Developmental exposure to bisphenol A (BPA) alters sexual differentiation in painted turtles (Chrysemys picta)

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Abstract

Environmental chemicals can disrupt endocrine signaling and adversely impact sexual differentiation in wildlife. Bisphenol A (BPA) is an estrogenic chemical commonly found in a variety of habitats. In this study, we used painted turtles (Chrysemys picta), which have temperature-dependent sex determination (TSD), as an animal model for ontogenetic endocrine disruption by BPA. We hypothesized that BPA would override TSD and disrupt sexual development. We incubated farm-raised turtle eggs at the male-producing temperature (26 °C), randomly assigned individuals to treatment groups: control, vehicle control, 17β-estradiol (E2, 20 ng/g-egg) or 0.01, 1.0, 100 µg BPA/g-egg and harvested tissues at hatch. Typical female gonads were present in 89% of the E2-treated "males", but in none of the control males (n = 35). Gonads of BPA-exposed turtles had varying amounts of ovarian-like cortical (OLC) tissue and disorganized testicular tubules in the medulla. Although the percentage of males with OLCs increased with BPA dose (BPA-low = 30%, BPA-medium = 33%, BPA-high = 39%), this difference was not significant (p = 0.85). In all three BPA treatments, SOX9 patterns revealed disorganized medullary testicular tubules and β-catenin expression in a thickened cortex. Liver vitellogenin, a female-specific liver protein commonly used as an exposure biomarker, was not induced by any of the treatments. Notably, these results suggest that developmental exposure to BPA disrupts sexual differentiation in painted turtles. Further examination is necessary to determine the underlying mechanisms of sex reversal in reptiles and how these translate to EDC exposure in wild populations.

1. Introduction

Many anthropogenic environmental chemicals are endocrine disrupting chemicals (EDCs) and can disrupt normal physiological and developmental signaling. Some EDCs act as xenoestrogens and may impact wildlife species through feminization or demasculinization, especially if exposure occurs during the developmental period. Bisphenol A (BPA) is one such synthetic estrogen mimic. At least 8, but up to as much as 15 billion pounds of BPA are produced annually (GrandViewResearch, 2014) in a variety of everyday plastics and epoxy resins making it one of the most ubiquitous EDCs (Vandenberg et al., 2010, 2013). In the environment,
Studies on aquatic species offer a way to monitor and better understand the potential impacts of contaminants like BPA on human and animal health. Turtles are important indicators of environmental health because of their extended juvenile period, high site fidelity, low metabolic rate, use of large fat deposits for egg development (Seler, 2006), and maternal transfer of lipid-soluble xenoestrogens during egg formation (Basile et al., 2011). Additionally, aquatic turtles reside in the water column and sediments of aquatic environments, which enhance the potential for exposure to BPA and other EDCs (Bhandari et al., 2014; Kasotos et al., 2013). Furthermore, female turtles nest in adjacent terrestrial sites potentially exposing their eggs to contaminants in the soils. As a first step in determining potential impacts of BPA on free-living aquatic turtle populations, it is imperative that we determine sensitivity under laboratory conditions.

One life trait of most turtle species is their temperature-dependent sex determination (TSD). Three TSD patterns are found in turtle species, with painted turtles (Chrysemys picta) demonstrating pattern 1a (Bull, 1980; Ernst and Lovich, 2009). In this pattern of TSD, the temperature at which the eggs develop during incubation determines the sex of the hatchlings such that cool temperatures produce males and warmer temperatures produce females (Bull, 1980; Ewert et al., 2004). Previous studies have shown that temperature determination can be overridden when reptile eggs are exposed to EDCs including: several legacy organochlorine contaminants in red-eared slider turtles (Trachemys scripta elegans; Bergeron et al., 1994; Matsumoto et al., 2014; Willingham and Crews, 1999); BPA and 17β-estradiol in Caiman latirostris (Stoker et al., 2002); and TCDD, o,p'-DDE, p,p'-DDE, indole-3-carbolin, and 17α-ethinylestradiol (EE2) in Alligator mississippiensis (Matter et al., 1998). Finally, Sheehan et al. (1999) determined 17β-estradiol, an endogenous estrogen and positive control for many EDC studies, induced sex reversal in turtles at every low vein and with evidence for the absence of a threshold dose. The authors suggest that other EDCs mechanically similar to 17β-estradiol may also show no threshold dose for sex reversal.

Most turtle sexual development begins with a bipotential gonad, which under the influence of temperature or chemicals, will develop into an ovary or testis. Initially Sox9 (sex determining region box 9) is expressed in the bipotential gonad (Morrish and Sinclair, 2002). However, with the development of Sertoli cells in the male testis, Sox9 expression is up-regulated (Spotila et al., 1998). If the gonad is to develop into an ovary, the existing Sertoli cells become lacunae (Mork and Capel, 2013), medullary structure containing Sertoli cells within the testicular medullary tubules (Barske and Capel, 2010; Bergeron et al., 1999; Crews et al., 1991; Pieau et al., 1999).

2. Materials and methods

2.1. Turtle egg incubation, dosing, and tissue collection

Freshly laid (<24 h post oviposition) painted turtle eggs were purchased from Louisiana Cypress Turtle Farms (Pierre Part, LA). Eggs were transported to the USGS Columbia Environmental Research Center (Columbia, MO) under the appropriate state and federal permits. This research was conducted under an approved Animal Welfare Plan (AWP) at the Columbia Environmental Research Center (CERC). All personnel involved in the animal care and use were required to adhere to the AWP which followed the spirit and intent of the policies and regulations that assure humane and ethical treatment of research animals. The AWP was reviewed by the CERC Animal Use Committee for compliance with the Animal Welfare Act and associated amendments, principles and guidelines. Additionally, we followed the NIH guidelines for the care and use of laboratory animals.

At the laboratory, we candled eggs to determine viability and developmental stage according to Mahmoud et al. (1973), weighed, and to minimize clutch effects and treatment bias randomly assigned the eggs to a treatment and a location within BPA-free incubation boxes. Eggs were placed in moistened vermiculite (1:1 v/v autoclaved vermiculite: water) and incubated at male-producing temperature. Additionally, we followed the NIH guidelines for the care and use of laboratory animals.

2.1.1. Egg incubation

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forty eggs at female-producing temperatures (30 ± 1°C) under otherwise identical conditions as non-treated controls or vehicle controls for comparisons with sex-reversed males. At hatch, we measured straight-line carapace and plastron lengths and carapace width using vernier calipers and we determined mass using a portable Ohaus Trooper balance. Hatchlings were then euthanized and liver and urogenital tissues were harvested. The right kidney and gonad were fixed in Bouin’s solution for 12h, washed in 70% EtOH over 3 days and then stored in 70% EtOH until histological analysis. The left gonad was collected for future gene expression studies.

2.2. Gonad histology

We serially dehydrated and embedded the urogenital tissues in paraffin. Sections (5–7 μm) were stained using Richard-Allan™ Hematoxylin 7211 and Richard-Allan™ Eosin-Y, Saturated (Thermo Scientific, Waltham, MA, USA). Histology was performed by Idexx Radil Bioresearch Laboratory (Columbia, MO) or by one of the authors (CMJ). Sex was determined by visual inspection of the histological slides independently and blindly by three of the authors (CMJ, SLD and DKH) and assessments made by comparison to images in Pieau and Dorizzi (2004), Wyneken et al. (2007). The following definitions were agreed upon by all observers. Testes were defined as a simple single-layered epithelial cortex and a well-formed medulla with testicular tubules; whereas ovaries exhibited a well-developed multi-layer cortex and a disorganized medulla occasionally with large lacunae (Fig 1). Gonads which had a thicker epithelial cortex with some medullary testicular tubules with varying amounts of disorganization were considered to be disrupted and categorized as ovarian-like cortex (OLC) sensu (Pieau et al., 1998). The term ovotestis or intersex was not used because it was unclear if the gonad at maturation would be capable of producing both eggs and sperm (Crews et al., 1991). We did not collect data on the presence or absence of Müllerian ducts.

2.3. Gonad Immunohistochemistry

Immunohistochemistry with gonadal markers was performed following methods similar to Mork and Capel (2013) on a subset of individuals (n = 7 per treatment). This subset of individuals represented multiple clutches to minimize any clutch effects and had complete histological sections through the entire gonad. Tissues were deparaffinized, rehydrated, and antigens retrieved by Sodium Citrate Heat-Induced Epitope Retrieval (Larsson, 1988). The slides were then submerged in blocking buffer (3.0% Bovine Serum Albumin and 3.0% Donkey Serum in PBS) and incubated for 1h. Primary antibodies, 1:500 dilution of SOX9 (EMD Millipore, Billerica, MA, USA) and 1:200 dilution of β-catenin (Sigma–Aldrich, St. Louis, MO, USA), diluted in blocking buffer were applied, and slides incubated overnight at 4°C. We then incubated the slides at 24°C for one hour with Cy-3 Conjugated Donkey anti-mouse diluted 1:100 in PBS (Jackson Immunoresearch, West Grove, PA, USA) and Alexa Fluor 488 Donkey anti-rabbit IgG diluted 1:300 in PBS (Abcam, Cambridge, MA, USA). Finally, Vectashield (Vector Laboratories, Burlingame, California, USA) and Prolong

Fig. 1. The effect of in ovo exposure of BPA and E2 during the temperature sensitive period on Chrysemys picta gonad structure. Photomicrographs of H&E images of full gonads at 10× (top row) and 20× (bottom row) showing details. Labeled are the single-layered epithelium (E) and testicular tubules (TT) characteristic of males and a thicker cortex (C) and lacunae (L) characteristic of females and those with an ovarian-like cortex (OLC). Scale bar = 50 μm.
2.4. Vitellogenin ELISA

We quantified vitellogenin protein using an enzyme-linked immunosorbent assay (ELISA) following methods similar to that of Irwin et al. (2001) on the same individuals used for IHC (*n* = 7 per treatment). Approximately one-third of each liver was weighed and placed in a 1.5 ml cryotube. Lysis beads, the same weight as the liver, were added to the cryotubes and 1 × TBS added at twice the original tissue mass. Cryotubes were placed in the Bullet Blender and mixed for <1 min. Homogenized livers were then centrifuged for 10 min at 3600 rpm at 4 °C. The supernatant was collected and stored at −80 °C until analysis. Standards were diluted in TBS (25 mM Tris, 0.3 M NaCl, pH 7.4) and loaded into a 96 well microtiter Nunc Maxisorb plates (50 µL per well). In addition to the samples, each plate contained standards, adult female box turtle plasma as a positive control (provided by SLD), and non-specific binding wells. All samples including positive controls were diluted 1:2500 and run in duplicate. The plate was incubated overnight at 4 °C. After incubation, plates were warmed to room temperature for 30 min and then washed three times with 200 µL of TBST (TBS + 0.2% Tween 20) and once more with TBS followed by blocking with 150 µL Blotto Tween (5% BSA, 0.2% Tween 20) for two hours at room temperature. The plates where then washed three additional times with 200 µL of TBST and once with TBS. We added 50 µL of primary antibody (provided by KWS) diluted 1:1000 in Blotto Tween and incubated the plates at room temperature for two hours. Plates were again washed three times with TBST and once with TBS. Secondary Antibody, Alexa Fluor® Goat anti-rabbit IgG (H + L) conjugated to alkaline phosphatase (Molecular Probes®), Life Technologies, Grand Island, NY, USA), diluted 1:1000 in Blotto Tween was added and incubated for two hours. Plates were then washed four times with TBST and four times with TBS. After the last wash, plates were developed using 0.1% (w/v) p-nitro-phenyl phosphate (PNPP) in developing buffer (0.1 M NaCl, 0.1 M Tris, 5 mM MgCl₂, pH 9.5) and incubated at room temperature for 15 min. Optical density was read with a Synergy4 BioTek Microplate Reader at 405 nm.

2.5. Statistics

Data were statistically analyzed using NCSS Version 8.0 (Kaysville, UT) with an alpha = 0.05. We used a Fisher’s Exact Test to compare hatching success among treatments and morphological measurements were analyzed using ANOVA for males and a t-test for females. The percentage of OLC or female was compared across treatment groups, and then between the three BPA treatments using Chi square Goodness of Fit tests. Because the vitellogenin data were not normally distributed, data were log transformed prior to an ANOVA. Figures were constructed using SigmaPlot Version 13.0 (Systat Software, San Jose, CA) and CorelDraw Version X3 (Ottawa, Canada).

### Table 1

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Eggs incubated</th>
<th>Eggs hatched</th>
<th>Percent hatched (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male control</td>
<td>46</td>
<td>35</td>
<td>76</td>
</tr>
<tr>
<td>Male control (EtOH)</td>
<td>47</td>
<td>42</td>
<td>89.4</td>
</tr>
<tr>
<td>Female control</td>
<td>20</td>
<td>17</td>
<td>85</td>
</tr>
<tr>
<td>Female control (EtOH)</td>
<td>20</td>
<td>18</td>
<td>90</td>
</tr>
<tr>
<td>E2 20 ng/g-egg</td>
<td>45</td>
<td>33</td>
<td>73.3</td>
</tr>
<tr>
<td>0.01 µg BPA/g-egg</td>
<td>44</td>
<td>42</td>
<td>95</td>
</tr>
<tr>
<td>1 µg BPA/g-egg</td>
<td>48</td>
<td>40</td>
<td>83.3</td>
</tr>
<tr>
<td>100 µg BPA/g-egg</td>
<td>48</td>
<td>39</td>
<td>81.3</td>
</tr>
</tbody>
</table>

3.3. Immunohistochemical expression of SOX9 and β-catenin

Immunohistochemistry supported our histological findings above. Control male gonads expressed a distinct pattern of SOX9 associated with the Sertoli cells (Sc) of the testicular tubules (tt) and β-catenin expression was present in the testicular tubules (Fig. 4). Temperature-determined female gonads did not have SOX9 expression and β-catenin expression was predominantly localized to the cortex. Unlike the temperature-induced females, the sex-reversed E2-treated individuals continued to express SOX9 along the perimeters of the testicular tubules, but a thickened cortical region with strong β-catenin expression was also clearly visible (Fig. 4). Exposure to each of the BPA concentrations produced varying degrees of disorganization in the testicular tubules as evidenced by the disruption of medullary SOX9 signaling. The degree of disorganization seemed to increase with increasing BPA concentration (Fig. 4). The expression of β-catenin was consistent with the thickness of the developing cortex. Those individuals with more cortical development (Fig. 4, 1 µg BPA/g-egg) appeared to have greater expression of cortical β-catenin; whereas those with a thinner cortex had less expression (Fig. 4, 0.01 µg and 100 µg BPA/g-egg), but still followed the pattern observed in both temperature-induced and E2-produced females.

3.4. Vitellogenin induction

Even though gonads from E2 and BPA-treated individuals exhibited evidence of full to partial sex-reversal, these histological and functional changes did not result in the predicted induction of hepatic VTG (F = 1.24, p = 0.31). Pre-reproductive female hepatic VTG concentrations were not significantly different from male hepatic concentrations. All values measured were an order of magnitude lower than the positive controls (not shown). Interestingly, there was more variation in the female and BPA high samples than the other treatments and control males; however, this could be a function of our small sample size (Fig. 5; n = 7 per treatment).

4. Discussion

Here we demonstrated that BPA exposures topicaly applied to painted turtle eggs incubated at male-producing temperatures resulted in the disruption of sexual development. As expected, exposure to E2 overrode the male-producing temperature-induced testicular differentiation and produced predominantly females. In ovo BPA exposure resulted in sexual disruption, as evidenced by disruption of the testicular tubules and formation and thickening of the cortex producing OLCs. Exposure to 100 µg/g-egg resulted in sexual disruption in 39% of our turtles, whereas a similar exposure of 138 µg/g-egg caused 100% sex reversal in 10 days and 6 month-old Caiman latirostris (Stoker et al., 2003). However, exposure to a lower dose of BPA resulted in very similar degrees of sexual disruption in both studies (40% in caiman at 1.3 µg/g-egg, 33% in our painted turtles at 1.0 µg/g-egg). The only paper examining the effects of BPA on turtles (Clairardin et al., 2013) determined that in T. scripta BPA interfered with estradiol metabolism. Trachemys eggs treated with 40 µg/g BPA early in development prior to the TSP had greater concentrations of yolk estradiol and estrone and lesser concentrations of estrone sulfate than untreated control eggs. This suggests that developmental exposure to BPA alters metabolism of maternal estrogens and possibly makes them bioavailable during later times in development, including the temperature sensitive period (TSP).

We applied the BPA and E2 topically to the eggs during the known TSP to ensure a controlled exposure to all embryos across the treatment groups. Although, this method does not exactly mimic natural exposure via maternal transfer and/or from the nesting substrate for the duration of incubation, it does target the most sensitive period for disruption of sex determination. The method we used in this study is similar to a number of other EDC studies in reptilian species (Crews et al., 1991; Matsumoto et al., 2014; Stoker et al., 2003). Although we did not explore mechanisms in the current study, our results of BPA-induced sex-reversal may be associated with altered hormone metabolism (Clairardin et al., 2013), in addition to binding to estrogen or other receptors (Reif et al., 2010), and thus both mechanisms and their relative importance warrant further study.

Because we collected samples at hatch, we were unable to determine if the observed effects persist into adulthood. However, our findings of BPA overriding TSD, even in this young cohort, suggest low doses of BPA can disrupt turtle sex...
Immunohistochemistry of *Chrysemys picta* gonads showing expression of SOX9 (green), β-catenin (red) and both overlaid at 100× and 200× (merged). Abbreviations are as follows: Sc = Sertoli cells, co = cortex, tt = testicular tubules. Scale bar = 100 µm at 100× and 50 µm at 200×. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
determination and future studies of hatchlings in older age classes is warranted to better understand possible effects at the population level. In this young age class, we observed that with increased concentrations of in ovo BPA, the percentage of OLCs tended to increase. Interestingly, a low dose of BPA (0.01 μg/g-egg) seemed to produce the same amount of disorganization within the gonad as the highest dose (100 μg-egg), suggesting that although BPA has previously been characterized as a weak estrogen with regard to its binding potency to estrogen receptors (Ben-Jonathan and Steinmetz, 1998; Vandenberg et al., 2009), it can produce gonadal disorganization and potential sex reversal across a wide range of exposures. Other research has demonstrated significant sex reversal in turtles (e.g., red-eared sliders) after topical exposure of eggs to a variety of EDCs (Bergeron et al., 1994; Crews et al., 1991; Matsumoto et al., 2014; Willingham and Crews, 1999). Although none of these prior studies examined BPA, all showed some degree of sex reversal, with up to 100% reversal in some instances. A number of these studies found a dose-dependent sex reversal (Bergeron et al., 1994; Crews et al., 1991; Willingham and Crews, 1999). Factors such as species, stage of development, length of exposure and dose seem to contribute to the variations in the degree of sex reversal observed in turtles.

Along with the histological examination of transforming gonads, it is essential to examine differentiation of gonadal cell types at the molecular level as these events can precede histological transformations. The expression of SOX9 and β-catenin together provide definitive information on the fate of a differentiating and developing gonad (Mork et al., 2014). Temperature-determined ovaries and E2-treated sex-reversed ovaries showed cortical enhancement of β-catenin expression and a reduction or absence of medullary SOX9 expression (Fig. 4). Recently, (Matsumoto et al., 2014) reported a similar reduction in Sox9 following exposure of otherwise male turtle embryos to EE2 or 4-hydroxy-2,4,6-trichlorobiphenyl. As predicted, testes continued to express β-catenin in the medulla (in association with the testicular tubules) and in the surface epithelium and SOX9 expression was clearly associated with the Sertoli cells. The gonads exposed to BPA produced altered expression patterns for these markers. Some gonads were clearly still male and histologically classified as such. Others were undergoing medullary reorganization with dissolution of testicular cords and varying amounts of cortical expansion. Typically during normal development, lacunae formation precedes cortical development (Wibbelts et al., 1991) so it is possible that with age further evidence of feminization of OLC gonads would be observed and thus our results may underestimate the true extent of changes from exposure to BPA. We are currently testing this hypothesis in a follow-up study.

The present results demonstrated that in ovo exposure to BPA causes sexual disruption in painted turtle embryos. We observed variation within treatments in the thickness of the cortex and the degree of testicular tubule disruption. Although turtle eggs are quite porous, the degree to which BPA can penetrate eggshells has not been studied. Another possible explanation for the degree of variation is that there may be individual differences in embryonic metabolism of these chemicals or possibly a distribution of sensitivities to BPA. Future studies should therefore examine uptake and pharmacokinetics of BPA in developing turtle embryos. Recently, Mork et al. (2014) demonstrated that although sexual differentiation of the gonad happens during the temperature sensitive period, there may be an earlier genetic or environmental sex-determining signal. Possibly, exposure at an earlier developmental stage would have resulted in a greater percentage of OLCs. Unfortunately, such a comprehensive temporal study would require a very large sample size and be cost-prohibitive.

One other endpoint tested in the current study was the induction of hepatic VTG. We chose this biomarker because it has been shown to be a powerful biomarker in fish (Marin and Matozzo, 2004) and has been demonstrated in turtles when assessing the impacts of EDCs on reproduction (Heck et al., 1997; Irwin et al., 2001; Palmer and Palmer, 1995). The lack of positive VTG induction in our study may be a result of the low dose of BPA tested or the low single dose of E2 as positive control (Irwin et al., 2001). Other studies with reptiles have failed to show VTG induction in the presence of a wide array of environmental contaminants (Irwin et al., 2001; Matter et al., 1998; Rie et al., 2005; Valverde et al., 2008). These collective findings may be an indication that VTG is not a good biomarker of estrogenized turtles and other reptiles and perhaps the use of other biomarkers should be further evaluated in these species.

Potential biomarkers may include biomolecular or epigenetic changes (such as altered DNA methylation status for select genes or altered expression of non-coding RNAs). The sequencing and annotation of the painted turtle genome (Shaffer et al., 2013) will assist in identifying these additional biomarkers that may be induced in BPA/E2 treated turtles and hold across other species (such as mammals) where currently no such reliable biomarkers have been discovered. The sequencing of this genome also allows for examination of potential molecular mechanisms driving the BPA/E2 induced sexual disruption in painted turtles.

Reptiles, including turtles, are one of the most threatened vertebrate taxa with habitat degradation, climate change, disease, and unsustainable harvest for food and the pet trade cited as the primary threats to their long-term survival (Gibbons et al., 2000). In addition to these commonly cited conservation challenges, it is imperative to explore other anthropogenic changes that may be driving shifts in population structure. Reliant on TSD, most turtles are increasingly threatened by climate change and environmental EDCs which both may lead to changes in the sex ratio of populations even prior to hatching (Bergeron et al., 1994; Janzen, 1994; Matsumoto et al., 2014; Sheehan et al., 1999; Willingham and Crews, 1999). Our findings of disruption during sexual differentiation following acute exposure to low doses of BPA may have far reaching consequences on sex ratios of wild populations and potentially exacerbate population decline. As sentinels for aquatic habitats, these findings have implications not only for turtle conservation and health, but for other aquatic organisms, and ultimately humans who also are reliant on a safe water supply (Bhandari et al., 2014).

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5. Conclusions

We demonstrated that developmental exposure to relevant concentrations of BPA or E2 caused a disruption in sexual differ-
entiation in painted turtle hatchlings. Histological analyses and
immunohistochemistry revealed disorganization of SOX9-express-
ing testicular tubules and the development of a β-catenin express-
ing ovarian-like cortex in the gonads of the BPA treated embryos.

Further studies are warranted to identify reliable biomarkers and
elucidate the underlying mechanisms responsible for these func-
tional and morphological changes.

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