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Teratogenicity and toxicity of the new BPA alternative TMBPF, and BPA, BPS, and BPAF in chick embryonic development

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ABSTRACT

Bisphenol A (BPA) is a widely known, yet controversial reproductive toxin, capable of inducing reproductive, developmental, and somatic growth defects across species. Due to scientific findings and public concern, companies have developed BPA alternatives remarkably similar to BPA. However, these alternatives have had much less testing and oversight, yet they are already being mass-produced and used across industries from plastics to food-contact coatings. The newest one, tetramethyl bisphenol F (TMBPF), is the least well-studied and has never been investigated in embryological models, however it continues to be mass produced and found in various products. Here, we used the chicken embryotoxicity screening test to compare the toxicities and potencies of several BPA analogs including TMBPF. We exposed developing chicken (*Gallus gallus domesticus*) embryos *in ovo*, from embryonic day 5 to 12 (E5–12), to increasing concentrations of BPA, bisphenol S (BPS), bisphenol AF (BPAF), and TMBPF, from 0.003 to 30 μM, and analyzed their developmental and toxic effects. The bisphenols significantly impaired development, growth, and survival in a dose-dependent manner, even at low, environmentally relevant concentrations of 3–30 nM. There was severely reduced growth and developmental delay, with exposed embryos averaging half the size and weight of control vehicle-treated embryos. The most common and severe dysmorphologies were craniofacial, eye, gastrointestinal, and body pigmentation abnormalities. The bisphenols caused dose-dependent toxicity with the lowest LC_{50}s (lethal concentration with 50% survival) ever demonstrated in chick embryos, at 0.83–2.92 μM. Notably, TMBPF was the second-most toxic and teratogenic of all chemicals tested (rank order of BPAF > TMBPF > BPS > BPA). These results underscore the adverse effects of BPA replacements on early embryo development and may have implications for reproductive health and disease across species, including pregnancy exposures in humans.

1. Introduction

Increased production and pollution of environmental toxins has led to ubiquitous exposure to toxic chemicals, many of which disrupt organismal development, growth, reproduction, and physiology (Flint et al., 2012; Mizell and Romig, 1997; Onundi et al., 2017; Vandenberg et al., 2007). Despite its well known environmental toxicity, the plastic chemical bisphenol A (BPA) is mass produced on a global scale. Its widespread use is due to its ability to increase durability and clarity within plastics and synthetic products, making it ideal for everyday consumer products such as food and liquid storage containers, cosmetic and personal care product packaging, medical, dental, and sports equipment, and others (Cao et al., 2011; Geens et al., 2012; Lassen et al., 2011; Lee and Peart, 2000; Liao and Kannan, 2013; Ozaki et al., 2004; Vandenberg et al., 2007; Winnebeck, 2013). Leaching of bisphenols from plastics and metal can coatings leads to ingestion and transdermal absorption into the skin (Bernier et al., 2017; Geens et al., 2012; Toner et al., 2018), as well as into the environment, causing bisphenols to be found in our water and soil systems (Chen et al., 2016a; Cousins et al., 2002; Flint et al., 2012; Fromme et al., 2002; Lee and Peart, 2000; Vandenberg et al., 2007; Yamazaki et al., 2015). This is particularly concerning given BPA's classification as an endocrine disrupting chemical (EDC) and its ability to interact with estrogen receptor- and androgen receptor-dependent signaling pathways (Gorini et al., 2020; Harnett et al., 2021a; Huang et al., 2016; Huang et al., 2021; Iwamuro et al., 2006; Kim and Park, 2019; Kitamura et al., 2005; Nomiri et al., 2019; Perera et al., 2017; Wang et al., 2017). Although there are conflicting studies in

Abbreviations: bisphenol A, BPA; bisphenol S, BPS; tetramethyl bisphenol F, TMBPF; bisphenol AF, BPAF.

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academia and industry, BPA likely influences reproductive health and embryo development across species, including human male and female fertility (Cabaton et al., 2011; Choi et al., 2016; Hanaoka et al., 2002; Huang et al., 2011; Hunt et al., 2003; Joint and WHO, 2011; Kim et al., 2001; Konieczna et al., 2015; Mok-Lin et al., 2010; Sabry et al., 2021).

As a result of many findings on the adverse effects of BPA, over the past decade authorities around the world have taken steps to reduce human exposure (European Commission, 2014; European Commission, 2011; Government of Canada, 2010). BPA is continuing to be phased out in many countries, causing companies to create dozens of "BPA-free" alternatives such as bisphenol F (BPF), bisphenol S (BPS), and bisphenol AF (BPAF), among others. These BPA alternatives are distinctly similar in structure to the parent compound BPA, with the original phenolic structure kept intact, changed only with the simple addition or removal of side groups (Fig. 1). Although these BPA alternatives have been much less studied, recent growing evidence suggests they are not the future of holistic health and safety. Similar to BPA, many of them are likely EDCs that interfere with hormone receptors and action. Numerous studies reveal the toxic potential of BPA analogs, showing various cellular, developmental, and reproductive disruptions across species (Arancio et al., 2018; Arancio et al., 2019; Cohen et al., 2021; Harnett et al., 2021a; Harnett et al., 2021b; Eladak et al., 2015; Ge et al., 2014; Huang et al., 2016; Moreman et al., 2017; Wang et al., 2017; Wu et al., 2017). Importantly, studies in various animal models and cell systems report that some analogs such as BPAF, are even more toxic, potent, and estrogenic than the parent compound BPA (Arancio et al., 2018; Cohen et al., 2021; Kojima et al., 2019; Liang et al., 2017; Michałowicz et al., 2015; Moreman et al., 2017).

Tetramethyl bisphenol F (TMBPF) is one of the most recently used BPA alternatives. It is the monomer of the Sherwin-Williams created compound valPure V70, now being used in polymer coatings for the linings of metal beverage and food cans (Valspar, 2017). In a new safety by design strategy, TMBPF was selected by using *in silico* computational structural analysis to search hundreds of chemicals that would share the same BPA-like properties of technical performance, durability, and integrity, but would lack its ability to interfere with estrogen receptors. With very limited independent research on TMBPF, a few recent company-sponsored studies conducted in collaboration with academic scientists, reported TMBPF lacks the same estrogenic activity and toxicity of BPA in rats and human cell lines, and may not have endocrine disrupting action (Maffini and Canatsey, 2020; Soto et al., 2017). Hence, TMBPF is being marketed as a 'low toxicity' BPA replacement monomer. However, we and others have found that TMBPF does have toxic effects on human and rat stem cells, and other human cell lines, and at very low concentrations, calling into question the safety of this new chemical (Cohen et al., 2021; Harnett et al., 2021a; Harnett et al., 2021b; Szafran et al., 2017).

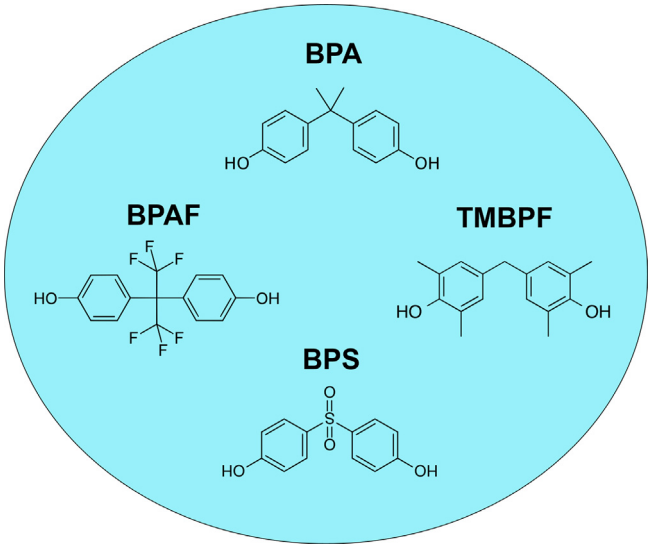

Fig. 1. BPA Analog Structures. The chemical structures of BPA and several new, commonly used BPA alternatives.

Notably, the food packaging industry is already using TMBPF in BPA-replacement metal food-contact coatings. These polymeric coatings prevent corrosion of the metal into the drink or food, but their monomer chemicals often end up migrating into the drink/food. Although coating formulations are proprietary to manufacturers, a recent study analyzed 4 popular metal beverage and food cans in the U.S. and found that one-third of the main migrant chemicals that leached from the cans into the liquid were TMBPF chemicals, making up some of the highest concentrations detected (Zhang et al., 2020). Clearly this newest BPA replacement is already being used across industries, with few published studies on it. To date, there are no investigations examining the *in vivo* effects of TMBPF in developing embryos, which could provide insight into the effects of this relatively untested BPA alternative in the earliest potentially sensitive windows of development.

Because BPA and BPA alternatives are ubiquitous and in everyday products, it is crucial to examine their effects on embryonic development. Indeed, we and others have found that BPA and certain BPA analogs cause severe developmental defects in early *Xenopus laevis* (African clawed frog) and zebrafish embryos, specifically inducing growth, body axis, craniofacial, and eye defects, and high mortality rates (Arancio et al., 2018; Arancio et al., 2019; Baba et al., 2009; Huang et al., 2016; Moreman et al., 2017; Pinto et al., 2019; Tomohiro et al., 2003; Wu et al., 2017). It is unclear if the teratogenic effects of BPA and these newer BPA analogs are specific to frogs and fish, or can be observed across species. Previous studies in chicken reported that a few BPA analogs caused increased embryo mortality, testicular feminization, and abnormal embryogenesis (Crump et al., 2016; Jessl et al., 2018; Mentor et al., 2020; Saito et al., 2012). However, no studies have examined the effects of these newer BPA analogs at environmentally relevant concentrations, side-by-side in embryonic development of chick or other species. As such, this was the primary objective of this work.

We investigated the potential acute toxic and teratogenic effects of BPA and newer BPA alternatives in chicken embryonic development. Specifically, we aimed to compare the effects and potency of TMBPF to the other bisphenols, at environmentally relevant concentrations. By further investigating the developmental and organismal effects of these chemicals using a well-characterized model system, we can better understand their ramifications during the earliest stages of development. Chicken embryos have been used as a good alternative toxicology model to mammalian systems and in predictions for human hazards, as they develop in an easily re-sealable shell that allows for easy access and manipulation of the developing embryo in an isolated system – negating the need for multiple exposures to many pregnant females and difficult analyses of fetal development *in utero* (Veselý and Veselá, 1991; Manakova et al., 2010). Despite chickens being oviparous and non-placental animals, chick embryonic development is more similar to mammals, and therefore humans, than amphibians, reptiles, or fish, primarily due to their development of an allantois (Veselý and Veselá, 1991; Manakova et al., 2010). They are also inexpensive, commercially available, mass-produced for human consumption, and their development has been very well-characterized and carefully staged (Eyal-Giladi and Kochav, 1976; Hamburger and Hamilton, 1951). In addition, the chicken's rapid development of 21 days and the ease of direct treatment to the embryo allow for multiple experiments to be completed in a relatively short time period. Here, we exposed chick embryos *in ovo*, from embryonic day 5 to 12 (E5–12) to increasing concentrations of BPA, BPS, BPAF, and TMBPF. Quantitative and qualitative analyses were then performed to

determine the analogs' effects on early embryo development, growth, and survival, as well as their rank order of potency.

2. Materials and methods

2.1. Chemicals and reagents

All chemicals were purchased from Sigma-Aldrich (Saint Louis, MO, USA; BPA, BPS, BPAF) and Tokyo Chemical Industry (Tokyo, Japan; TMBPF). Stock solutions of BPA (133027; >97% purity), BPS (103039; >98% purity), BPAF (90477; >99% purity), and TMBPF (M1099; >98% purity), at 300 mM were prepared in 95% ethanol in glass bottles. A 100X working stock was made for BPA, BPS, BPAF, and TMBPF, and diluted using a 40 ml dilution-factor to account for the estimated volume of the day 5 egg (yolk, fluid, albumin), to the final treatment concentrations on the day of injection. The final test concentrations of BPA, BPS, BPAF, and TMBPF were: 0, 0.003, 0.03, 0.3, 3, or 30 μM (part-per-billion to part-per-million). Preliminary rangefinder experiments were conducted with each analog to find ranges of concentrations at which toxic and teratogenic effects might occur. Previous studies completed by our lab on embryo development and stem cell cytotoxicity and apoptosis also provided insight on chemical ranges to test (Arancio et al., 2018; Harnett et al., 2021a). The final concentration of ethanol in all control vehicle and treatment solutions was 0.01%, a concentration known to have no adverse effects on embryo development (Timm et al., 2013).

2.2. Injection and exposure of chick embryos to BPA analogs

Brown fertile extra large Grade AA, cage-free chicken eggs (*Gallus gallus domesticus*) were obtained from local markets (Rock Island, Petaluma, CA). The eggs were incubated inside a HOVA Bator Genesis Deluxe Egg Incubator with automatic rotation (G.Q.F. Manufacturing Company Inc., Savannah, GA; 0720), at 37 °C and 70% humidity from E0 to E5, with the narrow end of the egg facing up and the air sac at the bottom of the egg. The day the eggs were removed from refrigeration and placed in the incubator was defined as embryonic day zero (E0). On E5, a single 4 μl injection was performed with a micropipet down through one small hole carved with a scalpel on the very top, superior aspect of the egg, directly exposing the embryos to control vehicle or various concentrations of the BPA analogs. After injection, the hole was covered with tape and parafilm, and the eggs were incubated at 37 °C and 70% humidity for seven days. On E12, the eggs were removed from incubation, and the embryos were extracted and separated from yolks for imaging, staging, and analysis. There are variable rates of embryo mortality and abnormalities that occur even in control embryos and vary from batch to batch, between parent chickens. Therefore, when the embryonic survival rate of the control eggs at E12 was lower than 85%, then the developmental toxicity data were discarded. All treatments were run in triplicate over several trials. Following all experiments embryos were immediately sacrificed by freezing at −80 °C for several days before disposal. No embryos were allowed to develop past E12. All animal experimental procedures were approved and performed in accordance with Saint Mary's College of California Institutional Animal Care and Use Committee (IACUC).

2.3. Embryo analysis and microscopic imaging

Extracted and rinsed embryos were weighed (in grams) using a TR-402 scale (Denver Instrument Company; Denver, CO), measured for rump-to-crown body length (in cm), and carefully assessed to stage and define morphological and developmental abnormalities. Each embryo was carefully imaged using either a Leica brightfield dissection microscope or higher resolution inverted Leica S6D Stereo Microscope and mounted MC190 camera (Leica Microsystems, JH Technologies, Fremont, CA), with a 64-bit Dell Latitude desktop computer using LAS imaging software (Leica Microsystems). The use of the Leica Stereo Microscope allowed for higher resolution images, time-lapse imaging, and analysis of the defects, specific body parts, and smallest and non-viable embryos. In quantification of survival rates, we counted the numbers of embryos that were non-viable, including those that were very small, lacked clear body form or heart, were severely underdeveloped, and weighed <2.8 g. As it was not always possible to assess which embryos were alive or dead, because signs of beating heart rapidly cease after separation from the yolk and some embryos had no clearly visible heart, we used this assessment as a way to quantify survival rates and LC_{50}s (lethal concentration with 50% survival). We did not analyze the embryos *in ovo* (by removing a piece of the shell) because preliminary trials revealed that some embryos were too small or underdeveloped to be examined through the opening, and this could have other potential effects on the developing embryo. The eggs used in these experiments were also brown-shelled, so we were unable to perform standard candle light tests to check for fertility and embryo growth throughout the exposure; the embryos were therefore analyzed at the end of the 7-day exposure period. All imaging and assessments were completed by a team of five observers, with cross-observer comparisons made on several embryos of each treatment in each trial.

2.4. Data analysis

Embryos were measured, weighed, and categorized as "normal" or "abnormal," and the specific abnormalities were recorded and carefully imaged. Images and videos were later used for embryonic staging and morphological analyses. The percentage of abnormal embryos was used as a way to compare and pool data across all trials. The LC_{50}s were calculated by plotting the percentage of viable embryos for all of the concentrations on E12, and solving for the concentration (x) at a y = 50 (% survival), from the linear regression equations. Using Microsoft Excel and the statistical program R, the Student's t-Test (two-tailed) and ANOVA (one-way, with repeated measures) were performed on all treatment groups versus controls, and P values of < 0.05 (*) and < 0.005 (**) were considered statistically significant. All results are expressed as mean percentages and mean ± standard error of the mean for at least 3–5 independent trials, with an average of approximately 20 embryos per treatment.

3. Results

3.1. BPA and BPA alternatives disrupt normal embryonic growth and development

We tested the effects of various concentrations of the potential environmental toxins BPA, BPS, BFAF, and TMBPF, on developing chick embryos. Embryos exposed from E5–12 to increasing concentrations of BPA or its analogs resulted in abnormal embryonic growth and development. There was a clear dose–response for each chemical, with higher concentrations of the chemicals resulting in severely decreased body length and body weight (Fig. 2). Growth restriction and delayed development were common, with exposed embryos being on average half the size and weight of control vehicle-treated embryos (Fig. 2). Qualitative and quantitative analysis of embryos revealed an overall decrease in body size and growth as concentrations increased, most dramatically observed in the embryos exposed to TMBPF and BPAF (Fig. 2A–C and Fig. 3). Control vehicle-treated embryos were an average length of 5.15 + 0.17 cm and average weight of 6.54 + 0.28 g, whereas 0.3 μM TBMPF-treated embryos were an average length of 3.92 + 0.59 cm and average weight of 3.97 + 0.82 g (length P value = 1.14×10^{-2}; weight P value = 4.31×10^{-4}). BPAF also had a strong, clear dose response, with 0.3 μM resulting in a signifi-

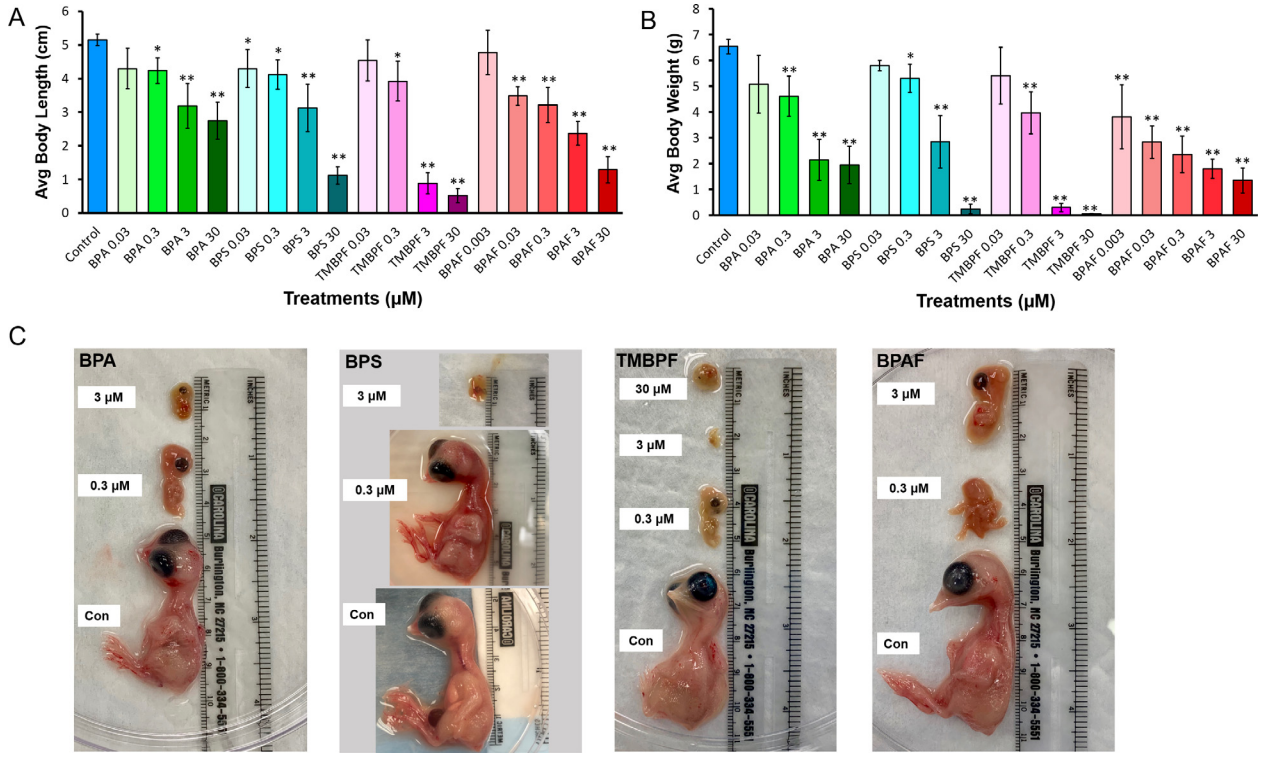

Fig. 2. BPA and BPA analogs disrupt normal embryo growth and development. Quantification of average body length (A) and body weight (B) for control and treated embryos on day 12 show the effect of the toxins (*$P < 0.05$; **$P < 0.005$). C) Representative embryos exposed to increasing concentrations of BPA, TMBPF, or BPAF, from embryonic day 4–12, Higher concentrations of the chemicals resulted in severely decreased body size, length, and weight, with many embryos halted in development and non-viable (n = 5–33 embryos/treatment; 3–6 trials).

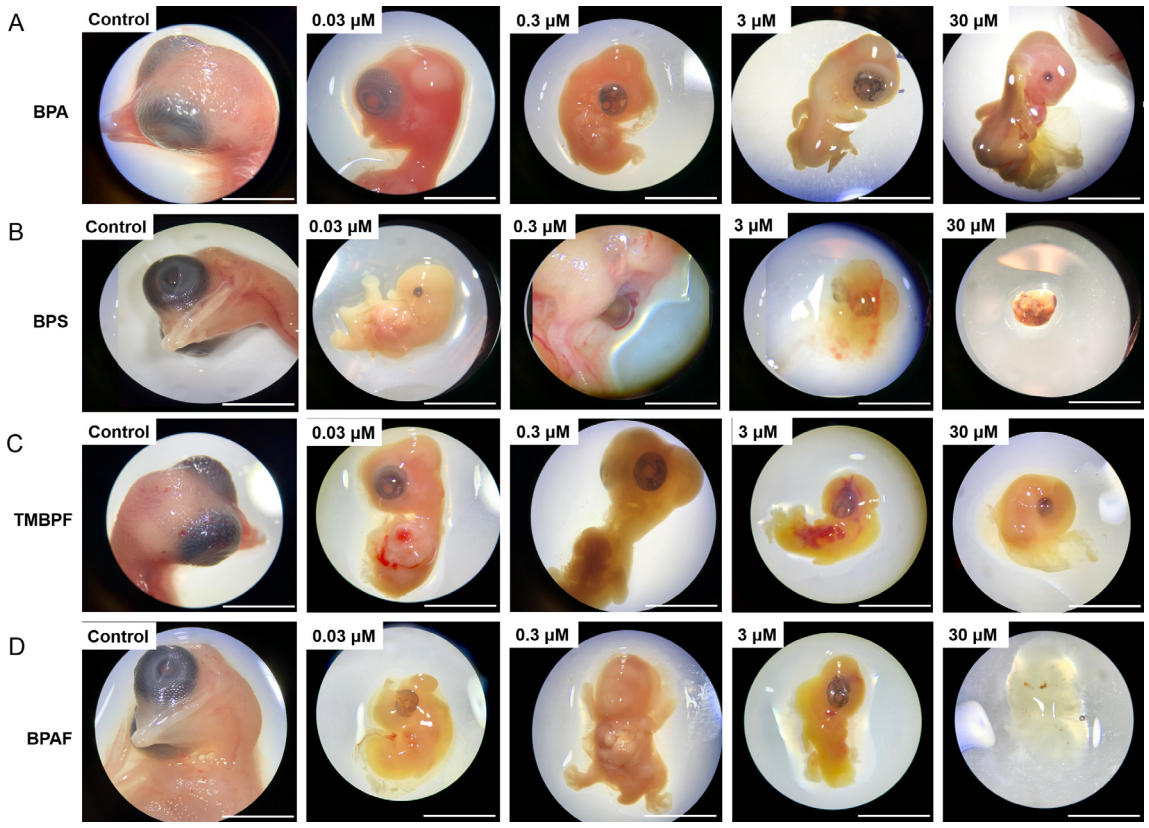

Fig. 3. Early severe embryological defects caused by BPA, BPS, TMBPF, and BPAF. Control embryonic day 12 embryos compared to embryos exposed to 0.03, 0.3, 3, and 30 μM of BPA (A), BPS (B), TMBPF (C), or BPAF (D). Representative images show stunted growth, halted development, and aberrant development of craniofacial structures, eyes, limbs, and hernial protrusions of the gastrointestinal organs. Nearly all exposed embryos had severe head and body malformations (magnification = 20×; scale bar = 10 mm).

cantly reduced body length and body weight of 3.21 + 0.52 cm and 2.36 + 0.71 g, respectively (length P value = 4.40 × 10^{-5}; weight P value = 9.49 × 10^{-8}) (Fig. 2A, B).

3.2. Dose-dependent effects of BPA and BPA alternatives on embryo survival

The effects of BPA and BPA alternatives were analyzed across many independent trials. To examine the embryonic abnormalities and viability, embryos were imaged under brightfield stereomicroscopes and their mortality rates quantified. We found dose-dependent toxicity with all of the tested bisphenols, as many embryos were non-viable or developmentally halted. There was significantly decreased embryo survival with increasing concentrations of each bisphenol (Table 1 and Supp. Fig. 1). At the lowest concentrations of BPA, BPAF, and TMBPF, survival rates ranged from 33 to 75%, compared to 100% survival in control-treated embryos. While at the highest concentrations of all compounds, survival rates severely decreased and ranged from 0 to 50% (Table 1). The calculated LC_{50}s for the compounds were 2.92 μM (BPA), 2.48 μM (BPS), 0.830 μM (BPAF), and 1.18 μM (TMBPF), establishing a rank order of potency of BPAF > TMBPF > BPS > BPA (Supp. Fig. 1).

Table 1
Survival rates of chick embryos exposed to various doses of BPA, BPS, TIVIBPF or BPAF.

Control	BPA (μM) 0.03	0.3	3	30
33 (100%)	6 (67%)*	10 (71%)*	4 (50%)*	4 (50%)
	BPS (μM)			
	0.03	0.3	3	30
	8 (100%)	10 (91%)	4 (40%)*	0 (0%)*
	TMBPF (μM)			
	0.03	0.3	3	30
	6 (75%)*	9 (64%)*	0 (0%)*	0 (0%)*
BPAF (μM)				
0.003	0.03	0.3	3	30
3 (75%)*	10 (62%)*	3 (33%)*	0 (0%)*	0 (0%)“

3.3. Dose-dependent teratogenic effects of BPA and BPA alternatives

There were clear dose-dependent teratogenic effects with higher concentrations leading to more drastic defects and a greater cumulative total of defects. Sublethal teratogenic effects of the bisphenols were categorized and quantified. It was found that 50–100% of the embryos exposed to BPA or its analogs resulted in abnormal development, even in embryos exposed to the lowest concentrations of 3 and 30 nM ((Figs. 3 and 4); BPAF 30 nM P value = 3.48 × 10^{-12}; TMBPF 30 nM P value = 9.58 × 10^{-8}; BPS 30 nM P value = 3.30 × 10^{-2}; BPA 30 nM P value = 1.26 × 10^{-3}). Further, embryos that were exposed to higher doses of the chemicals showed a correspondingly greater amount of gross developmental defects and delayed development (Figs. 3 and 4). It is of note that all BPAF and TMBPF concentrations tested resulted in 100% abnormal embryos, ranked as abnormal in at least one category of morphological defect. Note that while all of the embryos at the lowest doses were scored as abnormal, many of these had only one minor abnormality, which was often alterations to body pigmentation (Fig. 4C and D). In contrast, all control eggs injected with the 0.01% ethanol vehicle alone, resulted in < 10–15% of embryos with any developmental defects, which is the normal variation (Fig. 4). Gross anatomy and beating heart indicating viability of control embryos, could also be easily assessed and recorded via time-lapse imaging (see Supp. Video 1, showing beating heart of E12 control embryo).

The most common observed gross abnormalities included defects to the gastrointestinal tract, heart, eye, craniofacial structures, limbs, and body pigmentation (Fig. 3 and 4A–D); also see Supp. Fig. 2. Overall, embryos exposed to TMBPF had slightly less severe defects than those exposed to BPAF, but greater defects than BPA and BPS (Figs. 3 and Fig. 4A–D). The most notable developmental defects included extreme eye and craniofacial malformations (Fig. 5Aa–f and C), body pigmentation loss (Fig. 5B) and exceptionally stunted growth (Supp. Fig. 2), and malformed limbs (Fig. 5Aa). Other fairly common defects included abnormal, small hearts and malformed or enlarged gastrointestinal organs often with herniated protrusions (Fig. 5Ab; also see Supp. Fig. 3). One of the more common gastrointestinal abnormalities included an enlarged gizzard/ventriculus (stomach) (See Supp. Fig. 3. As shown in time-lapse imaging, BPS induced the greatest frequency of malformed, small hearts (Supp. Video 2; beating heart of embryo exposed to BPS). All these dysmorphologies became more

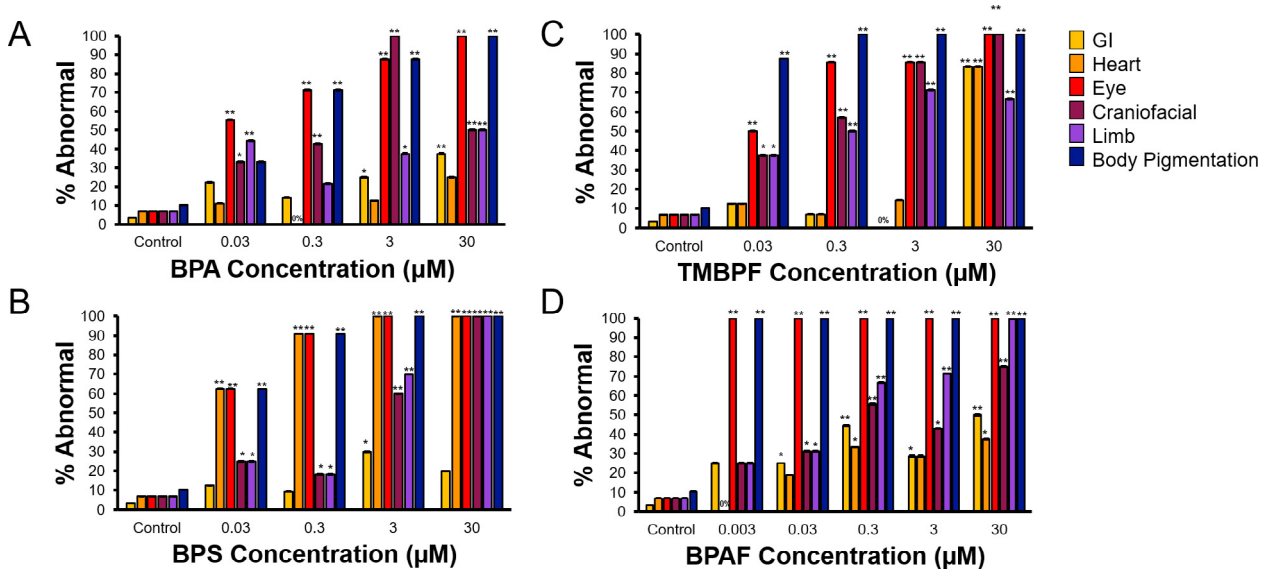

Fig. 4. Comparison of dose-dependent developmental defects induced by BPA, BPS, TMBPF, and BPAF in chick embryos. A–D) The mean percentages of abnormal embryos exposed to increasing doses of BPA, BPS, TMBPF, and BPAF, with gastrointestinal, heart, eye, craniofacial, limb, and body pigmentation defects. Legend indicates the observed categories of defects (*P < 0.05; **P < 0.005; n = 5–33 embryos/treatment; 3–6 trials).

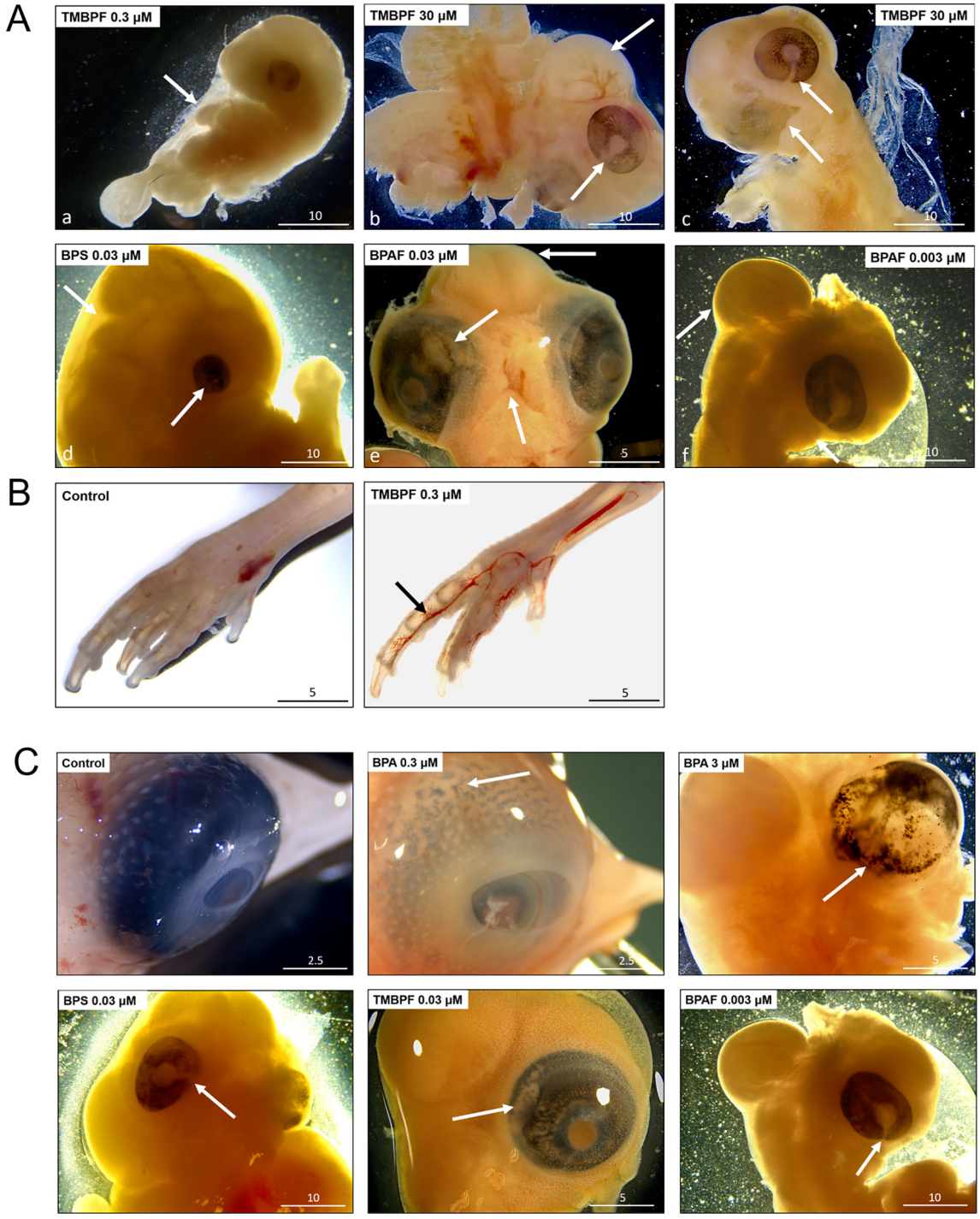

Fig. 5. Severe teratogenic defects caused by BPA and BPA analogs. A) Representative brightfield images of chick embryos exposed to various concentrations of BPA analogs show limb (a), craniofacial (b–f), eye (b–e), and gastrointestinal (b) defects (white arrows). B) Control-treated chick embryo foot compared to that of an embryo exposed to 0.3 μM TMBPF (black arrow indicates translucent skin showing blood vessels). Irregular pigmentation of the bodies and eyes was observed in the majority of exposed embryos (magnification = 25–50×; scale bars in mm). C) Control chick embryo eye on day 12 compared to the eyes of chick embryos exposed to varying concentrations of BPA and BPA analogs. Embryos display irregular iris and retina development and pigmentation, among other defects (white arrows; magnification = 25–100×; scale bars in mm).

frequent and severe as concentrations increased, indicating a clear dose–response with each bisphenol (Fig. 4A–D).

3.4. Specific embryonic defects induced by BPA and BPA analogs

Developmental defects were fairly consistent and dose-dependent within all treated embryos, however aberrant eye and craniofacial structures and body pigmentation were the most characteristic and severe as shown in Fig. 5 (see arrows). It was apparent that both low and higher concentrations of BPA and analogs resulted in these defects, although they became more frequent and extreme at the higher concentrations. Eye size, structure, and pigmentation were greatly affected in treated embryos, specifically in the formation of the iris and retina (Fig. 5C, see arrows). Body pigmentation defects

were also observed when examining the torso, limbs and feet of control and bisphenol-treated embryos (Fig. 5B). This was especially evident in embryos exposed to increasing concentrations of TMBPF and BPAF (see Supp. Video 3, showing pigmentation loss of the torso of a TMBPF-treated embryo). As shown in Fig. 5B, exposed embryos had more translucent integument with visible blood vessels (see arrow) and appeared more pinkish in color, whereas control embryos had normal skin pigmentation. Craniofacial defects including malformed heads, brains, faces, and beaks, were also common, especially in embryos exposed to the intermediate and higher concentrations. The specific dysmorphologies were particularly in the development of the mesencephalon of the brain, the cranium, and the beak (Fig. 5-Ac-f, see arrows).

4. Discussion

Here, we investigated the *in ovo* embryonic effects of several commonly used BPA alternatives on chick embryo development from E5 to E12. This is the first study to examine the effects of several of the newer BPA analogs including TMBPF, at environmentally relevant concentrations, and side-by-side in embryonic development of any species. We found that BPA, BPS, BPAF, and TMBPF were extremely teratogenic and toxic, causing severe dose-dependent growth and embryonic defects, even at low, physiologically relevant concentrations. Growth restriction and delayed development were common, with exposed embryos averaging half the size and weight of control vehicle-treated embryos. Specific patterns of defects were observed with craniofacial, eye structure and pigmentation, and body pigmentation abnormalities being the most frequent. TMBPF and BPAF both yielded near 100% abnormal or developmentally-halted, or non-viable embryos across all concentrations tested, with LC_{50}s in the high nM/low μM range. Notably, TMBPF was the second-most toxic and teratogenic of all chemicals tested, with a rank order of potency of: BPAF > TMBPF > BPS > BPA.

TMBPF has recently been used as a replica of BPA, still possessing the polymer capabilities, but without the known endocrine disruption caused by BPA (Maffini and Canatsey, 2020; Soto et al., 2017; Valspar, 2017). Most of the previously published studies on TMBPF were funded and/or completed by Sherwin Williams affiliates and academic scientists, with a declared conflict of interest. They reported no estrogenic activity, inhibitory androgenic activity, or toxicity in rats or human cell lines, even at concentrations as high as 1 μM and higher (Maffini and Canatsey, 2020; Soto et al., 2017). They concluded TMBPF was a “low-toxicity precursor” for epoxy coatings, and showed no similar actions to BPA. In contrast, we found TMBPF was severely toxic and teratogenic, even at low nM concentrations. TMBPF specifically induced embryonic defects, stunted growth, and high mortality rates at concentrations as low as 30 nM. The toxicity of TMBPF is similar to that of our recent work in rat and human adipose-derived stem cells, which showed that TMBPF was cytotoxic, potent, and apoptosis-inducing at concentrations as low as 10 nM (part-per-billion), making it about 100- to 1,000-fold more potent than BPA (Harnett et al., 2021a; Harnett et al., 2021b; Cohen et al., 2021). Similarly, here in chick embryos, TMBPF was much more teratogenic, potent and toxic than BPA (BPA LC_{50} of 2.92 μM; TMBPF LC_{50} of 1.18 μM).

Embryonic exposures to BPA and BPA analogs can directly result in long-term disease and acute and chronic health impacts related to reproduction, development, and somatic growth across species; therefore, understanding the developmental effects of these environmental chemicals is critical (Chianese et al., 2018; Kitamura et al., 2005; Vandenberg et al., 2007; Vandenberg et al., 2019). Many studies have reported that BPA has developmental effects *in vivo* at various concentrations across many species from invertebrates, to tunicates, amphibians, fish, chicken, mice, and monkeys (Brannick et al., 2012; Crump et al., 2016; Huang et al., 2011; Hunt et al., 2012; Iwamuro et al., 2003; Kontogiannatos et al., 2015; Matsushima et al., 2013; Mentor et al., 2020; Moreman et al., 2017; Qui et al., 2016; Saito et al., 2012; Tharp et al., 2012; Tomohiro et al., 2003; Wolkowicz et al., 2014). We and others have found that BPA and certain BPA analogs cause severe developmental defects in early *Xenopus* and zebrafish embryos, specifically inducing severe body axis and scoliosis-type spinal cord defects, decreased growth and body length and size, craniofacial malformations, eye dysplasia and pigmentation defects, loss of body pigmentation, gut and heart defects and edema, and high mortality rates (Arancio et al., 2018; Arancio et al., 2019; Baba et al., 2009; Huang et al., 2016; Moreman et al., 2017; Pinto et al., 2019; Tomohiro et al., 2003; Wu et al., 2017).

We were surprised to find that the bisphenols had such extreme teratogenic effects in chick embryos and at such low concentrations. Several of these BPA alternatives other than TMBPF, are reported to cause developmental effects across species. Similar to previous studies in *Xenopus*, we found chick embryos exposed to BPA analogs showed decreased growth and body size, craniofacial malformations, eye dysplasia, eye and body pigmentation loss, gut and heart malformations, and high mortality rates. Previous studies in chick, exposed embryos from E1 to E19 or E4 to E21 (hatching) to BPA, BPAF, BPS, or BPF at much higher concentrations ranging from: 75 μg/g–300 μg/g (329–1314 μM) for BPA; 210 nmol/g–200 μg/g (210–799 μM) for BPS; 210 nmol/g (210 μM) for BPF; and 2.1 nmol/g–520 nmol/g (2.1–520 μM) for BPAF (Berg et al., 2001; Crump et al., 2016; Jessl et al., 2018; Mentor et al., 2020; Saito et al., 2012). They found increased mortality and various defects including neural tube and head dysplasia, central immune organ defects, reduced growth and size, enlarged gallbladders, and decreased pipping success (breaking through the egg) (Crump et al., 2016; Mentor et al., 2020; Saito et al., 2012; Tian et al., 2014). Other investigations exposed chick embryos to 75, 150, and 300 μg/g (~0.328, 0.657, and 1.314 mM, respectively) of BPA from E1 to E19 and discovered several malformations (Jessl et al., 2018). We found similar effects as those reported by Jessl et al. and others including: celosomia leading to hernial protrusions of the gastrointestinal organs, limb abnormalities, and eye defects such as anophthalmia (eyes lacking) and microphthalmia (small eye size) often in combination with a craniofacial abnormality, particularly to the beak and head. Our results are in line with previous studies, although notably with shorter exposure times and significantly lower bisphenol concentrations than previously tested. We also found that the previously reported non-toxic TMBPF was one of the most teratogenic analogs, causing great dysmorphologies at even the lowest tested doses. Interestingly, with all analogs we found distinct eye and craniofacial dysmorphologies, enlarged stomachs, and disrupted eye and body pigmentation, that to our knowledge has only been reported in *Xenopus* and zebrafish, not in chicken.

There are likely several causes for these specific developmental defects, including EDC actions of BPA and analogs. Endocrine disruptors are chemicals that interfere with the balance of the endocrine system and its hormones, which can lead to developmental issues and birth defects (Monneret, 2017). These disruptors, even at low doses, have also been shown to interfere with the immune and nervous system, and reproductive organs in numerous species, including humans (Yang et al., 2007; Sanchez et al., 2018). BPA and BPA alternatives may bind to hormone receptors and both activate and/or antagonize them, likely through very similar EDC mechanisms given their almost identical chemical structures (Huang et al., 2016). As such, this can induce abnormal changes within cells, altered gene expression, and specifically cause effects on target tissues containing ERs and ARs including the reproductive, nervous, immune, and cardiovascular systems (Atay et al., 2020; Yang et al., 2007). Indeed, it has been shown in several species that BPA and analogs bind to the ERs, inducing physiological actions similar to estrogen and can lead to embryonic deformities in *Xenopus* (Arancio et al., 2018; Iwamuro et al., 2003; Tomohiro et al., 2003), zebrafish (Huang et al., 2016; Moreman et al., 2017;

Qui et al., 2020), and chick (Crump et al., 2016; Jessl et al., 2018; Mentor et al., 2020; Saito et al., 2012; Tian et al., 2014). Here, we observed a variety of deformities in chick embryos at various doses of BPA analogs, which may at least in part be caused by the affinity that each of these chemicals has for the different ERs (Saito et al., 2012).

An additional putative mechanism underlying the developmental delay, stunted growth, and dysmorphologies, include high levels of programmed cell death or apoptosis. Apoptotic mechanisms and pathways are well-characterized and understood to be crucial during embryological processes. Programmed cell death is largely responsible for the correct formation of various organs and structures throughout the embryo, leading to healthy development (Brill et al., 1999). The balance between cell death and proliferation is critical for normal development. However, apoptotic pathways can be victim to teratogens and other environmental toxins, such as BPA and its analogs (Brill et al., 1999; Harnett et al., 2021a; Huang et al., 2021; Tomohiro et al., 2003; Wang et al., 2017; Wu et al., 2017). As such, there is a high likelihood of overactive apoptotic pathways mediated by BPA and its alternatives, leading to many developmental abnormalities in the chick embryos. Crump et al. emphasized that the lipophilic nature of most cytotoxic BPA analogs allows them to pass through the cellular and mitochondrial phospholipid bilayers, causing them to accumulate in the negatively charged mitochondrial matrix, in-turn eliciting mitochondrial toxicity and subsequent apoptosis (Crump et al., 2021). Further, exposure to either BPA or BPS, or both together, resulted in significant activation of a checkpoint protein involved in germline apoptosis (Chen et al., 2016b). Other studies in frog embryonic cells (Tomohiro et al., 2003), mouse and rat cells (Pang et al., 2019; Ran et al., 2013), chicken embryonic cells (Crump et al., 2021), and human embryonic stem cells (Wang et al., 2019), have reported particularly high levels of apoptosis in neural and other tissues, which aligns with the teratogenic effects on the brain, spinal cord, and neural crest-derived melanocytes. We did not analyze apoptosis in specific tissues of the chick embryos. However, our previous work in both frog embryos and rat and human adult stem cells revealed that these BPA analogs induce very high levels of Caspase-6-mediated apoptosis, and severely disrupt neural development and pigmentation (Arancio et al., 2018; Arancio et al., 2019; Cohen et al., 2021; Harnett et al., 2021a; Harnett et al., 2021b). Here, we now find their potent teratogenic effects in chick development, with the most prominent effects on the head and brain, and melanocytes of the eyes and body. Future studies can investigate the apoptotic signaling pathways and mechanisms in these specific embryonic tissues.

Similar to some previous findings, BPA analog exposure dramatically decreased embryo survival. The lowest exposure concentrations resulted in 33–75% survival rates, whereas the highest exposure concentrations resulted in 0–50% survival rates, which is comparable to previous studies on E19 and E21 chick embryos, however at much lower concentrations here (Berg et al., 2001; Crump et al., 2016; Jessl et al., 2018; Mentor et al., 2020). Previous studies found dose-dependent increases in BPAF-induced mortality, particularly in the two highest tested concentrations of 2.10 nmol/g and 520 nmol/g (2.10 and 520 μM), with an LC_{50} of 185 nmol/g (185 μM), which is 200-fold higher than our calculated LC_{50} of 0.830 μM (Mentor et al., 2020). In other studies, chicks injected with various concentrations of BPS had an estimated LC_{50} of 279 μg/g (1.11 mM), which is about 500 times higher than our calculated BPS LC_{50} of 2.48 μM (Crump et al., 2016). Moreover, BPA exposures from 75 μg/g to 300 μg/g (329 μM–1.31 mM), resulted in chick mortality rates as high as 30% (Jessl et al., 2018), which is very similar to our 25–38% mortality rates, but with 1,000–10,000-fold lower concentrations of BPA and analogs here (30 and 300 nM). Overall, all of our LC_{50}s are much lower, 100–1,000-fold lower, than all previously reported LC_{50}s and lowest effective concentrations in chick. However, our results are in line with other cell-based and embryo studies revealing that BPAF is one of the most potent and toxic bisphenols ever tested (Arancio et al., 2018; Cohen et al., 2021; Harnett et al., 2021a; Kojima et al., 2019; Liang et al., 2017; Mentor et al., 2020; Michałowicz et al., 2015; Moreman et al., 2017). Indeed, we previously found that BPAF was 1,000-fold more potent and toxic than BPA, resulting in severe disruption of early cell cleavage division and embryo development in frog (Arancio et al., 2018; Arancio et al., 2019). Importantly, this work now shows that TMBPF's effects and toxicity are very similar to that of BPAF in chicken embryonic development.

Most studies report LC_{50}s and lowest effective doses for BPA and several BPA analogs in the range of ~ 0.009 nM to 200 μM, from cells to embryos (Arancio et al., 2018; Harnett et al., 2021a; Harnett et al., 2021b; Ma et al., 2015; Matsushima et al., 2013; Michałowicz et al., 2015; Moreman et al., 2017; Wolkowicz et al., 2014). The LC_{50}s calculated here in the low μM range from 0.83 to 2.92 μM, are slightly higher than those previously reported in frog embryos (LC_{50} for BPAF of 13 nM) (Arancio et al., 2018), and human and rat stem cells (LC_{50}s for BPAF of 1.2 nM and 4.8 nM, respectively; for TMBPF of 0.060 μM and 0.88 μM, respectively) (Harnett et al., 2021b), and very similar to those of zebrafish embryos (96-hr LD_{50} for BPAF of 4.73 μM) (Moreman et al., 2017). The great differences in bisphenol potencies and toxicities across species indicates that there are cellular- and species-specific differences in vulnerability to different environmental toxins including the different BPA replacement chemicals. Indeed, many studies report vastly different toxic concentrations and some of this is likely due to variation in the embryonic window of exposure, presence or absence of a placental barrier, length of exposure, routes of exposure, and species-specific effects. Yet another possibility for some of the differences between current and previous results in chick embryos might be at least partially due to the methodologies used for estimation of the mean egg weight and/or volume in the egg, and its variability, which is imprecise. Therefore, these estimates and dilution effects may make the actual exposure concentrations slightly lower or higher than the calculated concentrations. This might explain at least some of the disparity between previous and current chick developmental toxicity results, LC_{50}s, and the range of specific effects observed.

The severity of adverse outcomes in chick embryos exposed to BPA analogs *in ovo* does not necessarily infer a developmental hazard to humans due to species differences in physiology and response. However, while there are definitely species-specific effects, and differences between chicken and mammalian development including the lack of a placental barrier, there may be some extrapolation of results to humans. Notably, these findings may have implications for human development in terms of the low concentrations of BPA analogs to which humans are exposed and the new analogs that likely pose the greatest risks for humans. The sublethal and lethal concentrations found here, in the low to high nM range, are environmentally relevant and very similar to concentrations that are typically found in the environment and in human body fluids, at around 1–20 nM (Flint et al., 2012; Joint and WHO, 2011; Liao et al., 2012; Rudel et al., 1998; Vandenberg et al., 2007; Winnebeck 2013; Yamazaki et al., 2015). Interestingly, craniofacial anomalies, similar to what we found in chick embryos, are one of the most frequent birth defects in humans worldwide, that have complicated genetic and environmental etiologies that are often unknown (Ahmed et al., 2016, Yoon et al., 2016). Therefore, *in utero* exposure to BPA replacements may pose risks for early embryo and fetal development in both animals and humans.

Indeed, numerous association studies have linked BPA and analogs to human reproductive diseases and disorders including increased miscarriage rates (Sugiura-Ogasawara et al., 2005; Zbucka-Kretowska et al., 2018), low fetal birth weight (Huo et al., 2015), birth defects such as male hypospadias (Pallotti et al., 2020), endometriosis (Buck Louis et al., 2013; Simonelli et al., 2017), polycystic ovary syndrome (Rutkowska and Rachon, 2014), breast and prostate cancer (Jenkins et al., 2009; Moral et al., 2008; Shafei et al., 2018; Zhang et al., 2014), and precocious puberty (Leonardi et al., 2017), in addition to

many neurological, immune, and metabolic disorders (Robinson and Miller, 2015; Kardas et al., 2016; Thongkorn et al., 2019; Li et al., 2018; Tewar et al., 2016; Legeay and Faure, 2017; Wang, et al., 2012; Wang, et al., 2019; Yang et al., 2020; Youssef et al., 2018). Further, several large studies have reported that in couples undergoing *in vitro* fertilization (IVF) there were significant correlations between higher urinary BPA concentrations and reduced sperm counts, and abnormal sperm motility and morphology in men, reduced oocyte yield and peak serum estradiol in women, and reduced success with IVF (Joint and WHO, 2011; Matuszczak et al., 2019; Mok-Lin et al., 2010). Further, bisphenols can readily cross the blood-placental barrier due to their lipophilic structure, and have been detected in fetal blood, cord blood, breast milk, and amniotic fluid with bioaccumulation in maternal-fetal-placental tissues (Aris, 2014; Lee et al., 2018; Nishikawa et al., 2010). Therefore, exposures before or during pregnancy to low, environmentally relevant doses of BPA replacement chemicals such as TMBPF and BPAF, might have the potential to cause pregnancy loss and birth defects in humans and other mammals. Furthermore, through increased contact with personal care products and other exposures, women and especially Black, Indigenous, and People of Color women tend to have greater overall daily exposures to BPA, BPA alternatives, and other environmental toxins (Branch et al., 2015; Fisher et al. 2019; Jagne et al., 2016; James-Todd et al., 2016; Zota and Shamasunder, 2017). Therefore, it is possible that the effects of these BPA alternatives might have even greater implications for human female reproductive health and fertility.

5. Conclusion

Early chick embryonic exposure to BPA, BPS, BPAF, or TMBPF has significant dose-dependent effects on embryo development, growth, and survival. These findings are relevant as all of the bisphenol analogs tested induced severe morphological defects, including eye, craniofacial, gastrointestinal, heart, and body malformations. The calculated lethal concentrations of the BPA analogs and sublethal potencies are environmentally relevant, highlighting the need for greater characterization of all BPA replacements and better regulation. These ubiquitous chemicals are potentially detrimental to aquatic and terrestrial systems and the development of exposed organisms. These and other results underscore the adverse effects of BPA replacements on early embryo development across species, and may have implications for human and animal reproductive health and disease. Because of these early developmental effects and toxicity, we need a greater understanding of harmful levels for animals and humans, the critical vulnerable periods of exposure, and the unique physiological effects and signaling pathways of these prevalent environmental compounds.

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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 in this paper.

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Appendix A. Supplementary data

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