manifested as eg testis-size asymmetry and ovarian-like cortex in the left testis. In the BPS-group, too few males were alive on day 19 to evaluate any effects on testis development. We found no effects by any treatment on ovaries or Müllerian ducts. BPAF and BPS increased the gallbladder-somatic index and BPAF, BPF and BPS caused increased embryo mortality. The overall lowest-observed-adverse-effect level for BPAF was 210 nmol/g egg based on increased mortality, increased gallbladder-somatic index, and various signs of testis feminization. This study demonstrates that the BPA replacements BPAF, BPF, and BPS are embryotoxic and suggests that BPAF is at least as potent as BPA in inducing estrogen-like effects in chicken embryos. Our results support the notion that these bisphenols are not safe alternatives to BPA.

Key words: bisphenols; BPAF; BPF; BPS endocrine disruption; chicken; development; testis.

Bisphenol A (BPA) is a high production volume chemical that is classified as an endocrine disrupting compound and a reproductive toxicant (Rochester, 2013). The widespread use of BPA has led to widespread environmental contamination and exposure of humans and wildlife (Corrales *et al.*, 2015). The extensive exposure to BPA together with adverse effects observed in experimental and epidemiological studies have led to increasing use of replacement bisphenols, all of which are far less studied than BPA (Pelch *et al.*, 2019a).

BPA exposure of rodents during early development causes long-term adverse effects such as altered gonad morphology in both sexes as well as pathological changes of uterus and oviducts in females (Newbold *et al.*, 2007; Williams *et al.*, 2014). In egg-laying vertebrates such as the zebrafish, various amphibian species, and the chicken, BPA exposure during reproductive organ differentiation resulted in altered testis morphology, impaired sperm function, and even male-to-female sex reversal (Berg *et al.*, 2001; Chen *et al.*, 2017; Kloas *et al.*, 1999; Tamschick

et al., 2016). In birds, *in ovo* exposure to BPA has been demonstrated to cause estrogen-like effects, ie feminization of the left testis (ovotestis) and decreased ovarian cortex thickness in chicken embryos, as well as malformations of the Müllerian ducts (embryonic oviducts) in female quail embryos (Berg *et al.*, 2001; Jessl *et al.*, 2018b). Disruption of estrogen and androgen signaling is thought to be major mechanisms for BPA's interference with sexual differentiation and development (Acconcia *et al.*, 2015; Kojima *et al.*, 2019).

Bisphenol AF (BPAF), bisphenol F (BPF), and bisphenol S (BPS) have been introduced as alternatives to BPA. These and other BPA analogs are found in the environment and in human specimens (Pelch *et al.*, 2019a). BPAF, BPF, and BPS have been shown to act as agonists on the human nuclear estrogen receptors ERα and ERβ and as antagonists on the androgen receptor (AR) in various *in vitro* assays with higher, similar, or lower potency compared with BPA (Kojima *et al.*, 2019; Pelch *et al.*, 2019b; Rosenmai *et al.*, 2014). Only a few studies have elucidated effects of BPAF, BPF, or BPS on reproductive organ development. So far the results suggest that the effects are similar to those by BPA and that also additional effects can occur (Eladak *et al.*, 2015; Li *et al.*, 2016; Shi *et al.*, 2017, 2018; Ullah *et al.*, 2019; Yang *et al.*, 2018).

Birds and mammals share many of the cellular and molecular mechanisms that regulate sex differentiation of the reproductive organs, but there are also important differences. In most avian species, the female reproductive tract develops asymmetrically in that the ovary and Müllerian duct on the right side regress during embryogenesis whereas those on the left side differentiate and develop into functional organs (Romanoff, 1960). In males, the gonads develop into bilateral testes and the Müllerian ducts regress. The female differentiation is directed by gonadal estrogens, making the avian embryo sensitive to compounds that interfere with estrogen signaling (Scheib, 1983). We and others have shown that exposure of bird embryos to estrogenic compounds causes feminization of gonads in males and retention and malformation of the Müllerian ducts/oviducts in both males and females (Berg *et al.*, 1999; Fry and Toone, 1981; Intarapat *et al.*, 2014; Jessl *et al.*, 2018b; Mattsson *et al.*, 2011). In the chicken model, disrupted sex organ differentiation provides a sensitive and relevant marker for estrogen exposure which can be studied in embryos without the influence of eg, maternal toxicity and without having to sacrifice animals at more advanced developmental stages (Berg *et al.*, 1998).

To avoid regrettable BPA substitutions, it is imperative that the replacement alternatives are thoroughly evaluated regarding toxicological properties. However, *in vivo* data on effects by bisphenols other than BPA on reproductive organ development is limited and virtually absent for avian species. We hypothesized that since the BPA replacements BPAF, BPF, and BPS show estrogenic activity *in vitro* they may also, just like BPA, disrupt testis and Müllerian duct development in bird embryos. To test our hypothesis, we exposed chicken embryos *in ovo* to BPA, BPAF, BPF, or BPS at an equimolar dose and then studied the degree of regression/morphology of the Müllerian ducts and analyzed gross and histological testis morphology. We also established dose-response relationships for BPAF.

MATERIALS AND METHODS

Exposure chemicals. Bisphenol AF (hexafluorobisphenol A; CAS: 1478-61-1; ≥ 97% purity), BPA (CAS: 80-05-7; ≥ 99% purity), BPF (bis(4-hydroxyphenyl)methane; CAS: 620-92-8; ≥ 98% purity), BPS (4,4′-sulfonyldiphenol; CAS: 80-09-1; ≥ 98% purity), and dimethyl sulfoxide (DMSO; CAS: 67-68-5; ≥ 95% purity) were purchased from Sigma-Aldrich, St Louis, Missouri. Exposure solutions were prepared by dissolving the bisphenols in DMSO.

Study design. The chicken embryo experiments were approved by the Uppsala Ethical Committee for Research on Animals (permit number C 90/15) and carried out in accordance with guidelines by the Swedish Board of Agriculture. Facilities for egg incubation and embryo dissection were approved by the Swedish Board of Agriculture (permit number 5.2.18-11059).

Four separate experiments were carried out. In the first 3 experiments (experiments 1–3), chicken embryos were exposed to BPAF at various doses (2.1, 21, 210, and 520 nmol/g egg) or to vehicle (DMSO). In experiment 4, embryos were exposed to BPAF, BPA, BPF, or BPS at an equimolar dose (210 nmol/g egg) or to DMSO. Doses (nmol/g egg and μg/g egg) and number of embryos examined in the different experiments are given in Table 1. The BPAF experiments 1 and 2 were initial studies with the aim of finding appropriate doses, and therefore all endpoints were not studied in these experiments (eg testis histology and liver-somatic index, LSI). In experiment 3, all doses were covered and all endpoints were included. To make the best possible use of available experimental results, data from experiments 1–3 were merged for endpoints that did not differ between the experiments (see Statistics section). The dose of BPAF, BPA, BPF, and BPS in experiment 4 was chosen based on the results from experiments 1–3; ie a dose where BPAF induced specific effects without causing substantial mortality.

Egg incubation and in ovo exposure. For experiments 1–3, fertilized chicken eggs (*Gallus gallus domesticus*) were purchased from Ova Production AB, Vittinge, Sweden. For experiment 4, eggs were instead purchased from Håtunalab AB, Bro, Sweden because Ova Production AB ceased selling eggs for research. For each experiment, 18–64 eggs were randomly selected and weighed to estimate the mean egg weight within the experiment; this value was used to calculate injection doses in the experiment. The eggs were incubated horizontally at 37.2°C–38.0°C and 60%–70% relative humidity and were turned automatically every 4 h. The day the eggs were placed in the incubator was defined as embryonic day zero (E0).

Sexual differentiation of the gonads initiates around E3.5 and the sexes can be distinguished in histological sections of the gonads around E6.5 (Smith and Sinclair, 2004). To expose the embryos during gonadal differentiation, the test substances were injected into the air sac of fertilized eggs on E4. Before injection, eggs were candled and those containing viable embryos were randomly allocated to the different treatment groups. The blunt end of each egg was wiped with ethanol and a small hole was drilled through the shell above the air sac. The injection solution (20 μl) was then deposited onto the inner shell membrane in the air sac using a Hamilton syringe with a disposable needle. The egg was immediately placed horizontally following injection and the hole was sealed with melted paraffin wax. Eggs were marked with coded ID numbers. When all eggs in an exposure group had been injected they were returned to random locations in the incubator.

Embryo dissection and sampling. Embryos were dissected on E19, which is 2 days before anticipated hatching. At this developmental stage, control females and males are easily distinguishable by the morphology of their reproductive organs. The genetic sex of all exposed females and a few randomly selected

Table 1. Substances, Doses, Mortality, and Number of Dissected Chicken Embryos in Experiments 1–4

Experiment	Substance	Dose (μg/g egg)	Dose (nmol/g egg)	Dead/Total No of Embryos (%)	Dissected Embryos (Females + Males)
1, 2, 3	Control	0	0	3/50 (6)	40 (22 + 18)
2, 3	BPAF	0.7	2.1	1/33 (3)	32 (15 + 17)
1, 2, 3	BPAF	7	21	8/45 (18)	36 (21 + 15)
3	BPAF	70	210	12/23 (52)***	11 (3 + 8)
1, 3	BPAF	175	520	23/34 (68)***	11 (5 + 6)
4	Control	0	0	9/37 (24)	28 (15 + 13)
4	BPAF	70	210	31/47 (66)***	16 (6 + 10)
4	BPA	48	210	5/27 (19)	22 (6 + 16)
4	BPF	42	210	25/47 (53)**	22 (13 + 9)
4	BPS	53	210	16/28 (57)**	12 (9 + 3)

Control: Embryos exposed to vehicle (DMSO) only. Exposure groups were compared with the control using Fisher's exact test with Benjamini-Hochberg correction. ***$p \leq .001$, **$p \leq .01$.

males was confirmed using a PCR-based method (Fridolfsson and Ellegren, 1999). The phenotypic females were genetically sexed to make sure that they were not genetic males that had been feminized by the treatment. Embryos were immediately euthanized by decapitation. Body weight (all experiments), liver weight (experiments 2–4), and gallbladder weight (experiment 4) were recorded. The LSI was calculated as 100 × liver weight/body weight. The gallbladder-somatic index was calculated as 1000 × gallbladder weight/body weight. A piece of liver was sampled for determination of genetic sex. When present, length of the right Müllerian duct was measured using a digital slide caliper (experiments 2–4). Frequencies of males with one or both Müllerian ducts retained were recorded. Gonads and Müllerian ducts were visually inspected under a stereo microscope and any abnormalities were noted. In particular, the appearance of the left testicle was observed to determine whether it was ovary-like in size and shape. Gonads were photographed *in situ* through a stereo microscope with a digital camera (Canon EOS 100D). In experiments 3 and 4, the left testis was excised and processed for histological evaluation as described below. Four control ovaries were included for comparison. Dissection of embryos from different treatment groups was performed in a randomized order. The dissection and all measurements (except body and liver weight) and evaluations were performed without knowledge of treatment group.

Gonad size determination. The size of the left and right gonad in males and of the left ovary in females was determined from the photos taken *in situ* using the open source software ImageJ (National Institute of Health) (Schneider *et al.*, 2012). The relative gonad size was defined as the area of the gonad in the photo divided by the mean area of the ovary in the controls within each experiment. In experiment 1, the surface area was measured in pixels without scaling. In experiments 2–4 the gonads were photographed together with a millimeter scale and the measurements were scaled accordingly. Scaling and normalization were done to reduce potential variation in camera zoom and distance between specimen and microscope objective within and between experiments.

Gonad histology. Left testes from males in experiments 3 and 4 were evaluated histologically. For comparison, 4 control ovaries were also processed and examined. The histological processing and evaluation was performed as described by Berg *et al.* (1999) with some modifications. Gonads were fixed in phosphate-buffered formalin (4% formaldehyde in 0.1 M phosphate buffer, pH 7.4; v/v) and thereafter dehydrated by treatment with ethanol in a series of increasing concentration (70%, 95%, and absolute ethanol; v/v) followed by soaking in xylene. The dehydrated specimen was embedded in Technovit 7100 (Heraeus Kulzer, Hanau, Germany). Cross sections (2 μm) were taken at 3 different levels of the gonad; at approximately 25%, 50%, and 75% of its length (total length ca 5 mm). Three sections were collected from each level and were mounted on a Superfrost glass slide. Sections were stained with hematoxylin and eosin, dehydrated with ethanol (95% and absolute; v/v), and soaked in xylene before covered with a coverslip. One section from each of the three levels was photographed and then evaluated. In experiment 3, the sections were photographed at 10-fold magnification using a Leica Leitz DMRXE microscope with a DFC550 camera and the software Leica Application Suite 4.2.0 (Leica Microsystems, Wetzlar, Germany). In experiment 4, the sections were scanned at 40-fold magnification with a NanoZoomer slide scanner and the obtained images were viewed and exported using the NDP.view 2 application (Hamamatsu Photonics, Hamamatsu City, Japan). The histological evaluations were performed with coded ID numbers of the sections and without knowledge of treatment group. Deviations from a normal histological structure of the testis were noted. Signs of feminization that were searched for included presence of a thickened ovary-like cortex, clusters of oocyte-like germ cells in meiotic prophase beneath the superficial epithelium, and presence of lacunae in the medulla. Sections with at least 10 oocyte-like cells in clusters were noted. Shape of the cells in the superficial epithelium was noted, ie squamous, cuboidal, or columnar epithelium. The cortex area was defined as the area of the superficial epithelium and any underlying ovarian cortex-like tissue. The total gonad section area and the area comprising cortex were measured using ImageJ. The fraction of the total area consisting of cortex was determined for each of the three tissue levels and then the average of these values was calculated. The diameter of the testicular tubules was on average determined at 30 locations (selected by applying a 0.02 mm^2 grid to the image and selecting the tubules closest to each crossing point of the grid) in each of the three tissue levels. An average value of the testicular tubule diameter was calculated for each of the analyzed males.

Statistics. To analyze whether there were any statistically significant differences between the control groups in the three first experiments with BPAF (experiments 1–3), each control group was compared with the other control groups using 1-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparisons post hoc test. For endpoints that showed differences

between the control groups, each treatment group was compared with the control group within the same experiment. If the controls did not differ, the data sets from the three experiments were merged and each treatment group was compared with the merged control group. Experiment 4 included BPAF, BPA, BPF, and BPS, and was analyzed separately from experiments 1–3; the treatment groups in experiment 4 were compared with the control group in that experiment. Continuous responses measured in both females and males (eg body and organ weights) were first analyzed regarding sex differences and interaction between sex and treatment using 2-way ANOVA followed by Bonferroni's multiple comparisons post hoc test. In cases where significant sex differences or interaction between treatment and sex were found, females and males were analyzed separately and otherwise they were combined. Following this assessment, each treatment group was compared with the control using 1-way ANOVA and Dunnett's multiple comparisons post hoc test. D'Agostino & Pearson omnibus normality test was used for assessing normality. Data that deviated from normal distribution within groups or showed differences in variance between groups, even after transformation, was analyzed using Kruskal-Wallis test followed by Dunn's multiple comparisons post hoc test. Fisher's exact test (1-tailed) with Benjamini-Hochberg corrected p values was used for comparing quantal data, eg mortality and frequencies of ovotestis. Differences were considered statistically significant if $p \leq .05$. Statistical analyzes were performed using GraphPad Prism 5 (GraphPad Software, Inc, San Diego, California) with the exception of the estimated median lethal dose (LD50), which was computed using the drc package in R (software version 3.5.2 with the RStudio version 1.1.463).

RESULTS

Mortality, Body Weight, Liver-somatic Index, and Gallbladder Weight

The mortality rates are shown in Table 1. The control mortality rates in experiments 1–3 were low; in total 3 out of 50 controls (6%) died before termination of the experiments on E19. Exposure to BPAF caused a dose-dependent increase in mortality. At the two highest BPAF doses (210 and 520 nmol/g egg), the mortality rate was significantly increased compared with that in the control. The estimated median lethal dose, LD50, with 95% confidence interval for BPAF in the dose-response experiments (experiments 1–3) was 185 nmol/g egg (100–344 nmol/g egg). In experiment 4, the mortality rate in the control was 24%, which is high but still below the suggested validity criterion of 30% in vehicle-exposed chicken embryos (Jessl *et al.*, 2018b). The mortality rate was significantly increased compared with the control mortality in the BPAF, BPF, and BPS groups, but not in the BPA group, in experiment 4.

Body weights and liver-somatic indices from the dose-response experiments with BPAF are listed in Supplementary Table 1. The body weight of control animals was significantly higher in experiment 3 than in experiment 1 and the LSI in control animals was higher in experiment 3 than in experiment 2 (not measured in experiment 1). The cause of these differences is not obvious, but could possibly be variations in incubation temperature or age of the laying hens. Because of these differences among control groups, each experiment was analyzed separately for these endpoints. No effect by treatment on body weight was found. The LSI was significantly reduced by BPAF at 210 nmol/g egg ($p < .001$) but not at the higher dose (520 nmol/g egg).

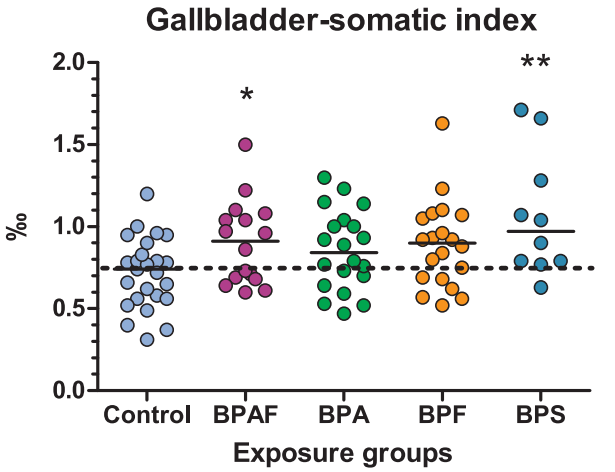

Figure 1. Gallbladder-somatic index of chicken embryos of both sexes in experiment 4. Chicken embryos were exposed to vehicle (DMSO; Control) or bisphenols at 210 nmol/g egg from E4 and were dissected on E19. Each individual is represented by a circle and the solid lines indicate the median values for each group. The dashed line indicates the control median. Following log-transformation, each group was compared with the control using 1-way ANOVA and Dunnett's multiple comparisons test. $^{*}p \leq .05$, $^{**}p \leq .01$.

Supplementary Table 2 presents body weights and liver-somatic indices from experiment 4. Body weight was not significantly affected by treatment in this experiment. As in experiment 3, the median (and mean) LSI was numerically lower in the group exposed to 210 nmol/g egg of BPAF than in the control group; however, in this experiment the difference was not statistically significant. The gallbladder-somatic index was significantly increased by BPAF and BPS at 210 nmol/g egg compared with the control group (Figure 1).

Müllerian Ducts

All females had a full-length left Müllerian duct that appeared morphologically similar between controls and treated individuals. In a vast majority of the females, the morphology of the right-side Müllerian duct did not appear to be affected by treatment; it was largely regressed with typically only a few millimeters of the caudal end remaining in females of all groups. The length of the right Müllerian duct was measured in females in the BPAF dose-response experiments 2 and 3 (Figure 2A) and in experiment 4 (Figure 2B). The length in the control group differed between experiments 2 and 3; therefore the experiments were analyzed separately. In the group exposed to BPAF at 2.1 nmol/g egg in experiment 3, there was one embryo with a remarkably long right Müllerian duct (approximately 20 mm long compared with 5.6 ± 0.8 mm in the control). However, there was no statistically significant effect in any of the treatment groups when compared with their corresponding control group. The Müllerian ducts were completely regressed in all males.

Gonad Gross Morphology

The gross morphology of the gonads was initially evaluated during dissection and the assessment was later confirmed, without knowledge of treatment group, using photos taken *in situ*. At the examined developmental stage (E19), the left ovary is wider and more flat than a testis and these organs can easily be distinguished in control animals. Gonads of a control male

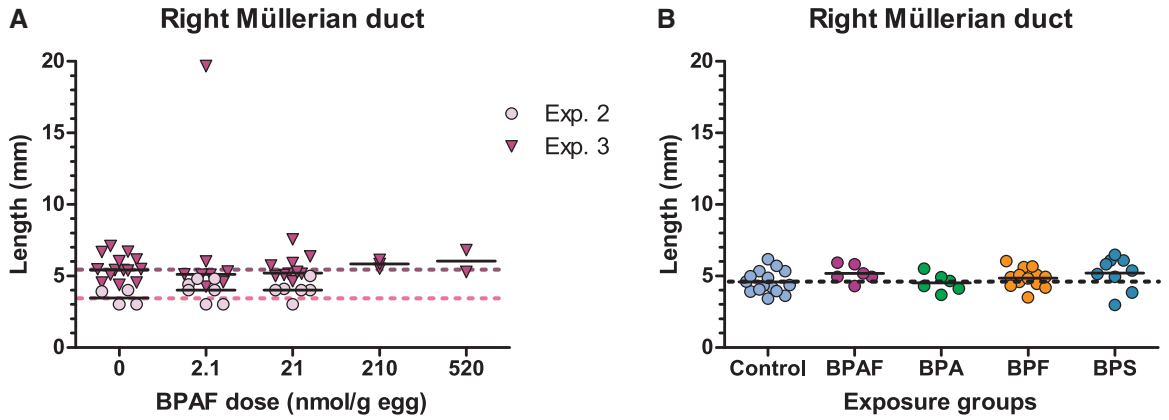

Figure 2. Length of the regressing right Müllerian duct in female chicken embryos. A, BPAF experiments 2 and 3, which were analyzed separately. B, Experiment 4 with BPAF, BPA, BPF, and BPS at 210 nmol/g egg. Chicken embryos were exposed to vehicle (DMSO) or bisphenols from E4 and were dissected on E19. Each data point represents one individual, and the solid lines show the median value for each experiment and treatment group. The dashed lines show the control medians in the different experiments. There was no significant effect of treatment in either of the experiments.

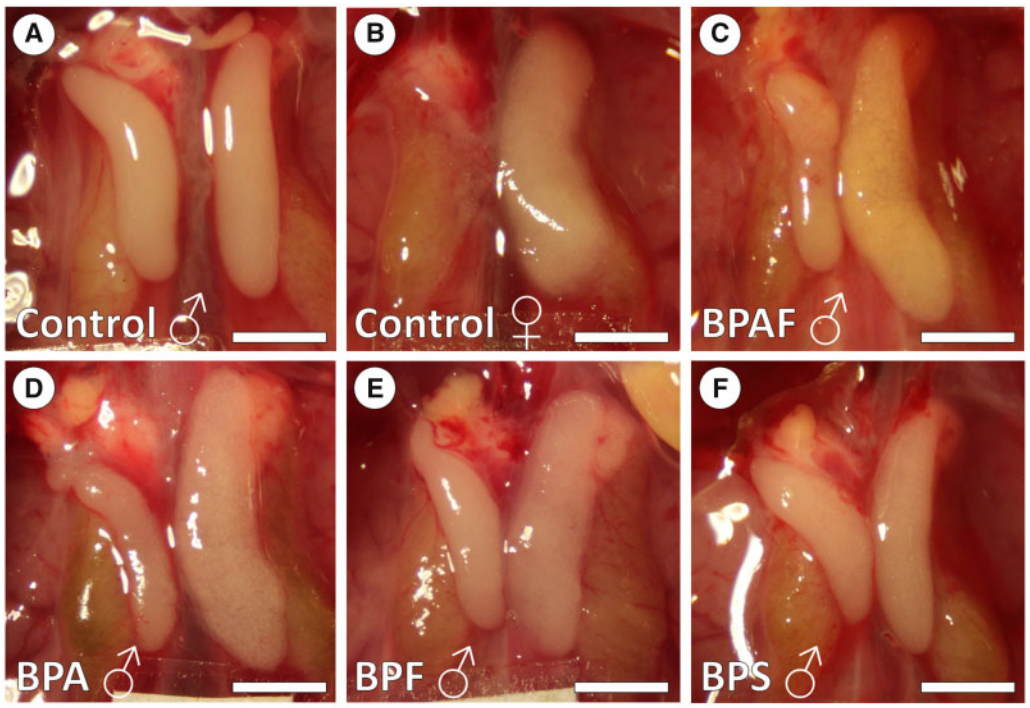

Figure 3. Gross morphology of chicken embryo gonads photographed *in situ*. A, Control male with uniform bilateral testes that appear smooth and cylindrical with tapered ends. B, Control female with a large left ovary and an almost completely regressed right ovary. The left ovary is variable in shape but is typically flattened and is wider and more irregular in shape than a testis. The surface of the ovary appears granulated under the microscope. C, Genetic male exposed to 210 nmol/g egg of BPAF. D, Genetic male exposed to BPA. E, Genetic male exposed to BPF. F, Genetic male exposed to BPS. The genetic males in C–E are partially feminized; the right testis is small and the left gonad has developed into an ovotestis. None of the 3 genetic males in the group exposed to BPS had a left gonad that was classified as an ovotestis. Scale bar: 2 mm.

and a control female are shown in Figures 3A and 3B, respectively.

In all female control embryos, the left ovary was well developed and the regressing right ovary was small or completely regressed (Figure 3B). No effect of BPAF, BPA, or BPF on gross morphology of the ovaries was observed. In the group exposed to BPS, 2 out of 9 females exhibited a left ovary that appeared thinner than normal (not statistically significant difference in frequency).

The control males had pairs of fairly symmetrical testes that appeared normal. The gross morphology of the right testis did not appear to be affected by any of the tested bisphenols. In contrast, the left testis showed varying degrees of feminization in some of the males exposed to BPAF, BPA, and BPF; it was typically larger than a normal testis and more or less ovary-like in shape. In this paper, we denote a left testis with such feminized shape as an ovotestis. No control males exhibited ovotestis; because experiments 1–3 did not differ in this respect, the results on this endpoint in these experiments were combined. As shown in Figure 4A, the frequency of ovotestis increased with increasing dose of BPAF and the effect was statistically

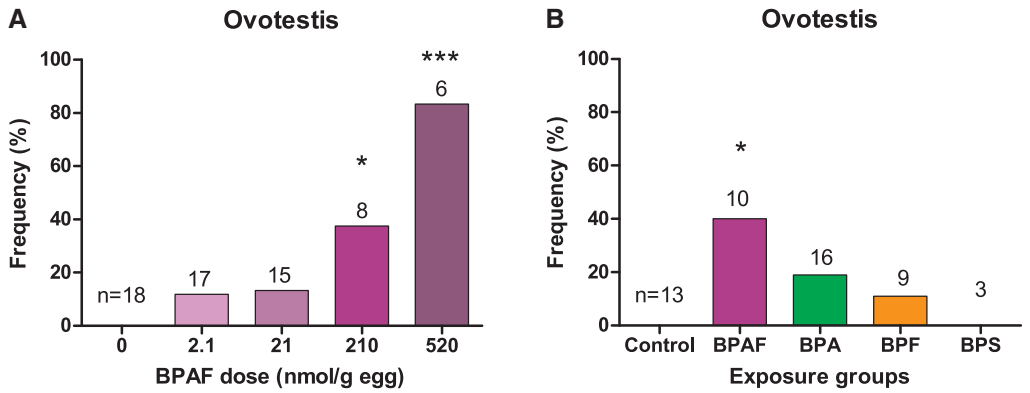

Figure 4. Frequencies of male chicken embryos with ovotestis. A, Combined results of BPAF experiments 1–3. B, Experiment 4 with BPAF, BPA, BPF, and BPS at 210 nmol/g egg. Chicken embryos were exposed to vehicle (DMSO; Control) or bisphenols from E4 and were dissected on E19. The numbers above the bars represent the total number of examined males in each group. The frequencies in the treatment groups were compared with the control using one-sided Fisher's exact test followed by Benjamini-Hochberg correction of p values; $^{*}p \leq .05$, $^{***}p \leq .001$.

significant at the two highest doses (210 and 520 nmol/g egg). The ovotestis frequency among males exposed to 210 nmol/g egg of BPAF was approximately 40% both in the dose-response experiments 1–3 and in experiment 4 (Figs. 4A and 4B). Three out of 16 males exposed to BPA and 1 out of 9 males exposed to BPF had ovotestis. The frequency of males with ovotestis was not significantly increased by BPA and BPF when compared with the vehicle control in experiment 4. Only three of the twelve dissected BPS-exposed embryos were males, and none of these had developed ovotestis.

Gonad Size

The size of the gonads was defined as the gonad area measured in photos taken *in situ* and data obtained was normalized to the mean size of the left ovary in the corresponding control group. Following normalization, data from experiments 1–3 were combined in the statistical analysis. The normalized size of the left ovary was 1.0 ± 0.1 (mean $\pm$ SD) in control females both in experiments 1–3 and in experiment 4. In the different treatment groups, the ovary size ranged between 0.9 and 1, and did not differ from the control (Figs. 5A and 5B). The size of the right ovary was only about 15% that of the left, and in many animals the right ovary was too small to be reliably measured and was therefore not further analyzed.

The size of right and left testis (including ovotestis) and the right/left testis size ratio are shown in Figure 5. In control males, both left and right testes were slightly smaller than half the size of a female left ovary. The combined data from experiments 1–3 showed no significant effect by any of the BPAF doses on right testis size (Figure 5A). In experiment 4, however, the right testis size was significantly reduced by BPAF at 210 nmol/g egg, but not by treatment with BPA, BPF, or BPS. Left testis size was significantly increased by BPAF at the highest dose (520 nmol/g egg) in the dose-response experiment (Figure 5B), but was not affected by BPAF, BPA, BPF, or BPS at 210 nmol/g egg in experiment 4 (Figure 5D). The right/left testis ratio was significantly reduced by BPAF at 210 nmol/g egg ($p \leq .05$) and 520 nmol/g egg compared with that of the control (Figs. 5C and 5F). The right/left testis ratio was also reduced by BPF (Figure 5F).

Gonad histology

In experiments 3 and 4, the left testis was evaluated regarding histological changes and the left ovary from 4 control females was included for comparison. Figure 6 shows histological sections of a left testis from a control male (A and G), an ovary from a control female (B and H), and left gonads from males treated with BPAF, BPA, BPF, and BPS (C–F and I–L).

The control testes were mainly composed of medulla with testicular cords (which will develop into seminiferous tubuli) separated by loose mesenchyme (Figs. 6A and 6G). The medulla was covered by a thin superficial epithelium, ie the cortex, with squamous to cuboidal cells. In some control males, small clusters of oocyte-like cells were found within or beneath the cortex.

All control ovaries looked normal for a 19-day-old embryo; they had a thick cortex containing numerous oocytes arrested in meiotic prophase and the ovarian medulla contained mesenchymal stroma and lacunae (Figs. 6B and 6H). The superficial epithelium of the cortex varied from squamous to high columnar but was mainly cuboidal to columnar.

BPAF, BPA, and BPF caused feminization that was detectable at the histological level in the left testis (Figs. 6C–E and 6I–K). The left testis of one of the three males in the BPS group was lost because of technical problems during sample preparation. The remaining two were analyzed and are included in the graphs (Figure 7) but were excluded in the statistical analyses.

The frequency of males having a left gonad with 10 or more oocyte-like cells in at least 1 out of 3 section levels was significantly increased by BPAF at the highest dose of 520 nmol/g egg in experiment 3 (Figure 7A). These clusters were generally larger and more common in both control- and BPAF-exposed males in experiment 4 than in experiment 3 (Figure 7A and B). The frequency was numerically higher in the exposure groups than in the control group, but there was no statistically significant difference in experiment 4.

The superficial epithelium of the left testis showed increased height following treatment with BPAF; the frequency of a left gonad showing columnar epithelium in at least one section level was increased by BPAF at the two highest doses in experiment 3 (210 and 520 nmol/g egg) and at 210 nmol/g egg in experiment 4 (Figs. 7C and 7D). The prevalence of columnar epithelium was also relatively high in males exposed to BPA and BPF but it was not significantly increased compared with the control (Figure 7D).

In control females, the relative cortex area in histological sections of the left gonad was $42 \pm 8\%$ (mean $\pm$ SD). In most control males the corresponding area was below 20%, ie, less than half of the female value (Figs. 7E and 7F). Exposure to

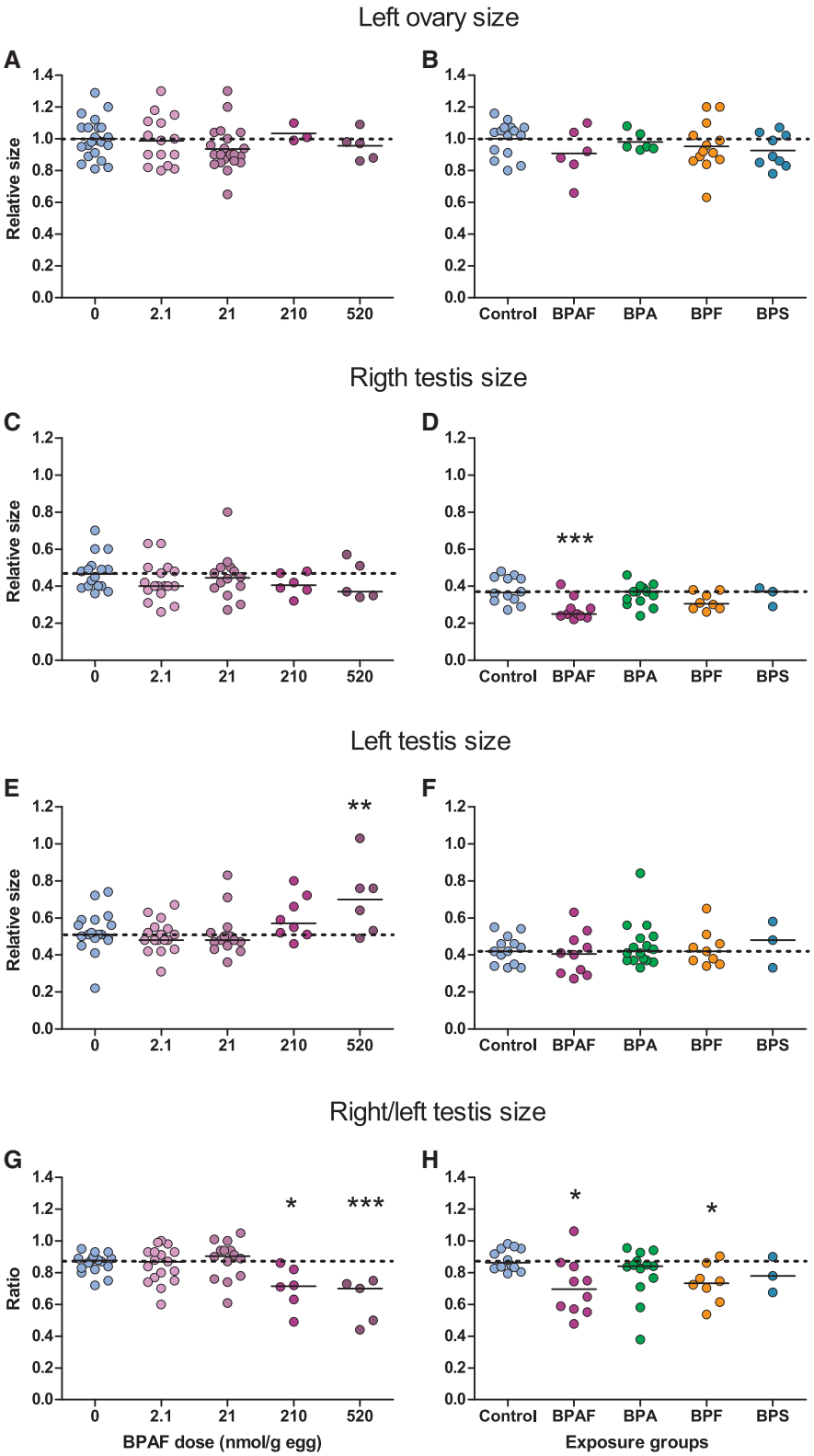

Figure 5. Gonad size and right/left testis size ratio in chicken embryos. The left graphs show combined results from the BPAF dose-response experiments 1–3, and the right graphs show effects of exposure to 210 nmol/g egg of BPAF, BPA, BPF, and BPS in experiment 4. Chicken embryos were exposed to vehicle (DMSO; Control), or bisphenols from E4 and were dissected on E19. Each individual is represented by a circle and the solid lines indicate the median values for each group. The dashed lines indicate the control medians. The gonad size was analyzed using photos taken *in situ* and defined as the surface area of the gonad divided by the mean surface area of the left ovary in the corresponding within-experiment control group. Each group was compared with the control using either 1-way ANOVA followed by Dunnett's multiple comparisons test or Kruskal-Wallis followed by Dunn's multiple comparisons test. $^{*}p \leq .05$, $^{**}p \leq .01$, $^{***}p \leq .001$.

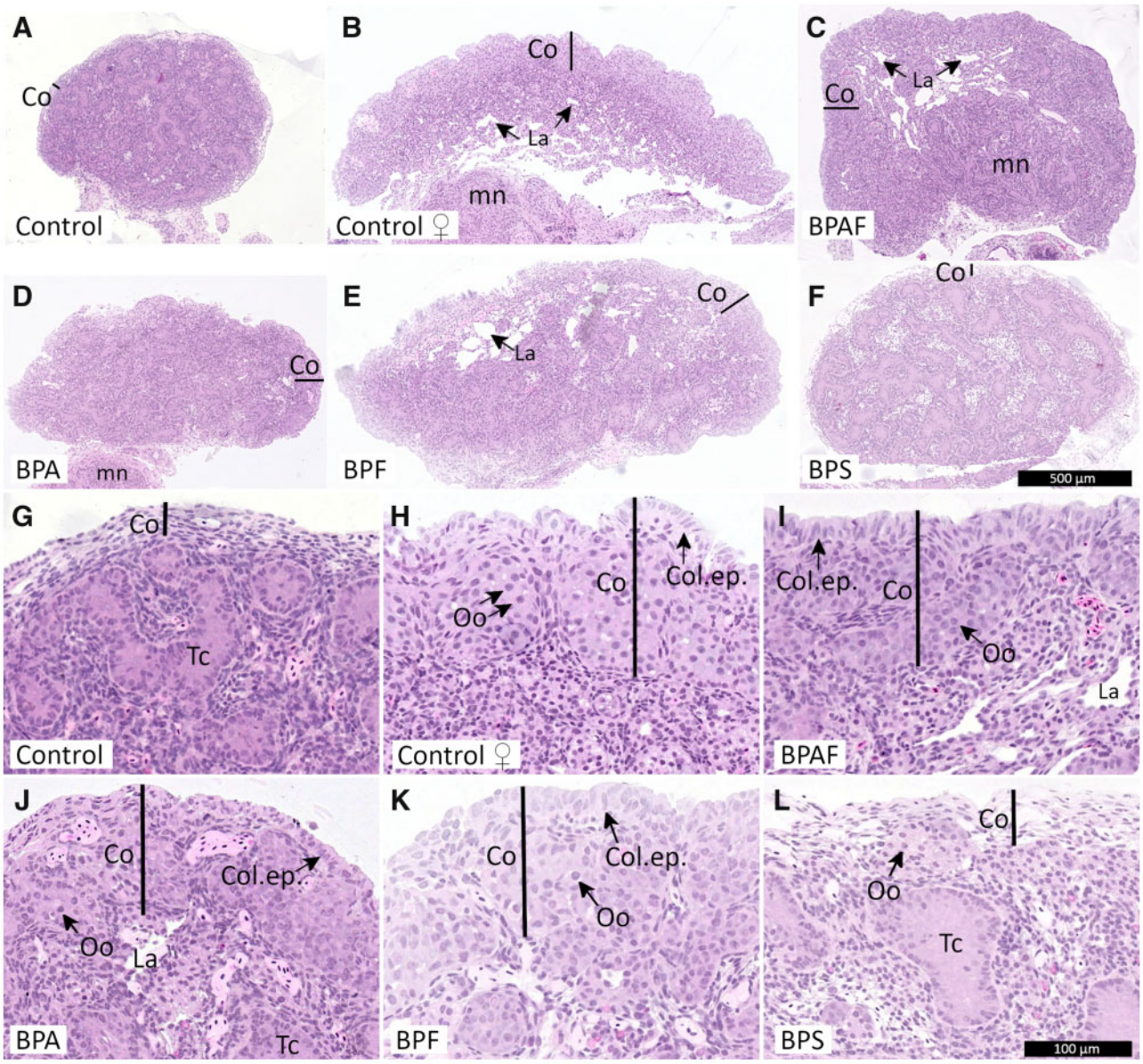

Figure 6. Histology of the left gonad from chicken embryos. Panels A–F show whole cross sections of gonads and G–L show the same sections at a higher magnification focusing on the cortical region. Panels A and G show a control testis, panels B and H show a control ovary and the other panels show testes/ovotestes from males treated with the different bisphenols (210 nmol/g egg). Note that the control testis (A and G) has a thin cortex (Co) enclosing a medulla filled with testicular cords (Tc), and that the control ovary (B and H) has a thick cortex containing numerous oocytes (Oo) and a medulla containing mesenchymal stroma and lacunae (La). The left testis of males exposed to BPAF, BPA, and BPF are partially feminized; they have a thickened ovarian-like cortex with oocyte-like cells (Oo) and a superficial epithelium with columnar epithelial cells (Col. ep.). Some of the testes from treated males also show ovarian-like lacunae. The gonads are located on the anterior surface of the mesonephros (mn). Stain: hematoxylin and eosin. Scale bar A–F: 500 µm. Scale bar G–L: 100 µm.

BPAF, BPA, and BPF at 210 nmol/g egg caused a significant increase in testis relative cortex area in experiment 4, whereas no significant effect of BPAF on this endpoint was seen in experiment 3 (Figs. 7E and 7F). It should be noted that one of the BPAF-exposed males in experiment 3 that showed a clearly feminized left testis had a cortex area > 50%.

The average diameter of the testicular tubules was reduced by BPA but was not significantly affected by BPAF or BPF (Fig. 7G and 7H).

All of the examined testes that were classified as ovotestis by the gross appearance also showed signs of feminization at the histological level, as described above. The most severely affected testes in the BPAF, BPA, and BPF groups displayed a thickened ovary-like cortex with high columnar epithelium and numerous oocyte-like cells and a medulla containing lacunae (similar to those in the ovarian medulla) and testicular cords that appeared irregular. Moreover, many of testes that were not classified as ovotestis by visual inspection still showed mild histological changes.

DISCUSSION

This study shows for the first time that the BPA replacement chemicals BPAF and BPF induce testes feminization in an avian embryo model.

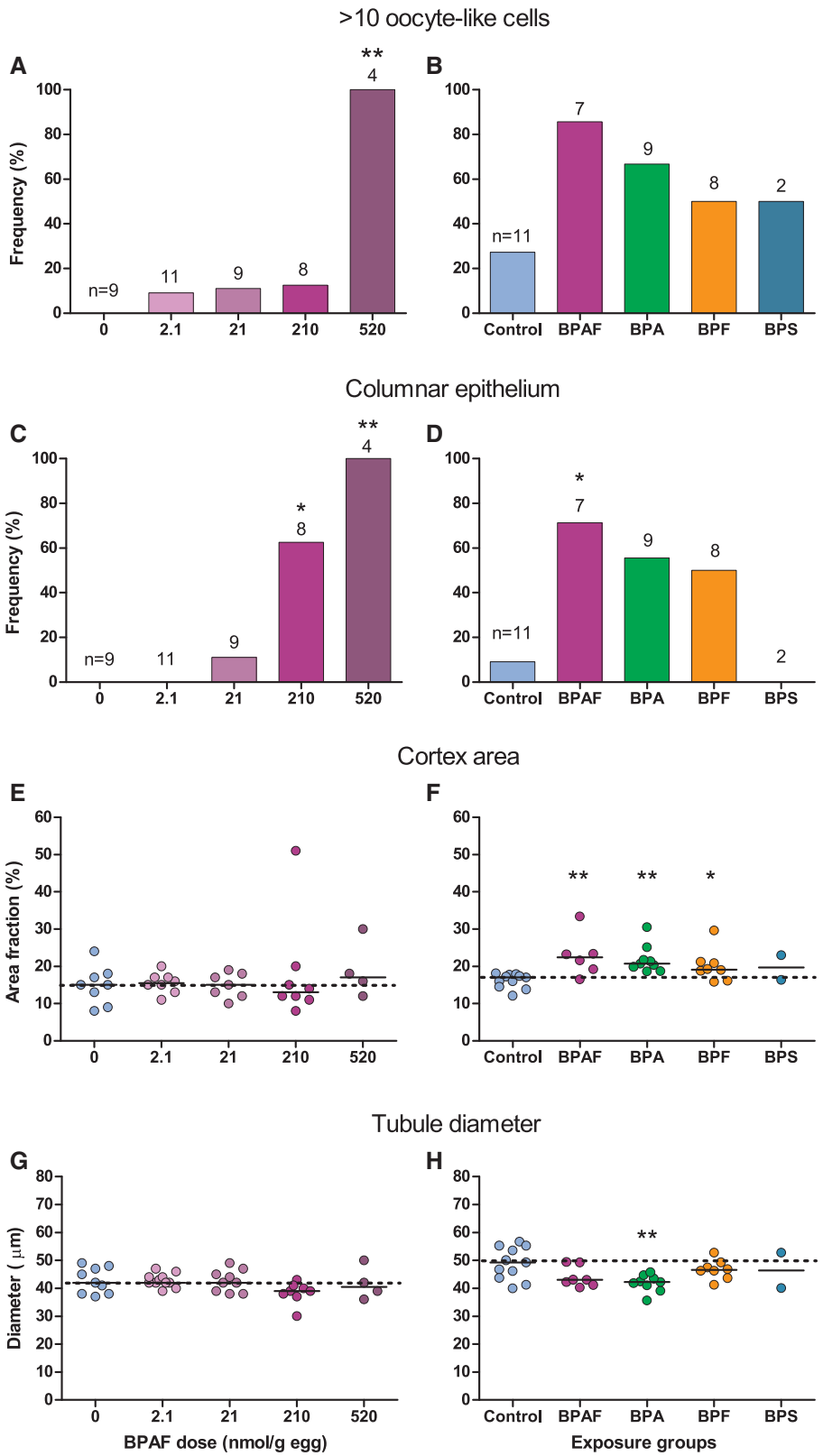

Figure 7. Histology of the left testis. The left graphs show results from the BPAF dose-response experiment 3 and the right graphs show effects of exposure to 210 nmol/g egg of BPAF, BPA, BPF, and BPS in experiment 4. A and B, Frequencies of males with at least 10 oocyte-like germ cells in at least 1 out of 3 histological levels. C and D, Frequencies of males with columnar epithelium in at least 1 out of 3 histological levels. E and F, Relative cortex area. G and H, Average testicular tubule diameter. Chicken embryos were exposed to vehicle (DMSO; Control), or bisphenols from E4 and were dissected at E19. The numbers above the bars in A–D show the total number of analyzed males in each group. In E–H, each individual is represented by a circle and the median value for each group is indicated by solid lines. The dashed lines show the control medians. Frequencies in the treatment groups were compared with the control frequency using one-sided Fisher's exact test followed by Benjamini-Hochberg correction of p values. The cortex area and the tubule diameter were analyzed with Kruskal-Wallis followed by Dunn's multiple comparisons test, comparing each treatment group with the control. BPS was not included in the statistical analysis due to the low number of replicates in this group ($n = 2$). $^{*}p \leq .05$, $^{**}p \leq .01$.

Testis Feminization

Male embryos exposed to BPAF, BPA, or BPF showed signs of testis feminization at the gross morphological and/or histological level. Ovotestis was not found among control males but was induced by BPAF exposure in a dose-dependent fashion (Figure 4). This phenotype was also noted in several of the males exposed to BPA and BPF, although the incidence was not statistically different compared with the control. BPAF and BPF, but not BPA, caused a significant decrease in right/left testis size ratio (Figure 5). The asymmetry may result from an estrogen-induced increase in size of the left testis, reduction in the size of the right testis, or a combination of these as indicated by the results of the present and previous studies (Jessl *et al.*, 2018b; Mattsson *et al.*, 2011; Shibuya *et al.*, 2005). Only three of the males exposed to BPS were alive by embryonic day 19; because of the low number of male embryos in this group we cannot draw any conclusions regarding potential effects of BPS on testis development.

The lack of significant effects on testis gross morphology by BPA at the tested dose (210 nmol/g egg) is consistent with a study by Jessl *et al.* (2018b), in which the right and left testis area of 19-day-old chicken embryos remained unaffected by BPA at doses up to 300 μg/g egg (1300 nmol/g egg). At the histological level, however, BPA caused formation of a thickened ovarian-like cortex with oocyte-like cells already at a dose of 75 μg/g egg (330 nmol/g egg) (Jessl *et al.*, 2018b), suggesting that histological changes occur at lower bisphenol doses than gross morphological changes. This was confirmed by the histological evaluation of the left testis in the present study showing that 210 nmol/g egg of BPA increased the cortex area and reduced the diameter of the testicular tubules (Figs. 6 and 7). Furthermore, the histological analyses showed that also BPAF and BPF increased the cortex area and that BPAF increased the number of oocyte-like cells in the cortical region and induced an ovarian-like columnar superficial epithelium. Thus, BPAF, BPA, and BPF all induced estrogen-like histological changes in the left testis of chicken embryos exposed from embryonic day 4.

Some of the control testes showed low numbers of oocyte-like cells and/or a few columnar epithelial cells. This was more common in experiment 4 than in experiment 3 which may be due to factors relating to the fact that the eggs in these 2 experiments were obtained from different suppliers. Low counts of oocyte-like cells may occur normally in testes of male bird embryos as this has been reported to occur in up to 45% of male quail embryos (Berg *et al.*, 1999, 2001). Nevertheless, a low background prevalence of oocyte-like cells and columnar epithelia in the embryos used facilitates detection of exposure-induced effects on these endpoints.

All testicular changes shown in the present study, ie ovotestis formation, increased testis size asymmetry, formation of ovarian-like cortex with oocyte-like cells and columnar epithelium, and reduced tubule diameter are known to be induced in male bird embryos by exposure to estrogenic compounds such as genistein, 17-beta-estradiol, ethinylestradiol (EE2) and diethylstilbestrol (DES) (Berg *et al.*, 1999; Intarapat *et al.*, 2014; Jessl *et al.*, 2018b; Shibuya *et al.*, 2005). Even though not all endpoints related to testis morphology were significantly affected by all the bisphenols in the present study, BPAF, BPA, and BPF at large showed similar effects. Thus, our results strongly suggest that the BPA substitutes BPAF and BPF can induce estrogen-like feminization of the testis in avian embryos in a similar manner as BPA.

Ovaries

We found no changes in size or shape of the ovaries in female embryos following exposure BPAF, BPA, or BPF in the present study. However, 2 out of 9 females exposed to BPS had ovaries that appeared thin and possibly atrophic. Similar to this observation, we recently found that the BPA metabolite 4-methyl-2,4-bis(4-hydroxyphenyl)pent-1-ene (MBP) not only caused thinning of the left ovary but also reduced its surface area (Mentor *et al.*, 2020). A small left ovary is consistent with effects from exposure to antiestrogens or inhibitors of estrogen synthesis and may be a sign of impaired female development (Burke and Henry, 1999; Jessl *et al.*, 2018a; Scheib, 1983). Whether the thin ovaries observed after exposure to BPS and MBP resulted from antiestrogenic effects remains to be elucidated.

No Effects on the Müllerian Ducts

Embryonic exposure to potent estrogens such as EE2 causes retention and malformation of the Müllerian ducts in both females and males in the chicken and quail (Berg *et al.*, 1999; Mattsson *et al.*, 2011). In the present study, we did not find any effects of the studied bisphenols on Müllerian duct gross morphology at the doses used. BPA at 200 μg/g egg (880 nmol/g) has previously been shown to induce testis feminization in chicken embryos and to induce Müllerian duct abnormalities in embryos of quail but not chicken (Berg *et al.*, 2001). This suggests that in the chicken embryo testis feminization is a more sensitive response to BPA than is Müllerian duct abnormalities.

Increased Gallbladder Weight

A significant increase in the gallbladder-somatic index was found in embryos treated with BPAF or BPS (Figure 1). This effect of BPS corroborates the finding by Crump *et al.* (2016) who showed that BPS, at a similar dose as in the present study, increased the gallbladder length in chicken embryos. The observation that all exposure groups in the present study had mean gallbladder-somatic indices that were numerically higher than that of the control indicates that disrupted bile acid homeostasis may be a common effect of bisphenol analogs in the chicken embryo. These notions call for further studies exploring the mechanisms behind the gallbladder enlargement and possible implications of this effect.

Mortality

All tested bisphenol analogs except for BPA caused a significant increase in mortality at 210 nmol/g egg (Table 1). Most of the deaths occurred during the first day after exposure, suggesting that the mortality was due to acute toxicity in the early embryo rather than to developmental hormone-related effects. The high mortality in the group exposed to BPS (57%) was unexpected considering that the pipping success by E22 was as high as 89% at a similar dose of BPS in the study by Crump *et al.* (2016). The main difference in experimental procedure that may explain this discrepancy was that the eggs were injected on E0 in the study by Crump *et al.* and 4 days later, ie on E4, in the present study. How the day of egg injection affects the embryo exposure and mortality remains to be clarified though.

BPA did not increase the mortality in the present study at a dose that induced testis feminization (48 μg/g; 210 nmol/g), whereas in the study by Berg *et al.* (2001), chicken embryo mortality was increased at a lower dose (67 μg/g; 290 nmol/g) than that required for testis feminization (200 μg/g; 880 nmol/g) (Berg *et al.*, 2001). The lowest dose at which BPAF significantly increased mortality (210 nmol/g egg) was also the lowest dose inducing testis feminization. Similarly, the tested BPF dose caused both increased mortality and mild testis feminization. Taken together, these results suggest that the doses of BPAF, BPA, and BPF required to impair reproductive organ

development in chicken embryos are similar to those causing increased mortality. However, the molecular events resulting in these different effects are likely to differ.

Relative Potencies and Environmental Relevance

In a recent study using an estrogen-responsive transgenic zebrafish, it was shown that the relative potencies of bisphenol analogs to activate ERs and to cause acute toxicity were BPAF > BPA ≥ BPF > BPS (Moreman *et al.*, 2017). This rank order for estrogenic potency correlates relatively well with rank orders found in a variety of *in vitro* assays (Pelch *et al.*, 2019a). It was not within the scope of the present study to compare dose-responses of the bisphenols. Nevertheless, our results show that at an equimolar dose, BPAF showed slightly higher embryo mortality, higher frequency of ovotestis in males, larger testis size asymmetry, and slightly more pronounced histological changes in the left testis than BPA and BPF. These results are in line with the notion that BPAF is the most potent of these compounds.

The internal exposure/effect concentrations were not determined in the present study, but previous studies on BPS and BPA suggest that the resulting whole embryo concentration is substantially lower than the nominal concentration applied into the egg. Less than 1% of radiolabeled BPA injected into the yolk of quail eggs on E3 was retrieved in the embryos on E6 and E9 (Halldin *et al.*, 2001). BPA-derived radioactivity was mainly distributed to the yolk, liver, gallbladder and allantoic fluid, suggesting that BPA was poorly absorbed but readily metabolized and excreted by the embryos. BPS showed a similar retention in chicken embryos; less than 1% of the nominal dose injected into the chicken egg on E0 was found in the embryo at the time of pipping (Crump *et al.*, 2016). The lowest observed effect dose of BPAF in the present study was 210 nmol/g egg, which would correspond to an internal concentration of 2.1 nmol/g embryo tissue (0.7 μg/g) assuming that 1% of the applied dose was retained in the embryo. This concentration is substantially higher than concentrations of BPA and its analogs currently found in wildlife (low ng/g) but is only slightly more than one order of magnitude higher than levels of BPA found in human fetal liver (up to 0.123 μg/g) (Cao *et al.*, 2012; Chen *et al.*, 2016; Corrales *et al.*, 2015).

CONCLUSION

In conclusion, BPA and its structural analogs BPAF and BPF disrupted reproductive organ differentiation in chicken embryos at 210 nmol/g egg causing effects that are consistent with an estrogenic mode of action. BPS at the same dose caused increased mortality and increased weight of the gallbladder. Thus, our result provides further evidence that BPAF, BPF, and BPS are not safe alternatives to BPA and that BPAF is at least as potent as BPA.

SUPPLEMENTARY DATA

Supplementary data are available at *Toxicological Sciences* online.

DATA AVAILABILITY

The datasets generated and analyzed during the current study are available from the corresponding author on reasonable request.

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DECLARATION OF CONFLICTING INTERESTS

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

AUTHOR CONTRIBUTIONS

Conceptualization: B.B., A.Ma, A.Me, and M.J.; Formal analysis: A.Ma and A.Me; Investigation: BB, A.Ma, A.Me, M.J., and M.W.; Project administration: A.Ma and A.Me; Writing—original draft: A.Ma; Writing—review and editing: B.B., A.Ma, A.Me, M.J., and M.W.; Visualization: A.Ma; and Funding acquisition: B.B.

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