Sex-specific influence of maternal exposure to bisphenol A on sodium and fluid balance in response to dipsogenic challenges in rats

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ABSTRACT

The plasticizer bisphenol A (BPA) is one of the highest volume chemicals produced worldwide. Human exposure to BPA occurs almost constantly. BPA is an endocrine disruptor that interferes in estrogen receptor functions. This is important for the developing brain, which is particularly sensitive to the estrogenic effects of BPA. Body fluid balance is maintained by a complex network of systems that regulate sodium and water intakes and electrolyte excretion. The development of these control systems occurs during early life and therefore, may be susceptible to changes in the uterine environment. The aim of this work was to study the effects of two low BPA doses in the dam, during pregnancy and lactation, on adult offspring drinking and sodium and urine excretion after dipsogenic challenge. Dams were exposed to BPA in drinking water to mimic the most likely route of human exposure. The results showed that BPA did not disrupt spontaneous fluid balance, but altered sodium and fluid intakes in the BPA offspring under osmotic challenges. In experiments, both 24h fluid deprivation and sodium depletion modified fluid ingestive response in BPA offspring compared to control offspring. The increased preference for 2.7% NaCl solution in male BPA offspring is similar to female control offspring, altered ingestive behavior appears to be due to feminization of males and "hyperfeminization" of female BPA offspring, as they drink more than female control offspring. Our results indicate that exposure to low doses of BPA in early life may disrupt the development of sex-specific drinking behaviors by altering the steroid programming of the brain, and this disruption affects males and females differently.

1. Introduction

Bisphenol A (BPA) is a major component of polycarbonate plastics and is used in the manufacture of a wide range of consumer products, including bottles, baby bottles, canned food, thermal paper and dental compounds. Human exposure to BPA occurs almost constantly; it is regularly detected in human biological samples such as urine, plasma, placenta, breast milk, neonatal blood and amniotic fluid (Azzouz et al., 2016). Multiple in-vivo studies have highlighted non-monotonic dose responses on exposure to low doses of BPA, with the response curve falling below the control at low doses and increasing at high doses (called a U-shape or J-shape; Lagarde et al., 2015). This chronic exposure to BPA has been linked to a number of non-communicable diseases, although the exact mechanisms involved are as yet unknown (Giulivo et al., 2016). By definition, non-communicable diseases are long in duration and occur as a result of interactions between various environmental, biological or social factors (WHO, 2019). According to the

European Food Safety Agency (2018), there is limited knowledge of the impact of the current level of human exposure to BPA, although it is known that it could adversely affect human health.

The hydromineral balance of body fluids serves to maintain optimal levels of osmolarity and blood volume, thus preventing cardiovascular disease; changes in this balance are known to produce behavioral and physiological compensatory responses (Johnson & Thunhorst, 1997; Santollo & Daniels, 2015). Estrogen is a steroid hormone that is also involved in the control of hydrosaline balance in numerous species, including humans and rodents (Stachenfeld, 2008). It detects signals from the central nervous system and hormonal systems that indicate specific responses to fluctuations in osmotic balance or blood volume (Curtis, 2015). BPA—a "xenoestrogen"—exhibits estrogenic activity, allowing it to bind to estrogen receptors and disrupt their activity. This endocrine-disrupting capacity could interfere with any aspect of hormone action (McLachlan, 2016). Early life is a period of vulnerability, and any disturbance to natural processes during this time can

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irreversibly alter the structure and/or functions of physiological systems. The concentration of a hormone is as critical for unimpaired development as is the moment when the hormone is released (Miranda & Sousa, 2018). Consequently, early development is particularly sensitive to BPA perturbations that may have adverse health effects later in life. This is especially true for the developing brain, which is particularly sensitive to the estrogenic effects of BPA during early life. In fact, several human studies have correlated BPA exposure in early life with behavioral problems later in childhood, suggesting that BPA permanently alters brain development and has long-lasting effects on the neural network (Mustieles & Fernández, 2020; Nesan et al., 2018, 2021; Patisaul, 2020).

We previously studied the effects of short-term and long-term BPA exposure in adult Wistar rats. Our analysis suggested that exposure to low doses of BPA had minor effects on daily fluid intake and the selection of saline solutions. Fluid and electrolyte balance in BPA-treated rats were largely adequate, but were impaired when met with osmotic challenges such as furosemide-induced depletion. Additionally, we observed that these minor effects may cumulatively lead to physiologically relevant long-term effects, specifically against osmotic challenges, e.g., in cardiovascular health (Nuñez et al., 2018, 2021). This study aims to determine how developmental exposure to low doses of BPA affects ingestive behavior involved in the maintenance of hydromineral balance later in life.

2. Materials and methods

2.1. Chemicals

Bisphenol A (BPA Sigma-Aldrich, purity ≥99%, CAS no. 80–05-7) was dissolved in ethanol and subsequently diluted with tap water to defined final concentrations (ethanol comprising 1% of the final solutions).

2.2. Animals

All experimental procedures followed the guidelines set forth in the Care and Use of Animals and The University of Oviedo's Experimental Animal Use Committee approved all experimental procedures (PROE 15/2016). Adult female and male Wistar rats (aged 4 months) were housed individually under standard conditions at 22 ± 3 °C with light/dark periods of 12h and a minimum relative humidity of 40%. Rats were maintained on 2014 Teklad global 14% protein rodent maintenance diet (Envigo Laboratories, Barcelona, Spain), which does not contain alfalfa or soybean meal (chow diet). The composition of the diet was as follows: calories from protein, 18%; calories from fat, 11%; calories from carbohydrate, 71%; and an energy content of 2.9 kcal/g. Tap water was supplied ad libitum in glass bottles with rubber stoppers. Fluid intakes were measured by weighing the drinking bottles on an electric balance during the early light phase once a week. The spillage and evaporation is < 0.5 g/day (Núñez Martínez et al., 2016). We did not attempt to correct for this source of error. However, we kept records of visible spillage. The individual body weight and food intakes were recorded weekly. Rats were acclimatised to housing conditions for at least 15 days.

2.3. Exposure

Prior to mating, vaginal cytology was performed on the female breeders for 10 consecutive days to document the regularity of estrous cycles. Following this, a female in proestrus and a randomly assigned male were co-housed for 1 day. Mating was confirmed by the presence of a vaginal plug. The day of copulation was marked as GD 0 (see Fig. 1). Each dam was randomly assigned to one of 3 different treatment groups: Control (n = 4), BPA5 (n = 4) or BPA10 (n = 4). Control rats had access to tap drinking water (containing 1% ethanol, vehicle), whereas the BPA groups received tap drinking water containing BPA (5 mg/L or 10 mg/L; BPA Sigma-Aldrich, purity ≥99%, CAS no. 80–05-7) to mimic the most
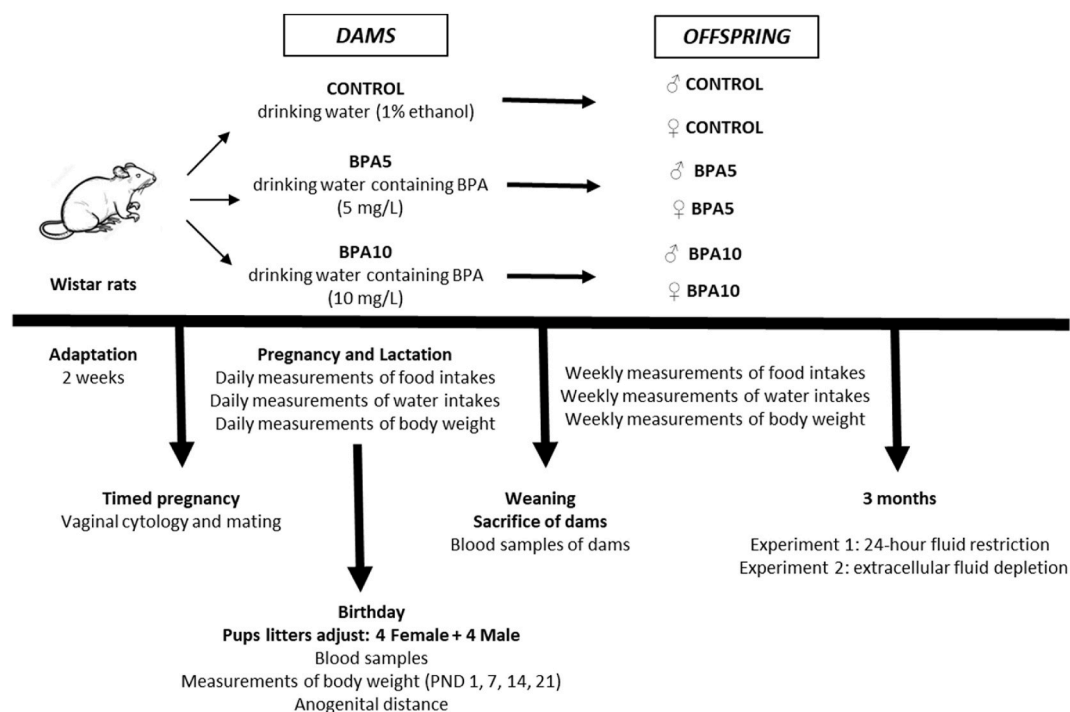

Fig. 1. Schematic depicting the experimental design. Control Wistar rats (N = 4) had access to drinking water (containing 1% ethanol, vehicle), whereas the BPA Wistar rats received drinking water containing BPA (BPA5, 5 mg/L, N = 4 or BPA10, 10 mg/L, N = 4) during gestation and lactation. At day 1 after birth, pups were weighed and litter size was culled to 8 to ensure no interlitter nutritional variability. We selected the heaviest pups of each litter (4 females and 4 males when possible). N = 16 animals/group/sex. Several measurements were obtained during gestation and on postnatal days (PND) 1,7, 14 and 21. Body weights, food intakes, and water intakes were recorded weekly. All of the following experiments were performed in these animals when they are adults, when they reached 3 months of age.

likely route of human exposure (Desai et al., 2018; Galyon et al., 2017). Water and BPA solutions were freshly made and changed once every week during gestation and lactation stages. All dams had free access to the standard diet designed to support gestation and lactation (2018 Teklad global 18% protein rodent maintenance diet; Envigo Laboratories, Barcelona, Spain) which does not contain alfalfa or soybean meal (chow diet). The composition of the diet was as follows: calories from protein, 24%; calories from fat, 18%; calories from carbohydrate, 58%; and an energy content of 3.1 kcal/g. Maternal body weights (BWs), food and water intake were recorded daily. The dams that returned to pre-pregnancy weight would be marked as aborted. The gestational index was calculated as % of pregnancies yielding live litters (Table 1).

At day 1 after birth, pups were weighed and litter size was culled to 8 to ensure no interlitter nutritional variability. We selected the heaviest pups of each litter (4 females and 4 males when possible, n = 16 rats/ group/sex). The rest of the litter was decapitated and their blood was collected and pooled by sex to create sufficient volume to determine plasma BPA level (see Fig. 1). The analyses of plasma BPA levels were conducted at the Environmental Testing Unit of the University of Oviedo. A highly sensitive gas chromatography-mass spectrometry (GC-MS) method for the determination of BPA plasma samples was used (Azzouz et al., 2016). Pup body weights were recorded at 1, 7, 14 and 21 days of age. Anogenital distance (AGD) was defined as the distance between the base of the genital papilla and the rostral end of the anal opening (Hotchkiss et al., 2004). At 21 days of age, the AGD was measured. After weaning (21 days old), offspring were housed by sex and litter, with three or four pups from the same litter per cage, fed standard laboratory diet and tap water. Dams were sacrificed by decapitation and blood centrifuged at 4 °C to obtain plasma for BPA measurement. Plasmas were stored at −20 °C. BWs, food intakes, and water intakes were recorded weekly. All of the following experiments were performed in these animals when they were adult at 3 months of age.

2.4. Experiment 1: 24 h' fluid restriction

Water deprivation produces thirst by causing both intracellular and extracellular dehydration. Water and 2.7% NaCl solution were supplied simultaneously from two separate drinking bottles, reversing daily their location for 7 days habituation. We then performed a water deprivation test on day seven to assess water and 2.7% NaCl solution induced intakes. The rats were maintained for 24h on standard food diet with no drinking fluids available. Thereafter, fluid intakes after 24h water deprivation were measured. 2.7% NaCl solution and water were made available. Intakes of the two fluids were recorded after 5, 10, 15, 30, 60 and 120 min (Nuñez et al., 2018, 2021).

2.5. Experiment 2: extracellular fluid depletion

Furosemide treatment causes hypovolemic dehydration. Rats were weighed in the morning and placed in standard metabolism cages with stainless steel funnels. Furosemide (Seguril, Sanofi-Aventis Laboratories, Barcelona, Spain) was injected (10 ml/kg body wt sc) to induce natriuresis and diuresis. After 1 h, access to water was provided. Food was not present. Next morning, 19 h later, overnight water intakes were recorded. The rats were then returned to their home cages and water and 2.7% NaCl solution were recorded every 30 min for 4 h. In tests, urine was collected for the 1st h after furosemide injection. Urine for the remainder of the overnight period was also collected, and urine volume was recorded in the morning simultaneously with overnight water intakes (Nuñez et al., 2021; Thunhorst & Johnson, 2003). This urine volume was calculated as 1 g = 1 ml. Samples were refrigerated for later analysis of sodium content.

2.6. Urine analysis

Urine volume was measured (UV). Urinary concentration of sodium (Una) was determined by reflectance spectrophotometry and used for calculation of urinary excretion of sodium (UnaV). Relative water balances were calculated by subtracting the total UV collected from the total amount of fluid (water + 2.7% NaCl solution) ingested over the course of Experiment 2. Relative sodium balances were calculated by subtracting total UnaV from the total amount of sodium ingested in the form of 2.7% NaCl solution over the course of Experiment 2. We use the term "relative" for the balance measures, inasmuch as respiratory and fecal losses of water and sodium were not considered.

2.7. Statistical analysis

The results are expressed as the mean ± the standard error of the mean (SEM). Statistical software (IBM SPSS v. 24, IBM Analytics) was used for statistical analyses. Chi-square tests of homogeneity were applied to the number of pregnancies, abortions and sex ratios. Gestational weight gain, offspring weights, and litter size at every time point were analyzed using One-way Analysis of Variance (ANOVA) followed by Tukey's multiple comparisons post hoc test. The Shapiro–Wilk test showed that the variables follow a normal distribution of data. Weaning BWs, food intakes, and water intakes were analyzed using a linear mixed effect model, with group and sex as fixed effects and dam as a random effect. All drinking data were normalised for body-weight. The 2.7% NaCl preference was calculated as the ratio of 2.7% NaCl solution intake to total fluid intake and it is expressed as a percentage. Data were analyzed using a repeated-measures ANOVA with sex (female, male), group (control, BPA5, BPA10) and time as factors. Given that separate ANOVAs of cumulative drinking intake at each time point can potentially increase false positives (Fitts, 2006), data were analyzed as cumulative and non-cumulative data, and since there were no differences in the outcome of the hypothesis testing, such findings were presented as cumulative data as it is more usual. All values are reported as significant at the $p < 0.05$ level.

Table 1
Pregnancy outcomes of control and BPA-treated dams. Pre-weaning measures of control and BPA-exposed offspring. Note: BPA, bisphenol A; PND, postnatal day. Control dams had access to drinking water (containing 1% ethanol, vehicle), whereas the BPA dams received drinking water containing BPA (BPA5, 5 mg/L or BPA10, 10 mg/L). Gestation index = (# of dams with live litters/# of pregnant dams x 100). Pre-weaning measures were obtained from offspring perinatal exposure to vehicle or BPA. Values are expressed as the mean ± SEM. *p < 0.05, control and BPA5 groups vs. BPA10 group. [a]p < 0.05, males vs. females.

	Control	BPA5	BPA10
Total pups/litter	10.80 ± 1.38	10.00 ± 0.46	12.33 ± 0.91
Sex ratio (% males)	46.00*	40.13	39.44
Abortions (% of dams)	0	0	0
Gestational index (%)	100	100	100
Pup body weight (g) at PND1			
Male	5.73 ± 0.17	6.07 ± 0.22	5.69 ± 0.12
Female	5.28 ± 0.16	5.68 ± 0.13	5.56 ± 0.12
Body weight (g) at PND7			
Male	15.07 ± 0.36*	16.52 ± 0.59	16.97 ± 0.36
Female	14.23 ± 0.52*	15.93 ± 0.52	16.54 ± 0.48
Body weight (g) at PND14			
Male	30.45 ± 0.78	32.66 ± 1.45	32.20 ± 1.00
Female	29.56 ± 0.96	31.02 ± 1.29	32.40 ± 1.12
Body weight (g) at weaning			
Male	48.94 ± 1.23	51.53 ± 2.16	52.87 ± 1.41
Female	47.02 ± 1.43	48.32 ± 1.61	52.41 ± 0.95
Anogenital distance (mm) at weaning			
Male	16.18 ± 0.33[a]	15.93 ± 0.18[a]	15.69 ± 0.63[a]
Female	11.13 ± 0.25*	11.33 ± 0.30*	12.90 ± 0.59

3. Results

3.1. Developmental measurements

Maternal body weight, food intake and water consumption were monitored daily during the pregnancy and lactation periods (Fig. 2). In all cases, BPA treatment did not affect these parameters. Gestational duration and total number of pups per litter also remained unaffected (Table 1). In dams, the incidence of post-implantation loss (0% abortions) and the gestational index (100%) did not differ significantly between the control and BPA groups. However, the sex-ratio differed, and a lower percentage of male offspring was observed in one particular litter in each BPA group (BPA5 and BPA10) containing 7 females as compared to only 3 males.

3.2. BPA effects on plasma BPA levels

The average water consumption over the course of gestation and

lactation was similar in BPA-treated and control rats (Fig. 2). There was no observable effect on water intake associated with BPA exposure. The daily intake of BPA in adult humans is known to be approximately 500 µg/kg/day (Taylor et al., 2011). The European Food Safety Authority (2011) has set a specific migration limit for BPA at 600 µg/kg. In our study, BPA-treated rats were exposed to a BPA dose of 600 (BPA5 dams) or 1200 (BPA10 dams) µg/kg/day for 3 weeks during gestation and 1200 (BPA5 dams) or 2500 (BPA10 dams) µg/kg/day during lactation. This dosage was selected based on a previous study where maternal and newborn serum levels were within the lower range of observed human levels with normal BPA exposure (Desai et al., 2018; Galyon et al., 2017). Also, BPA doses were below the EPA recommended NOAEL doses of 5 mg/kg/day of BPA (EPA, 2018). BPA-treated rats had higher (F (2, 6) = 4.57, p = 0.09) BPA levels in plasma samples (BPA5, 35.74 ± 15.35 ng/mL, p < 0.05; BPA10, 77.90 ± 22.20 ng/mL, p < 0.05) as compared to the detectable levels in control rats (6.95 ± 3.42 ng/mL). Insufficient plasma volume from newborns necessitated pooling of samples and hence only the mean values for each group are reported. Newborns of BPA dams had higher plasma BPA (BPA5, 45.56 ng/ml; BPA10, 73.9 ng/mL) as compared to levels in newborns of control dams (13.9 ng/mL). The limit of quantification for this method (GS-MS) was 1 ng/mL for BPA.

3.3. Post-weaning measurements

Fig. 3 displays weight gain, food and water intakes of male and female offspring. No significant difference was observed in any of the post-weaning measures.

3.4. Experiment 1: 24-h fluid restriction

The water intake, 2.7% NaCl solution intake and 2.7% NaCl preference at 120 min after a 24h period of water deprivation is shown in Fig. 4. A significant observation was that fluid intake for females (p < 0.001) as well as males (p < 0.001) increased during the test. Females always drank more water and 2.7% NaCl solution than males. Significant sexual dimorphism (p < 0.01 or p < 0.05) was observed for water intake, 2.7% NaCl solution intake and 2.7% NaCl solution preference in all groups, except for the 2.7% NaCl solution preference of BPA5 group, which was the same for both sexes. Water intake did not differ among groups. The 2.7% NaCl solution intake was significant higher (p < 0.05) in the BPA10 group as compared to the BPA5 group for females and males. The 2.7% NaCl solution preference was significantly higher (p < 0.01 or p < 0.05) in the BPA10 group when compared to the control and BPA5 groups for both sexes. Preference values for females in the control group were very similar to BPA10 males.

3.5. Experiment 2: extracellular fluid depletion

The total amount of fluid ingested during the 4h salt appetite tests is presented in Fig. 5. A significant effect of time (water, p < 0.001; 2.7% NaCl solution, p < 0.001) revealed that fluid intake for all groups increased during the tests. Females always drank more water and 2.7% NaCl solution than males. A significant sex dimorphism (p < 0.01) occurred for water intake and 2.7% NaCl solution intakes in all groups. Water intake did not differ among groups. The 2.7% NaCl solution intake was significantly higher (p < 0.05) in the BPA10 group than in the BPA5 group in male rats at times 3,4,5,6, 7 and 8; no differences were found in case of female rats. The average preference for 2.7% NaCl was similar for males and females. In male rats it was significantly lower in the BPA5 at times 4, 5, 6, 7 and 8 when compared to the control and BPA5 groups; no differences were found in females.

In the 20h period after the furosemide injections and before the drinking tests, BPA groups tended to drink the same amount of water and excrete the same volume of urine as the control rats. Because the females drank significantly more (p < 0.01) than the males, they had
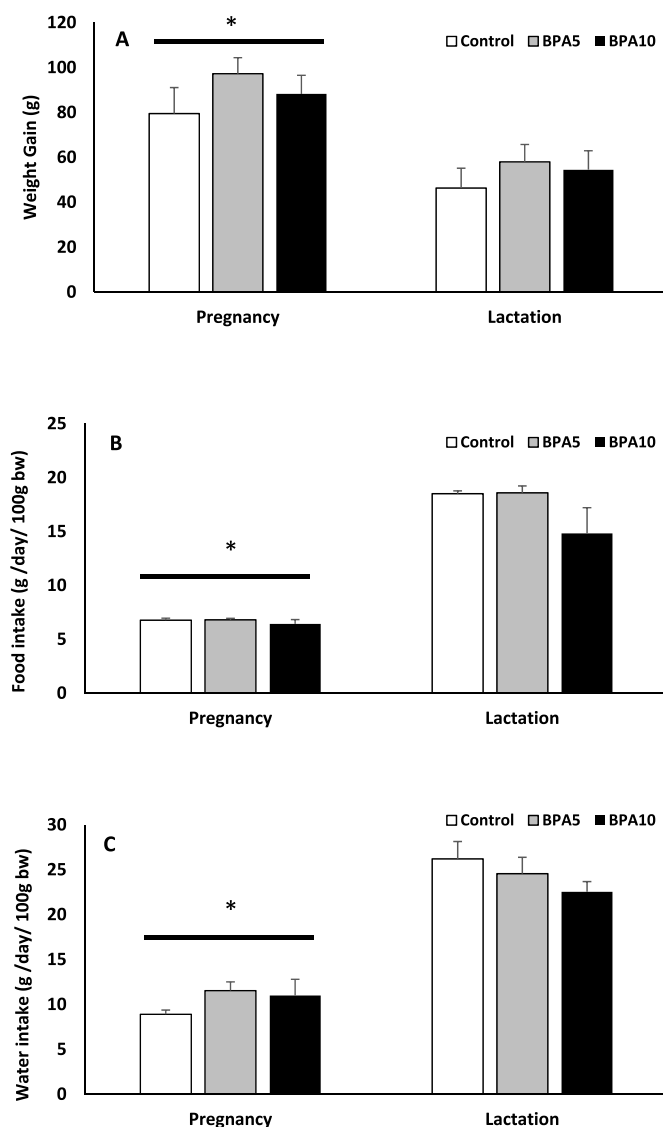
Fig. 2. Weight gain (g), food (g/day/100 g b.w.) and water (g/day/100 g b.w.) intakes of control and BPA-treated dams during gestation and lactation. Control dams had access to drinking water (containing 1% ethanol, vehicle), whereas the BPA dams received drinking water containing BPA (BPA5, 5 mg/L or BPA10, 10 mg/L). N = 4 dams/group. Values are means ± S.E.M. *P < 0.05, lactation vs. gestation.
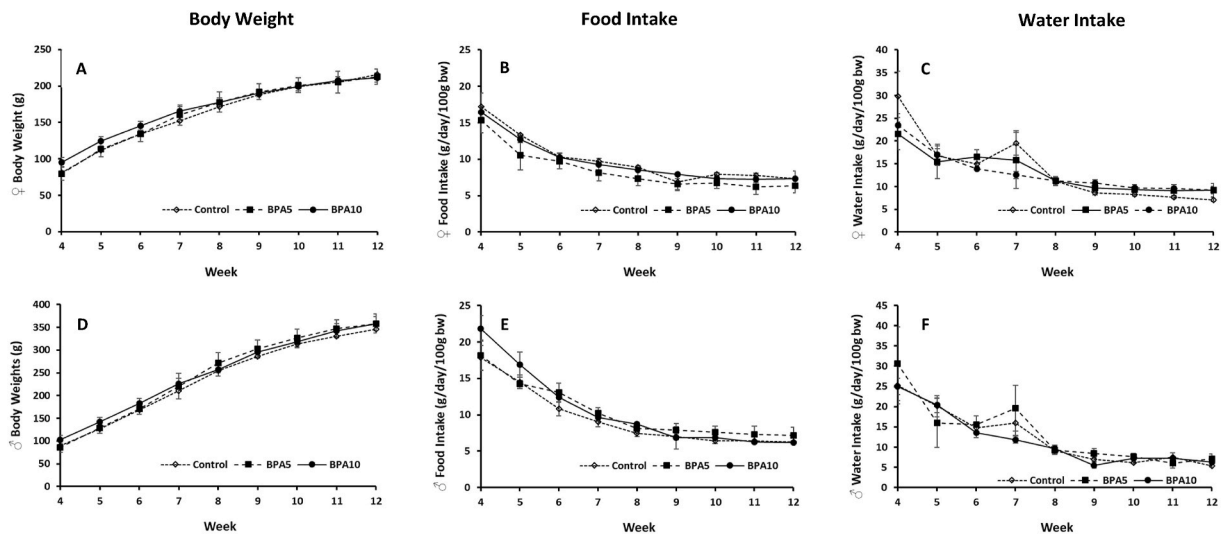
Fig. 3. Post-weaning changes for 12 weeks in body weight (g), food intake (g/day/100 g b.w.) and water intake (mL/day/100 g b.w.) of female and male offspring exposed to water (containing 1% ethanol, vehicle) or water containing BPA (BPA5, 5 mg/L or BPA10, 10 mg/L) during gestation and lactation. N = 16 animals/group/sex. Values are means ± S.E.M.
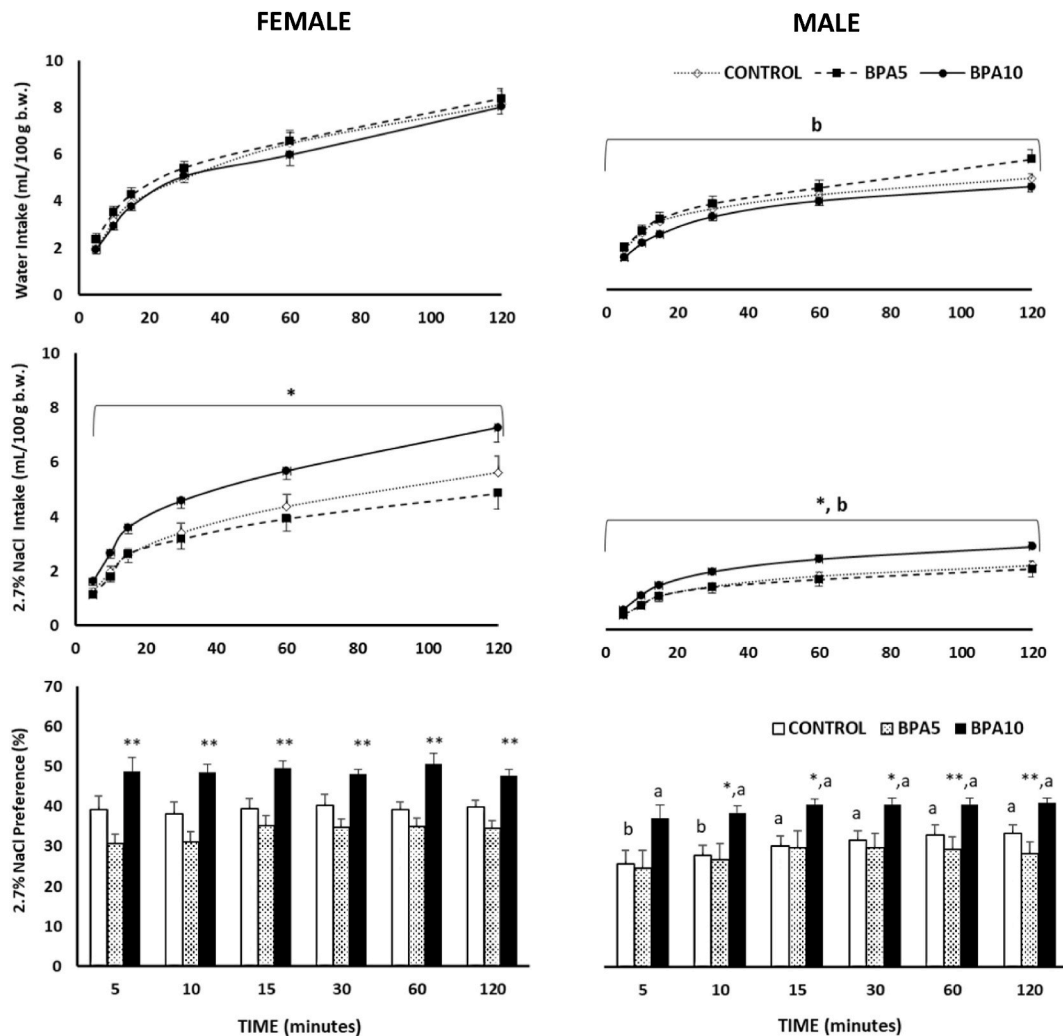
Fig. 4. Cumulative intake of water and 2.7% NaCl solution intakes (mL/100 g b.w.) and 2.7% NaCl preference at 120 min after a 24-h period of water deprivation in control, BPA5 and BPA10 offspring. N = 16 animals/group/sex. Results are expressed as the mean ± SEM. [b]$p < 0.01$ or [a]$p < 0.05$, females vs. males. In water and saline intake: *$p < 0.05$, BPA10 group vs. BPA5 group. In 2.7% NaCl solution preference: **$p < 0.01$; *$p < 0.05$, BPA10 group vs. control and BPA5 groups.
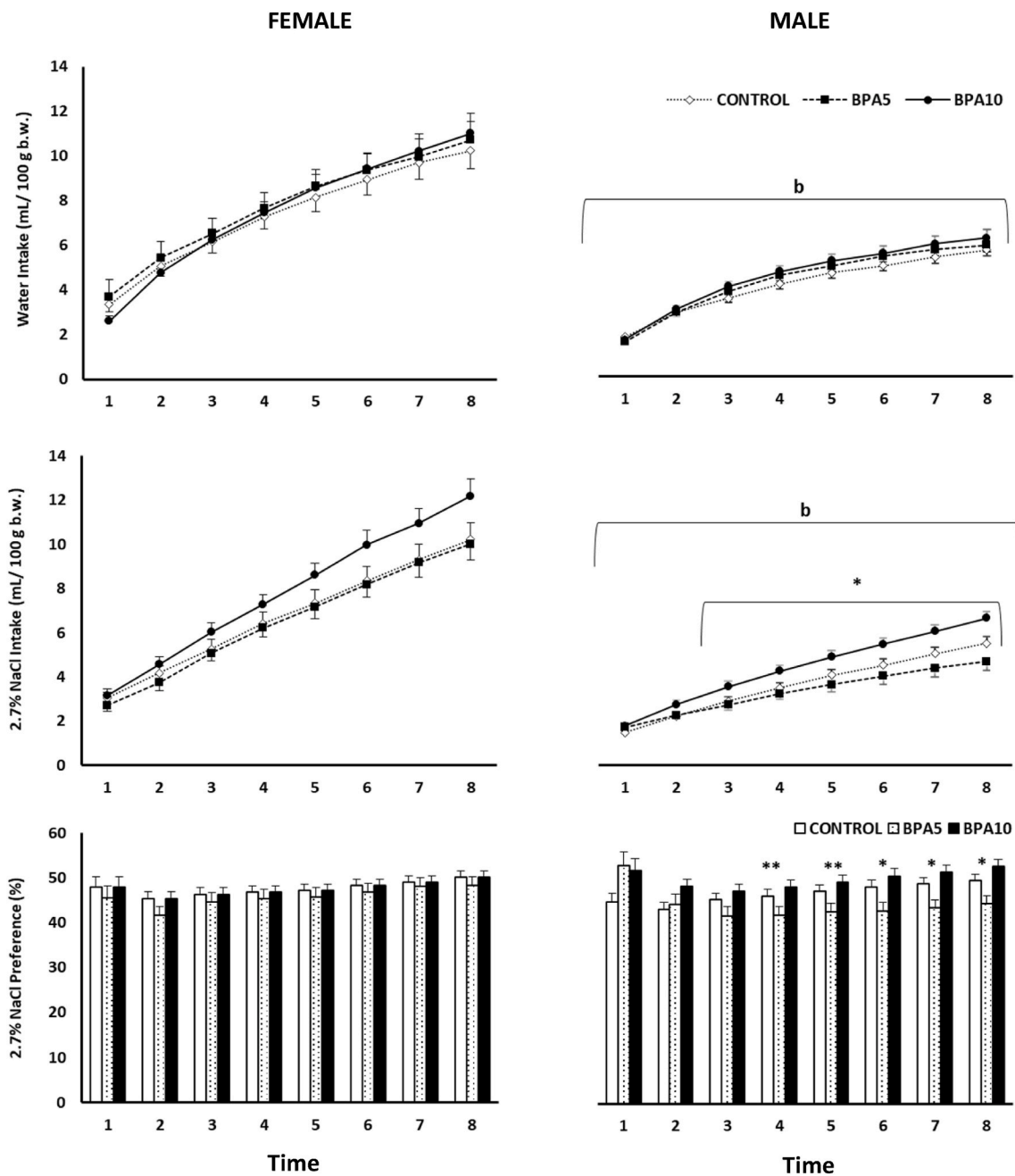
Fig. 5. Cumulative intake of water and 2.7% NaCl solution intakes (mL/100 g b.w.) and 2.7% NaCl preference in response to sodium depletion during the 4-h salt appetite test in control, BPA5 and BPA10 offspring. N = 16 animals/group/sex. Results are expressed as the mean ± SEM. In water and saline intake: *p < 0.05, BPA10 group vs. BPA5 group. ^{b}p < 0.01, females vs. males. In 2.7% NaCl solution preference: **p < 0.01; *p < 0.05, BPA10 group vs. control and BPA5 groups.

significantly greater (p < 0.01) water balance than the males across all groups. UV was different between control and BPA10 rats (p < 0.05) in females and between control and BPA5 rats (p < 0.05) in males (Table 2). The predominantly lower UV values of rats were accompanied by smaller overnight water intake, hence water balance immediately before tests was similar among all groups. Rats drank enough fluid during the 4h test to replace more than the amount lost during the 20h depletion.

Urine collection was done for the first hour after the furosemide injection and was repeated 19 h later. In the first hour, Una and UnaV were both significantly greater (p < 0.01) in females than in males in the control group and the BPA10 group, but not in the BPA5 group. Nineteen hours later, Una and UnaV were significantly greater (p < 0.05) in females than males only in the BPA5 group. When compared within the

same sex, UV, Una, and UnaV were similar among groups and no significant differences were observed (Table 3). Una over the entire 19h depletion period was significantly lower (p < 0.01) in all groups. It appears that Una in the rats decreased at some point after the first hour of depletion, presumably when the effects of furosemide began to decline. The Na intake and Na balance were significantly higher (p < 0.05) in females than in males for all groups, but there was no difference among groups. Rats drank enough 2.7% NaCl solution during the 4h test to replace more sodium than the amount lost during the 20h depletion. We did not collect urine during the 4h drinking tests, so we cannot ascertain the cumulative water and sodium balances at the end of the testing period. However, it is evident that all groups maintained their capacity to ingest sufficient water and 2.7% NaCl solution to restore deficits accrued during the 20 h preceding the drinking tests.

Table 2
Relative water balance. Values are expressed as the mean $\pm$ SEM. Urine volume (UV), water intake and relative water balance were measured in the 20-h preceding the salt appetite test, total fluid intake during the 4-h salt appetite test, and relative water balance at the end of testing. UV was different between control group and BPA10 group (*p $<$ 0.05) in females and between control group and BPA5 group (*p $<$ 0.05) in males. [b]p $<$ 0.01, males vs. females.

		20-h UV (mL/100 g bw)	20-h water intake (mL/100 g bw)	Pretest water balance (mL/100 g bw)	4-h fluid intake (mL/100 g bw)	Relative water balance (mL/100 g bw)
Control	*Female*	6.81 $\pm$ 0.41*	13.23 $\pm$ 3.56	−6.69 $\pm$ 3.73	20.46 $\pm$ 1.45[b]	25.73 $\pm$ 3.72[b]
	Male	7.15 $\pm$ 0.53*	9.04 $\pm$ 1.73	−1.69 $\pm$ 1.51	11.28 $\pm$ 0.47	13.28 $\pm$ 1.52
BPA5	*Female*	9.06 $\pm$ 0.59	13.77 $\pm$ 2.03	−4.55 $\pm$ 2.12	20.73 $\pm$ 1.26[b]	24.70 $\pm$ 2.28[b]
	Male	9.71 $\pm$ 0.96	10.51 $\pm$ 1.83	−0.76 $\pm$ 1.72	10.68 $\pm$ 1.56	11.39 $\pm$ 1.68
BPA10	*Female*	9.54 $\pm$ 1.18	8.60 $\pm$ 1.27	0.88 $\pm$ 0.32	23.04 $\pm$ 1.44[b]	22.40 $\pm$ 1.28[b]
	Male	7.92 $\pm$ 0.34	6.58 $\pm$ 0.46	1.34 $\pm$ 0.38	12.99 $\pm$ 0.56	11.83 $\pm$ 0.56

Table 3
Relative sodium balance. Values are expressed as the mean $\pm$ SEM. Urine volume (UV), urinary Na concentration (Una) and urinary Na excretion (UnaV) were measured in 1h (A) and 20h (B) after furosemide injection. Na intake during the 4-h salt appetite test, and relative Na balance at the end of testing are showed (C). [a]p $<$ 0.05, [b] p $<$ 0.01, males vs. females. *p $<$ 0.05, BPA5 group vs. BPA10 group.

A		1h UV (mL/100g bw)	1h Una (mmol/L/100g bw)	1h UnaV (mmol/L/100g bw)
Control	*Female*	2.57 $\pm$ 0.12	58.35 $\pm$ 7.06	0.33 $\pm$ 0.05
	Male	1.86 $\pm$ 0.18[b]	27.65 $\pm$ 1.68[b]	0.19 $\pm$ 0.02[b]
BPA5	*Female*	2.36 $\pm$ 0.27	46.73 $\pm$ 4.66	0.22 $\pm$ 0.03
	Male	2.30 $\pm$ 0.18	28.73 $\pm$ 2.04[a]	0.23 $\pm$ 0.02
BPA10	*Female*	2.50 $\pm$ 0.15	44.37 $\pm$ 4.39	0.25 $\pm$ 0.01
	Male	1.99 $\pm$ 0.15[b]	23.57 $\pm$ 2.12[a]	0.19 $\pm$ 0.01[b]

B		19h UV (mL/100g bw)	19h Una (mmol/L/100g bw)	19h UnaV (mmol/100g bw)
Control	*Female*	0.10 $\pm$ 0.02	10.85 $\pm$ 2.38	0.09 $\pm$ 0.02
	Male	0.12 $\pm$ 0.02	8.50 $\pm$ 1.77	0.12 $\pm$ 0.02
BPA5	*Female*	0.09 $\pm$ 0.02	10.59 $\pm$ 4.42	0.09 $\pm$ 0.20
	Male	0.18 $\pm$ 0.04[a]	12.12 $\pm$ 3.04	0.18 $\pm$ 0.04[a]
BPA10	*Female*	0.08 $\pm$ 0.01	11.32 $\pm$ 4.94	0.08 $\pm$ 0.01
	Male	0.13 $\pm$ 0.03	5.75 $\pm$ 1.45	0.13 $\pm$ 0.03

C		4h, 2.7% NaCl Intake (mL/100g bw)	4h, Na Intake (mmol/100g bw)	4h, Test Na balance (mmol/100g bw)
Control	*Female*	10.09 $\pm$ 1.14	4.63 $\pm$ 0.52	4.18 $\pm$ 0.49
	Male	5.53 $\pm$ 0.32[b]	2.74 $\pm$ 0.28[b]	2.42 $\pm$ 0.29[b]
BPA5	*Female*	11.80 $\pm$ 1.16	5.41 $\pm$ 0.53	5.09 $\pm$ 0.53
	Male	4.71 $\pm$ 0.41*,[b]	2.61 $\pm$ 0.39[b]	2.15 $\pm$ 0.39[b]
BPA10	*Female*	12.18 $\pm$ 0.76	6.46 $\pm$ 0.82	6.10 $\pm$ 0.83
	Male	6.69 $\pm$ 0.30[b]	3.06 $\pm$ 0.20[b]	2.73 $\pm$ 0.18[b]

4. Discussion

Body fluid balance is maintained by a complex network of systems that regulate fluid and electrolyte excretion, and sodium and fluid intake. The development of these control systems occurs during the fetal period and therefore, may be susceptible to changes in the uterine environment. According to the hypothesis of the fetal origins of adult disease, prenatal development is a critical window of vulnerability during which the individual is particularly sensitive to environmental stressors (Barker, 1990; Barker & Osmond, 1986). In utero conditions can permanently change body structure and function, which could cause disease in later life. Consequently, alterations in metabolic pathways during fetal development may contribute to specific pathological conditions, including neurodevelopmental and cardiovascular diseases in adult life (Alastalo et al., 2013; Fleming et al., 2021; Langley-Evans et al., 1996; Law et al., 2002; Pandolfini et al., 2021; Philips et al., 2017).

It is known that BPA can be transferred from the mother to the fetus during pregnancy and lactation, since this chemical has been detected in breast milk, amniotic fluid, placental tissue and fetal serum (Aris, 2014; Mendonca et al., 2014; Vandenberg et al., 2010). Thus, there is a growing concern about prenatal BPA exposure and its connection to adult diseases (Schug et al., 2011; Alonso-Magdalena et al., 2015; Hsu et al., 2019). In the present work, we studied the effects of two low maternal BPA doses, during pregnancy and lactation, on ingestive behavior and fluid and sodium excretion after dipsogenic challenges in the adult offspring.

4.1. Developmental measurements

No treatment-related effects on body weight gain, food intake and water consumption were observed in BPA-treated dams in agreement with previous studies (Nuñez et al, 2021). Gestational index (the ratio of the numbers of dams with live litters to the number of pregnant dams) was similar for all groups when using lower doses of BPA (5 µg/kg/day), according to Kaimal et al. (2021). In the offspring, no treatment-related effects on growth were found, although the effects of different gestational BPA doses in body weights in postnatal rat pups were reported by CLARIFY-BPA studies (Uchtmann et al., 2020); doses below 250

µg/kg/day lowered body weight, but no differences were found in pups' body weight for higher doses, in line with our results. Offspring survival and total number of pups per litter did also not differ. However, sex ratio was altered, a greater number of females were observed in the BPA-treated dams' litters. Hahn and Hays (1963) reported that the sex ratio can be significantly altered by giving exogenous ovarian steroids, but we did not find similar post BPA treatment results in the literature. The lower percentage of male BPA5 and BPA10 offspring as compared to control offspring may have resulted from one particular litter of each BPA group containing 7 females and only 3 males.

4.2. Post-weaning measurements

Previous research confirm that the behavioral profile of BPA-treated males was feminized (Adriani et al., 2003; Gioiosa et al, 2007; Jones and Watson, 2012). The offspring of BPA-treated dams showed the same ingestive behavior and sexual dimorphism as the control offspring, suggesting no estrogenic BPA effect on the brain pathways that control spontaneous water intake. Sex differences in fluid intake have been established by various studies since Richter and Brailey (1929), and appear to be the result of organizational effects of gonadal hormones during development. The difference in water intake between females

and males almost disappears when the intake is calculated per 100 g of body weight. Our results clearly confirm this well-established paradigm.

4.3. Experiment 1: 24h fluid restriction

The 24h water deprivation is associated with hypertonicity and hypovolemia systemic, and induces dehydration that is a mixture of both intracellular and extracellular water loss (Santollo et al., 2017). A water-deprived animal efficiently corrects its intracellular dehydration if it ingests only water, but remains hypovolemic (i.e., negative sodium balance) with high plasma renin activity and angiotensin II. Therefore, it is not surprising that it also ingests sodium. The stimulated dipsogenic response, for both water intake and salt intake, significantly increased immediately after the 24h water deprivation in both control and BPA offspring. This behavior indicates that dipsogenic mechanisms were unaltered in BPA offspring, regardless of BPA dose. Similarly, sex differences were preserved in this response, which is in agreement with previous studies that have described how intact adult female rats are more responsive than adult male rats to stimuli acting through the pathways of extracellularly induced thirst and salt intake (Kaufman, 1980; Chow et al., 1992; Quirós Cognuck et al., 2020). On the other hand, differences in taste sensitivity between males and females may contribute to salt intake difference, because concentration of estrogens decreases gustatory sensitivity to sodium (Curtis & Contreras, 2006).

Water intake of BPA offspring after 24h fluid restriction was similar to that of control offspring, but 2.7% NaCl solution intake and 2.7% NaCl solution preference were higher in BPA10 offspring. Ferguson et al. (2003) and Flynn et al. (2005) reported a significant increase in consumption of a 3.0% NaCl-flavored solution in male and female offspring of dams treated with compounds with estrogen-like activity. The 2.7% NaCl solution preference of BPA5 offspring is particularly interesting because there was no significant difference between females and males. Additionally, the increased preference for 2.7% NaCl solution in male BPA10 offspring is very similar to the behavior of female control offspring. These results indicate that this ingestive behavior appears to be due to feminization of males (they are matched to female offspring) and "hyperfeminization" of female BPA offspring as they drink more than female control offspring. Again, the hormonal role appears decisive, decreasing saline sensibility and discrimination in offspring. Sexual dimorphism in taste preferences has already been established, and the influence of estrogens exposure during the neonatal period has been demonstrated through salt intake (Krecek et al., 1972). Sexual differences also exist in adult rats which had been gonadectomised at the age of 10 days (Curtis et al., 2004).

Exposure to BPA interferes with the dimorphic development of the neuronal networks controlling brain functions (Desai et al., 2018; Mackay et al., 2013; Rubin et al., 2006; Wolstenholme et al., 2011) and alters dimorphic ingestive behavior (Negri-Cesi & Bisphenol, 2015). Our results do not show any masculization signal in female ingestive behavior, but showed male feminization, because maternal BPA exposure altered the sexual dimorphism in saline intake stimulated by water deprivation and sodium depletion. Therefore, BPA could be capable of altering important events during critical periods of brain development of fetuses, which in turn, can affect their fluid and sodium intakes much later in life.

4.4. Experiment 2: extracellular fluid depletion

The furosemide experiment compared the salt appetite and renal responses of offspring during a single episode of sodium depletion. The ingestion of sodium after furosemide-induced depletion is mediated largely by increased renin secretion and the resultant increased levels of circulating angiotensin II (Meyer et al., 1968). During the two-bottle test, sodium-depleted rats exhibited higher 2.7% NaCl solution intake associated with decreased water intake, suggesting a greater preference for 2.7% NaCl solution. When sodium-depleted rats had access to 2.7%

NaCl solution, male offspring consumed less sodium fluid overall (i.e., water + 2.7% NaCl solution) than female offspring. Therefore, all rats reliably compensated for their increased sodium deficit. Nonetheless, male offspring drank sufficient fluid during testing to achieve a positive water balance. This is consistent with several reports of increased sodium intake by female rodents. These differences have been attributed to differential gonadal hormone levels in male and female rats, with previous data demonstrating that testosterone inhibits (Krecek et al., 1972) and estrogens enhances sodium intake (Curtis et al., 2004; Curtis & Contreras, 2006ent did not affect fluid intake in furosemide-induced response of female offspring, although female BPA10 offspring showed a slight increase in 2.7% NaCl solution intake compared to control and BPA5 offspring. In contrast, male offspring showed a difference in 2.7% NaCl solution intake as a response to maternal BPA treatment, with BPA10 offspring showing higher 2.7% NaCl solution intake as compared to BPA5 offspring. BPA5 offspring showed lower intake compared to control and BPA10 offspring. Both male and female BPA10 offspring showed a similar increased 2.7% NaCl solution intake. No significant differences in preference were observed. It is known that furosemide-induced sodium depletion alters salt palatability; it increases the hedonic properties and decreases the aversive properties of the taste of sodium (Sorge et al., 2002; St John, 2017).

The analysis of urine volume and its sodium concentration allowed us to study kidney function in this sodium depletion experiment. One hour after depletion, female control and BPA10 offspring exhibited increased excretion of urine and sodium as compared to male offspring; this sexual dimorphism was missing in BPA5 offspring. During the 19 h after depletion, male and female offspring showed an expected decrease in urine and sodium excretion, because the effects of furosemide manifest rapidly, lasting for two to three hours (Ogawa et al., 1984). For male BPA5 offspring, while diuresis after the furosemide injections was higher as compared to male control offspring and male BPA10 offspring, natriuresis was not. The sodium balance was similar in control offspring and BPA offspring, showing that renal handling of sodium is not altered; this indicates correct functioning of the underlying mechanisms for sodium renal handling, which adapt perfectly to higher sodium intake.

4.5. Conclusions

Behavioral responses are critical for defending the body against any threats to the optimal water-salt balance which can lead to long-term changes in the cardiovascular system, even in BPA exposure below the safe limit for humans as prescribed by regulatory agencies (Gore et al., 2019; Ma et al., 2019). In our experiment, both 24h fluid deprivation and sodium depletion—induced by acute furosemide treatment—produced a modified fluid ingestive response in BPA offspring compared to control, although a BPA did not influence spontaneous thirst. The causal relationship between early BPA exposure and changes in salt preference remains unclear. Similar results obtained by different protocols have also reported increased activity of the maternal renin-angiotensin system (Galaverna et al., 1995; Leshem, 1998; Argüelles, Brime, López-Sela, Perillán, & Vijande, 2000). Recently, it was found that the expression of Angiotensin II was upregulated after BPA treatment in vitro (Gao et al., 2019). Therefore, future studies of the potential impact of BPA on the renin-angiotensin system are warranted.

Gestational BPA exposure in animal models demonstrates that the hypothalamus is a particularly susceptible brain region, which is also responsible for the regulation of neuroendocrine interactions and a variety of behaviors including ingestive behavior (Nesan et al, 2018). Sodium appetite appear to be located in the sub-fornical organ, amygdala, brainstem, and circumventricular organs (Morris et al., 2008). These cerebral areas increase in volume in the offspring of mothers treated with different doses of BPA, suggesting that the developing brain is vulnerable to endocrine disruption (Arambula et al., 2017). Nesan et al. (2021) confirm that gestational low-dose BPA exposure impacts neurogenesis in the hypothalamic nucleus. Our results show that exposure to

low doses of BPA in early life may disrupt the development of sex-specific behaviors by perhaps altering the steroid programming of the brain, and this disruption affects males and females differently. This has been consistently demonstrated in current Endocrine Disrupting Chemicals (EDCs) research. This indicates the relevance of analyses that include both sexes and assess sexually differentiated/dimorphic responses as outcomes of developmental exposure to EDCs. Using a model of maternal treatment with low BPA doses, our results suggest that BPA does not disrupt body fluid balance under spontaneous conditions, but alters sodium and fluid intakes in the exposed offspring under osmotic challenges. Therefore, early-life exposure to BPA should be considered as a possible risk factor for the future development of cardiovascular diseases.

Authors' contributions

Paula Nuñez: Conceptualization, Methodology, Investigation, Formal analysis, Data curation, Funding acquisition, Writing-review & editing. Juan Arguelles: Formal analysis, Writing -review & editing. Carmen Perillan: Conceptualization, Methodology, Investigation, Formal analysis, Writing -review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported.

Ethical approval on animal research

All experimental procedures followed the guidelines set forth in the Care and Use of Animals, and The University of Oviedo's Experimental Animal Use Committee approved all experimental procedures (PROE 15/2016).

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