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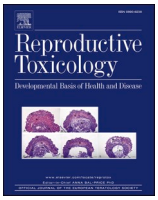

Teratogenic and neuro-behavioural toxic effects of bisphenol A (BPA) and B (BPB) on *Xenopus laevis* development

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ABSTRACT

Bisphenol A (BPA) is a plastic additive with endocrine disruptive activity, classified in 2017 by EU ECHA as substance of very high concern. A correlation between environmental exposure to BPA and congenital defects has been described in humans and in experimental species, including the amphibian *Xenopus laevis*. Among BPA analogues, bisphenol B (BPB) is used as alternative in different not-EU countries, including US, but seems to share with BPA its endocrine disruptor properties. Aim of the present work is the evaluation of the effects of BPB versus BPA exposure in a *X. laevis* developmental model. A windowed exposure (R-FETAX method) was applied covering the developmental phylotypic period (teratogenicity window), or the late tailbud stages (neuro-behavioural toxicity window, corresponding to the spontaneous swimming acquisition period). Samples were monitored for lethal effects during the full test period. External morphology evaluation and deglutition functional test were applied in any group. Abnormal tadpoles were also processed for cartilage staining. In groups exposed during neuro-behavioural toxicity window the swimming test was also applied. Lethality and malformations were obtained only in samples exposed during the teratogenicity window; these data were modelled using PROAST software and BPB relative potency resulted about 3 times higher than BPA. The day-by-day evaluation revealed that lethality was correlated to embryonic abnormal development of gills and apoptosis in gill primordia. Teratogenicity was never detected in groups exposed during the neuro-behavioural toxicity window, where some significant neuro-behavioural deficits were detected in tadpoles exposed to the highest tested concentrations of BPA and BPB.

1. Introduction

Bisphenol A (BPA) is a diphenylmethane derivative, used from decades in the production of polycarbonate plastics, epoxy resins and various other plastic-based consumer products; some, but not all, recycled plastics may contain BPA too [1].

Due to its ability to mimic oestrogen binding [2–4] and to exhibit anti-androgenic activity [5–7], BPA is classified among endocrine disruptors and is considered by ECHA a “substance of very high concern” [8]. Moreover, BPA passes through the blood-brain barrier and its exposure was linked with multiple neuropsychological dysfunctions, neurobehavioral disorders and neurodegenerative diseases [9]. A direct or indirect release of BPA into the environment has been demonstrated at any level of plastic product life cycle (production, consumption, disposal) [10]. Humans are directly or indirectly exposed to BPA, through ingestion, inhalation and dermal, and vertical maternal-to-embryofoetal exposure [11]. In light of that, EU authorities updated the specific migration limit for BPA at 0.05 mg/kg of food [12] while many EU member countries completely banned BPA in baby feeding bottle, food contact material and thermal paper production. In addition, recently the European Food Safety Agency (EFSA) updated the tolerable intake limit, setting it at 0.04 ng/kg body weight/ day [13].

As far as materno-embryofoetal health is concerned, vertical transmission has been demonstrated: BPA is able to cross the placental barrier and has been detected in human maternal/foetal serum and in placental tissues [14]. A correlation between environmental exposure to BPA and congenital defects has been described both in humans [15–17] and in different vertebrate experimental species [18–26]. In the amphibian

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Xenopus laevis developmental model, lethal and teratogenic effects of BPA were documented by Iwamuro et al., [27], with the reported estimated lethal concentration for 50% samples (LC_{50}) 21 μM. Ge and colleagues described, side to extremely severe teratogenic effects (stunted body, bent notochord, short or bent tail axis, deformed brain and/or eyes, cardiac or abdominal oedema, miscoiled gut, and lengthened abdomen), concentration-related behavioural deficits evaluated by touch response at 48, 60, and 72 h and by autonomous swimming tracking at 96 h. Effects were correlated to the observed apoptosis detected at 96 h by acridine orange staining and TUNEL techniques in groups exposed to 10 and 20 μM BPA: in malformed samples, muscle tissue and notochord were the main targets, with concentration-related signs of apoptosis [23]. Relatively to teratogenesis, no data are available on molecular mechanisms and a precise structure characterisation is lacking. Literature on mammals and humans shows a BPA-related increase in hyperactivity in mice, rats and children exposed *in utero* to BPA [28]; anxiety-related behaviour and impairment in aversive and spatial memory in rats [29]; a correlation between BPA maternal plasmatic levels and central and peripheral nervous system malformations [15] or abnormal behaviour [30] in humans. Till now, however, independent teratogenicity and neurotoxicity protocols, necessary in order to avoid confounding overlapping data, were never applied.

Due to the regulatory restrictions in BPA use, several BPA analogues were synthesised but their safety is under debate. Among BPA analogues, bisphenol B (BPB) is used as BPA alternative in plastic production in different not-EU countries, including US. Although BPB is not manufactured or used as a chemical in Europe (it is not registered under the European Registration-Evaluation-Authorization and Restriction of Chemicals (REACh) Regulation), it has been detected also in several European food products, such as various canned foods, [31–34] and in commercial milk samples [35]; by consequence it is not surprising that detectable BPB plasma levels were found also in EU population [36,37]. BPB shares a strong structural similarity with BPA (Fig. 1) and this seems to drive their common endocrine disruption activity [38]. In spite of the evidence that BPB meets the WHO definition currently used in a regulatory context of endocrine disrupting chemical [38], at the moment studies on developmental effects of this molecule are lacking. Moreover, studies on BPB-related teratogenic or neuro-behavioural effects are few and limited to the zebrafish model, and a comparison with the parental compound (BPA) was till now never performed [39].

Aim of the present work is the characterisation of teratogenic and neuro-behavioural effects of BPB and a comparison of BPB to BPA effects, using the developmental *X. laevis* model (R-FETAX methodology). A windowed exposure protocol was applied covering: i) the teratogenicity window, corresponding to the developmental stages common to all vertebrate embryos (phylotypic period); ii) the neuro-behavioural toxicity window, corresponding to late tailbud stages (spontaneous swimming acquisition period).

The "teratogenicity window" covers NF stage 10–26, considered in *X. laevis* the period common to any vertebrate at both morphological and molecular point of view [40–42]. The "neuro-behavioural toxicity window" covers the spontaneous swimming acquisition period (tadpole model, NF 38–46), reported indicative to evaluate neuro-developmental disorders [43]. As reported by literature, till time of hatching (NF stage 37/38), tadpole locomotion is still inflexible, with a stereotyped organisation; while by larval stage 42, approximately 1 day later, a gradually acquisition of motor versatility enables animals to move efficiently and coordinately through thy environment [44].

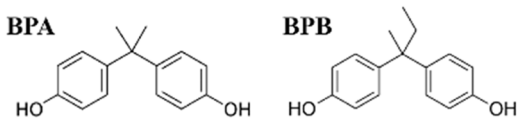

Fig. 1. BPA and BPB chemical structures. Note the extreme similarity between the two molecules.

2. Materials and methods

2.1. R-FETAX

R-FETAX methodology was applied according to Battistoni et al. [45]. Briefly, amphibian *X. laevis* adults (Nasco, USA), maintained under controlled conditions in an automatic breeding system (TecnoPlus, Techniplast, Italy), were naturally mated overnight. Collected embryos were cleaned by gentle swirling in a 2.25% L-cysteine solution with an arranged pH of 8.0 and rinsed several times in FETAX solution (625 mg/L NaCl, 96 mg/L $NaHCO_3$, 30 mg/L KCl, 15 mg/L $CaCl_2$, 60 mg/L $CaSO_4 \cdot 2\,H_2O$, and 70 mg/L $MgSO_4$). Normally cleaved embryos at the mid-blastula stage (stage 8, according to Nieuwkoop and Faber [NF] stadiation [46]) were selected and maintained at 23 °C during the whole testing time (6 days, corresponding to final NF stage 46, reached in historical not exposed tadpoles). Different exposure groups were set to cover specific developmental windows: i) teratogenicity window (NF stage 10–26, from day 0.5 to day 1.5), corresponding to the developmental stages common to all vertebrate embryos (gastrula-early morphogenesis, also known as phylotypic period, representing the window for species-agnostic teratogenesis purposes); ii) neuro-behavioural toxicity window (late tailbud stages corresponding to NF 38–46, from day 2 to day 6), covering spontaneous swimming acquisition period (Fig. 2). Stage identification was performed referring to Nieuwkoop and Faber and Zhan stadiation tables (www.xenbase.com).

2.2. Range-finding test

Test chemicals (Sigma, Italy) were dissolved in DMSO (Sigma, Italy). Stock solutions were added to FETAX medium (4 μL/mL) in order to reach the final concentration of BPA 0–10–20–25–30–35 μM or BPB 0–5–7.5–10–15–20 μM.

Samples (at least a triplicate of 5 embryos/group) were exposed during the teratogenicity window (NF 10–26). During the full six-day test period, samples were monitored using a cold-light stereomicroscope (Zeiss) to check lethal effects.

2.3. Main tests

A total of nine concentration levels ranging from 0 to 35 μM for BPA, and from 0 to 15 μM for BPB were tested in samples (at least a triplicate of 5 embryos/group) exposed during the teratogenicity window (NF 10–26). BPA 0–10–20–25 μM and BPB 0–5–7.5–10 μM were tested during the neuro-behavioural toxicity window (NF 38–46) (at least a

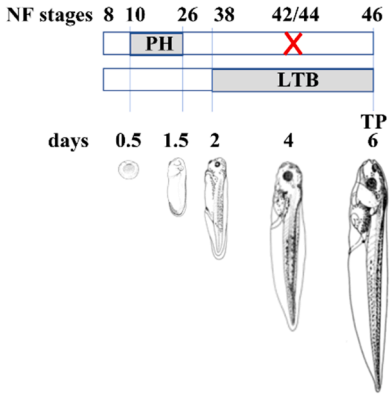

Fig. 2. R-FETAX protocol: grey boxes represent the exposure windows, white boxes the maintenance in FETAX solution, the red cross the timing of observed BP-related lethality (approximately at NF 42/44 stages) after exposure during the phylotypic period (NF 10–26). PH= phylotypic period; LTB= late tailbud stages; TP= tadpole.

triplicate of 3 embryos/group) (Fig. 2). Embryo-lethality was evaluated throughout the test monitoring samples daily using a cold-light stereomicroscope (Zeiss). At the end of the test (day 6) the functional deglutition test was applied according to Battistoni et al., 2022b: maintaining larvae for 2 h at 23 ± 0.5 °C in FETAX solution containing 25 μg/mL red polystyrene microparticles (1 μm diameter, Sigma). Tadpoles, anaesthetised with MS-222 (Sigma, Italy; 0.01% in FETAX solution), were evaluated for gross morphology and for presence/absence of red staining at the level of the intestine (deglutition test positive/negative) under a camera-equipped cold-light illuminated dissecting microscope (Leica). At the end of the evaluation, samples were photographed, euthanized by anaesthetic overdose (MS-222 0.1% in FETAX solution at 4 °C), fixed in ethanol 50% (Sigma, Italy) and dehydrated in ethanol 70%. Cartilage staining was performed on abnormal tadpoles according to Di Renzo et al. [48], applying flat mount technique for a detailed cartilage evaluation [48].

Neuro-behavioural evaluation was performed according to Battistoni et al. [47] on tadpoles from groups exposed during the spontaneous swimming acquisition period (neuro-behavioural toxicity window, NF stages 38 − 46, representing the transition from dormant life to progressive free-swimming locomotion at the onset of active feeding) [49]. Briefly, before deglutition test, single tadpoles were transferred into a 27 mm arena, on a cold-light under-illuminated stereomicroscope (Zeiss). After 30 s acclimation time, 60 s videos were taken from above using a 1080p HD 30 fps digital camera and subsequently analysed using the AnimalTracker plugin [50]. Free images were processed using programme ImageJ [51]. Activities in the inner circle (with a diameter 0.75 of the arena diameter fixed as 1) and in the outer ring (0.25 of the arena) were analysed to obtain immobility time (sec), total distance (mm), distance (mm) in the outer ring and the inner circle, swimming speed (total distance (mm)/ [test time - immobility time] (sec)).

2.4. Additional tests (evaluation of fine morphology and apoptosis) using a new superfast-R-FETAX procedure

As in groups exposed to BPA or BPB during the teratogenicity window lethal effects mainly occurred at day 4 (approximately NF stage 42–44), extra groups were processed to evaluate the cause of the observed lethality. 15 embryos/group were exposed during the teratogenicity window (NF 10–26) to concentration levels of BPs effective in 40% cases or more (BPA 25 μM and BPB 7.5–10 μM) or to the solvent alone (DMSO). At the end of exposure (day 1.5, NF stage 26) or at developmental day 2.5 (NF 40 stage, 24 h after the end of exposure and 2 days before the compound-induced lethality) samples were evaluated for viability using a cold-light stereomicroscope (Zeiss) and processed to visualise apoptotic cells and fine morphology. To detect apoptosis, the acridine orange vital staining [52], partially modified, was applied. Embryos were maintained for 5 min in 5 μg/mL acridine orange (Sigma, Italy) in FETAX, washed 3 × 5 min in FETAX solution, and viewed under a fluorescence stereomicroscope (EX=450–490 nm; LP=520 nm) (Leica). Apoptotic cells appeared green fluorescent. Samples were fixed in a mixture of 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffered solution at pH 7.4 and processed for the ultrastructural analyses. After washes in cacodylate buffer, tadpoles were post-fixed in 1% Os04 for 2 h in dark condition at 4 °C, dehydrated with an ascending ethanol series, and critical-point dried in a Balzers Unions CPD 020 apparatus (Balzers Unions, Lichtenstein). Under a stereomicroscope, the whole samples were mounted onto standard aluminium stubs, gold-sputtered, and observed under a FE-SEM Sigma (Zeiss, Jena, Germany) at 7 kV, WD 20–10 cm.

2.5. Statistical analysis and mathematical modelling

Quantal data were analysed using the Chi- square for trend. Continuous data, expressed as mean and standard deviation, were analysed using ANOVA followed by Tukey's post hoc test. The level of significance was set at $p < 0.05$. The benchmark dose (BMD) approach was applied using PROAST (70.3 version), a software package developed by the Dutch National Institute for Public Health and the Environment (RIVM) (www. proast.nl) for the statistical analysis of dose-response toxicological data. Data on teratogenicity and lethality were pooled and modelled to characterise the single dose-response curves, setting the benchmark dose (BMD) at 50% benchmark response. After modelling the results obtained for each molecule, the log-likelihood ratio test was applied to assess the equal steepness assumption and the relative potency factor (RPF) derived, showing the relative potency of BPB versus BPA. The exponential model family equations were selected to describe the dose-response curves and obtain the RPFs. Swimming data were not modelled due to large standard deviation of the obtained data points.

3. Results

3.1. Range-finding test

Concentration-related lethal effects were evident in groups exposed to BPA or BPB (Table 1). BP-related lethality mainly occurred far from exposure, typically at day 4 (approximatively NF 42–44, at the last tailbud stages) (Table 1).

3.2. Main developmental toxicity test: exposure during the teratogenicity window (NF stage 10–26)

3.2.1. R-FETAX: evaluation at day 6 (NF stage 46)

Dose-related lethal and teratogenic effects were evident in groups exposed to BPA or BPB (Table 2). Teratogenic effects detected in living tadpoles were classified as anterior defects (round head, shortened and reduced gill basket, ventral oedema), only in few cases associated to bent or wavy tail (Fig. 3a-c). Deglutition test was positive for all tadpoles, showing no differences among groups and indicating functionally normal facial articulation. After alcyan blue cartilage staining, in comparison to unaffected tadpoles, abnormal external samples showed facial skeletal elements reduced in size (without any fusion) and smaller and shorter gill basket (Fig. 3d-e). The main experiment confirmed observations described in the range-finding test: BP-related lethality typically occurred two days after the end of exposure (day 4, NF 42–44, the last tailbud stages). To explain this delayed effect, an indirect cause of lethality (involving severe defects at primordia of organs later becoming essential for survival) was hypothesised. This hypothesis was further tested applying the new superfast-R-FETAX approach.

Table 1
Range-finding test: lethal effects observed in groups exposed to BPA or BPB during teratogenicity window (phylotypic period, NF 10–26). Statistics (Chi-square for trend, calculated on frequencies) shows dose-relationship in both BPA and BPB tested series.

Concentration (μM)	Dead (%)	Concentration (μM)	Dead (%)
DMSO (BPA 0) *(N = 74)*	8.1	DMSO (BPB 0) *(N = 74)*	8.1
BPA 10 *(N = 15)*	0.0	BPB 5 *(N = 30)*	0.0
BPA 20 *(N = 15)*	13.3	BPB 7.5 *(N = 15)*	33.3
BPA 25 *(N = 30)*	26.7	BPB 10 *(N = 30)*	50.0
BPA 30 *(N = 15)*	86.7	BPB 15 *(N = 15)*	100.0
BPA 35 *(N = 15)*	100.0	BPB 20 *(N = 15)*	100.0
p=	**< 0.0000001**	**p=**	**< 0.0000001**

Table 2
Main test: lethal, teratogenic (abnormal) and total (dead + abnormal) effects observed in groups exposed to BPA or BPB during the teratogenicity window (phylotypic period, NF 10–26). Statistics (Chi-square for trend, calculated on frequencies) are shown.

Concentration (μM)	Dead (% of exposed)	Abnormal (% of living tadpoles)	Total (% of exposed)	Concentration (μM)	Dead (% of exposed)	Abnormal (% of living tadpoles)	Total (% of exposed)
DMSO (BPA 0) *(N = 111)*	3.6	0.0	3.6	DMSO (BPB 0) *(N = 111)*	3.6	0.0	3.6
BPA 10 *(N = 15)*	0.0	0.0	0.0	BPB 3.25 *(N = 17)*	0.0	17.6	17.6
BPA 12.5 *(N = 16)*	12.5	6.7	18.8	BPB 5 *(N = 65)*	6.2	0.0	6.2
BPA 20 *(N = 50)*	4.0	27.1	30.0	BPB 6.5 *(N = 39)*	17.9	18.8	33.3
BPA 24 *(N = 15)*	13.3	23.1	33.3	BPB 7.5 *(N = 48)*	27.1	2.9	29.2
BPA 25 *(N = 36)*	13.8	16.1	27.8	BPB 8 *(N = 14)*	42.9	37.5	64.3
BPA 27 *(N = 16)*	25.0	8.3	31.3	BPB 9.5 *(N = 27)*	96.3	100	100
BPA 30 *(N = 38)*	39.5	21.7	52.6	BPB 10 *(N = 45)*	66.7	20.0	73.3
BPA 35 *(N = 15)*	100	-	100	BPB 15 *(N = 15)*	100	-	100
p=	**< 0.0000001**	**0.000000976**	**< 0.0000001**	**p=**	**< 0.0000001**	**0.00005125**	**< 0.0000001**

3.2.2. Superfast-R-FETAX: evaluation at the end of exposure (day 1.5, NF 26) and at day 2.5 (NF stage 40)

At the end of exposure (day 1.5, NF 26) morphological evaluation and acridine orange vital staining did not show any differences in embryos exposed to BPs when compared to unexposed samples. By contrast, at day 2.5 (24 h after the end of exposure and 1.5 days before lethality), acridine orange staining revealed apoptotic areas at the level of underdeveloped gill primordia (Fig. 4), suggesting the branchial apparatus as the main target of BPA and BPB; SEM detailed evaluation showed mild to severe branchial bud hypoplasia, with the most severe branchial defect being gill primordia agenesis (Fig. 5). This evidence seemed to confirm the hypothesis of lethality as a secondary event due to severe abnormalities at structures (gills) becoming essential for survival at the end of late tailbud period (NF 42–44). Considering these results, lethal effects were reclassified as teratogenicity-related lethal effects and combined data on lethality and teratogenicity (Table 2, dead + abnormal) were used for modelling.

3.2.3. Data modelling

Data on total affected tadpoles (dead at day 4 plus malformed at day 6) obtained in groups exposed during the "teratogenicity window" were modelled by PROAST software. Single dose-response curves (Fig. 6a-b) were obtained applying exponential models. Log-likelihood ratio test showed the equal steepness assumption not rejected ($p = 0.58$) and RPF of BPB versus BPA was derived by dose-response curve comparison: BPB resulted 3.42 times (CI 3.20–3.68) more potent than BPA in inducing teratogenic effects (Fig. 6c).

3.3. Main developmental toxicity test: exposure during the neuro-behavioural toxicity window (NF 38–46)

3.3.1. R-FETAX: evaluation at day 6 (NF stage 46)

Groups exposed at tadpole stages (NF 38–46) did not differ in terms of lethality and teratogenicity from DMSO-group (Table 3). Deglutition test was positive for all tadpoles, showing, also for this parameter, no differences among groups. By contrast, the statistical analysis of data obtained by functional swimming test suggested significant altered tracking profiles in tadpoles exposed to BPA 25 μM and BPB 10 μM (increased distance in the inner circle) (Table 4, Fig. 7). Due to the poor sample size and the high variability, these data were excluded from modelling and should be considered just preliminary, needing an ad hoc refined evaluation including increasing the sample size and investigating eventual involved pathogenic pathways.

4. Discussion

BPA is one of the best characterised endocrine-disrupting chemicals, exhibiting both oestrogen-like and anti-androgenic activity [2–7]. *Xenopus* embryo model is widely used in embryotoxicity research fields [53–56], and has been also selected to test BPA developmental toxic effects. BPA exposure induced lethal and teratogenic effects with the reported estimated lethal concentration for 50% samples (LC_{50}) 21 μM [23,27]. Ge and colleagues described, side to extremely severe teratogenic effects (stunted body, bent notochord, short or bent tail axis, deformed brain and/or eyes, cardiac or abdominal oedema, miscoiled gut, and lengthened abdomen), concentration-related behavioural deficits evaluated by touch response at 48, 60, and 72 h and by autonomous swimming tracking at 96 h. Effects were correlated to the observed apoptosis detected at 96 h by acridine orange staining and TUNEL techniques in groups exposed to 10 and 20 μM BPA [23].

Recent restriction on the use of BPA stressed the need for entry of its analogues, including BPB, in the market. BPB-related teratogenic and neuro-behavioural effects in the zebrafish model are described by Yang et al. [39] exposing embryos during the whole test period: pericardial and yolk oedema, curvature and fin defects, decreased swimming ability were detected. In this work the relative potency of BPB versus its parental compound, BPA, was not evaluated.

Aim of our work was the comparison of effects induced by BPA and its analogue BPB. Teratogenic effects were evaluated in samples exposed during the "teratogenicity window", covering NF stage 10–26 considered in *X.laevis* the phylotypic period (pharyngula). By definition, pharyngula is common to any vertebrate at both morphological and molecular point of view [40–42]. Anuran-specific morphogenetic phases (NF stage 27–37) were not considered, because these events are difficult to compare with mammal morphogenesis. Neurocognitive behavioural effects were evaluated exposing samples during the "neuro-behavioural toxicity window", covering the spontaneous swimming acquisition period (tadpole model, NF 38–46), reported indicative to evaluate neuro-developmental disorders [43]. As reported by literature, till time of hatching (NF stage 37/38), tadpole locomotion is still inflexible, with a stereotyped organisation; while by larval stage 42, approximately 1 day later, a gradually acquisition of motor versatility enables animals

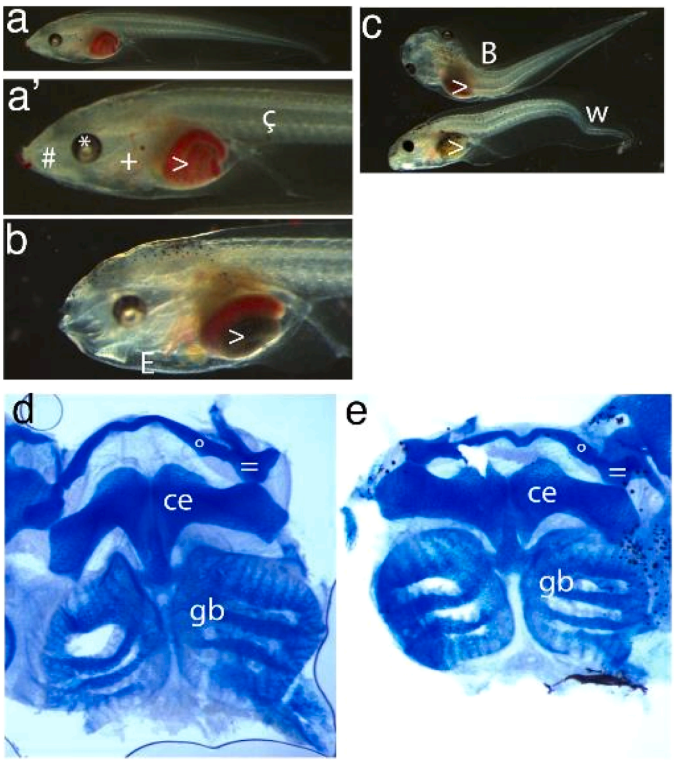

Fig. 3. Main experiment: phenotypes observed at the end of R-FETAX in groups exposed at phylotypic stages (NF 10–26, teratogenicity window).
External morphology: a-a') normal phenotype of NF 46 tadpole. Note the linear encephalon (dotted line), the eye (*) representing the limit border between the anterior craniofacial region (#) and the branchial basket (+), the coiled intestine (>, index of NF 46 developmental stage reached) and the tail (ç). b) abnormal phenotype showing round head (dotted line) and ventral oedema (E). Normal coiling intestine (>). c) severe abnormal phenotypes with multiple defects, including round head, oedema and bent (B) or wavy (w) tail. Normal intestine coiling (>) confirms NF 46 stage reached. a-a') DMSO-exposed tadpole; b) BPB 7.5 μM-exposed tadpole; c) BPB 7.5 μM-exposed tadpole. Red stain in the intestine= deglutition test positive. a-c) Magnification 8x; a'-b) Magnification 20x.
Cartilage evaluation d-e (flat-mount technique, magnification 40x): structure of facial and branchial skeletal elements in a DMSO-exposed tadpole (d) and in a BPB 7.5 μM-exposed tadpole classified as abnormal at the external evaluation (e). In e facial elements are reduced but not fused (= maxilla, ° mandible, ce ceratohyal cartilage); gill basket (gb) is shorter and smaller than normal.

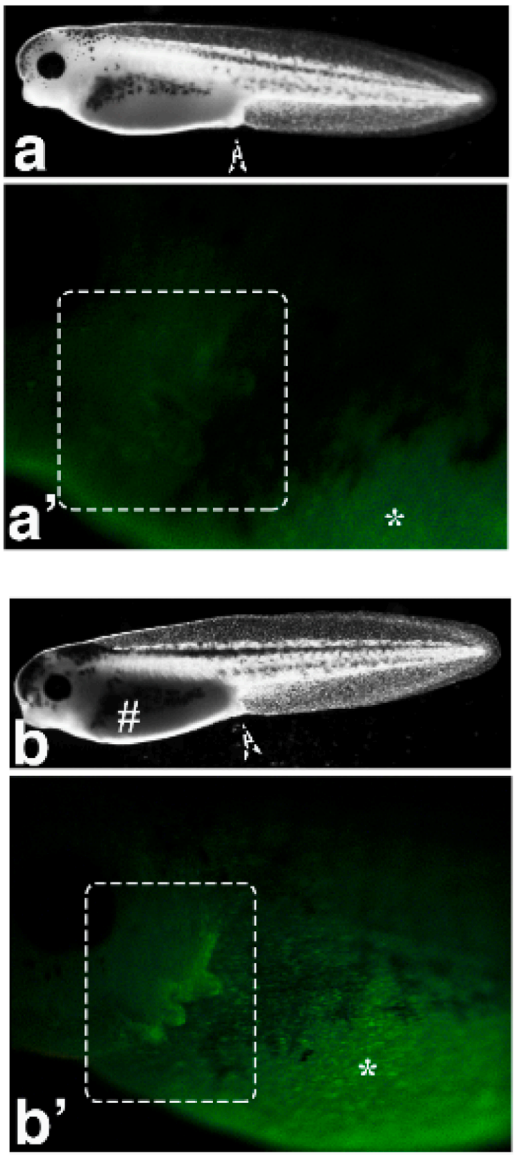

Fig. 4. Superfast- R-FETAX. Morphology of tailbud exposed during the teratogenicity window (NF 10–26) and evaluated 1.5 days later (at NF 40) using a cold-light stereomicroscope (a, b). By definition, NF 40 stage is characterised by "mouth broken through, length of gills about twice their breadth, the anterior branched and posterior one sometimes also showing a branch, blood circulation in gills beginning, outlines of proctodeum and tail myotomes forming angles of 90 degrees (>)". A strict correlation between abnormal gill primordia and apoptosis was evident: acridine orange staining shows fluorescent bright green apoptotic areas in atypical gill primordia (b'). Unspecific yolk auto-fluorescence was visible at the ventral region (*, where the yolk mass (a-b, #) is abundant in intestines) in all samples. a-a', DMSO normal sample, b-b' BPA 25 μM sample with reduced gill primordia, characterised by marked fluorescence. Dotted boxes indicate the branchial region. Magnification: a-b 20x; a'-b' 80x.

to move efficiently and coordinately through thy environment: in a brief 24-h period, motoneurons differentiate, both in firing properties and peripheral innervation fields, in a manner that promotes the control of movement direction and speed during 3D navigation through the environment [44].

Our results confirm BPA and BPB as teratogenic agent, reclassifying lethal effects as a consequence of teratogenicity itself: lethality resulted secondarily induced by specific severe malformations at the branchial apparatus, visible one day after the end of exposure. Relationship between branchial arch derivatives in amphibians and mammals is shown in Table 5 and suggest that early pregnancy exposure to BPA or BPB could elicit foetal effects with craniofacial and neck elements involved. In an in vivo rat study, BPA maternal treatment during organogenetic period was correlated to cleft palate [57]; in humans, a correlation between maternal environmental exposure to BPA and foetal severe multiple malformations [15] has been reported. No data are available on BPB.

The specific BP-related teratogenic mechanism of action is not fully understood. According to previous works on BPA effects [23,58,59], we showed a correlation between BP-reated teratogenic effects and apoptosis. Considering that BPA induces apoptosis via oxidative stress in different cellular models [60,61], a complex teratogenic adverse outcome pathway can be hypothesised and needs further detailed ad hoc experiments investigating the linked molecular events.

As far as the relative potency of BPB versus BPA is concerned, the present work clearly shows BPB sharing the same teratogenic activity than BPA, with a marked higher potency (BPB approximately three folds more potent than BPA for both endpoints). Structurally, BPB has an ethyl group on the central carbon atom instead of a methyl group found in BPA and shows more hydrophobic nature (a more hydrophobic nature

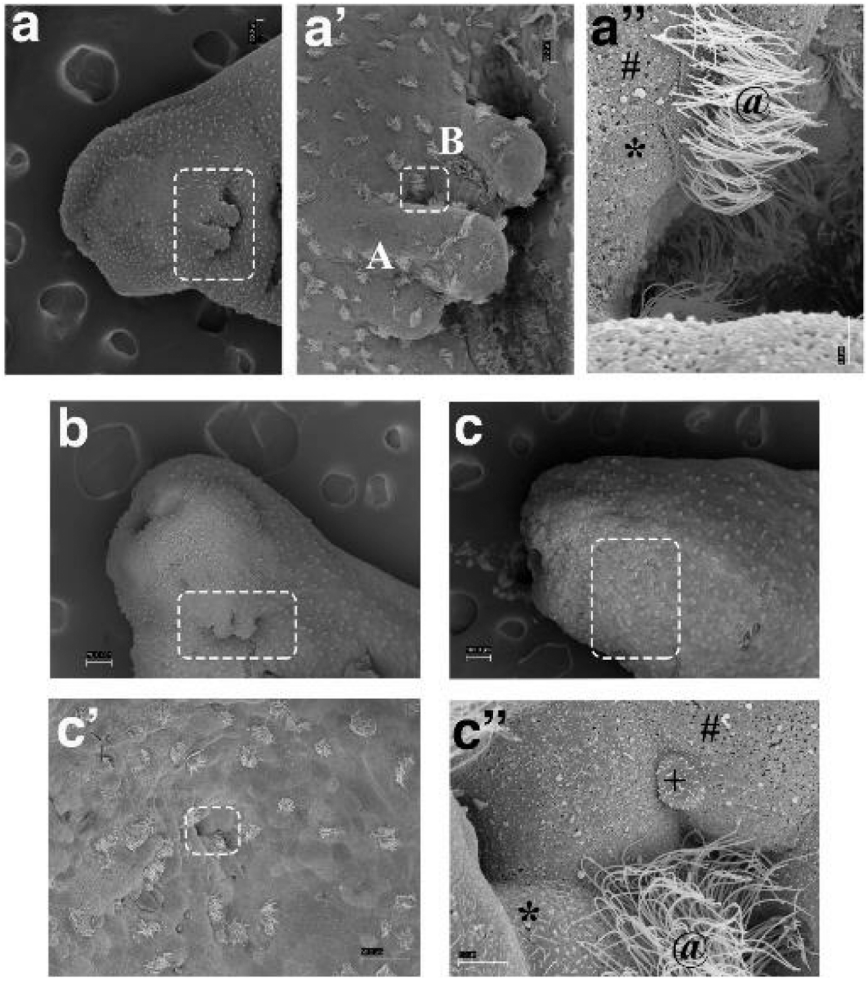

Fig. 5. Superfast- R-FETAX. SEM fine morphology of tailbud exposed during the teratogenicity window (NF 10–26) and evaluated 1.5 days later (at NF 40). a-a') normal gill primordia: both gill rudiments (A, B) are typically branched and nipple-shaped, the branches of the anterior one well elongated. Extremely shortened and unbranched (b) or absent (c-c') gill primordia were observed in samples exposed at NF 10–26 to BPA 25 μM or BPB 7.5–10 μM. High magnification SEM images show the normal skin at NF 40 stage with three cell lines visible: keratinocytes (*), mucosal cells (#) and ciliated cells (@) both in DMSO-exposed normal samples (A'') and in BPs-exposed samples (c''). In c'' (sample with gill primordium agenesis) note an apoptotic cellular debris (+) about to be extruded from the surface. Dotted boxes indicate the gill primordium regions and the area of subsequent magnification. a-a'-a''), DMSO normal sample; b) BPB 7.5 μM; c-c'-c'') BPB 10 μM. Magnification: a-b-c) 250x; a'-c') 1k x; a''-c'') 10k x.

increases its oestrogenic activity) [62]. Finally, literature demonstrated that, in comparison to BPA, BPB shows slow aerobic and anaerobic biodegradation, possesses more acute toxicity and it is slightly more cytotoxic than BPA and that it can possess genotoxic potential too [62]. These considerations and our data support that the use of BPB as BPA substitute should require further focused assessments.

Finally, our present work shows BP-related neuro-behaviour defects in tadpoles exposed during neuro-cognitive sensitive stages. The present work excludes indirect effects on swimming performances (samples were not affected by developmental delays, tail defects or other abnormalities), therefore a direct effect of BPA and BPB on swimming performances was demonstrated. The involved specific pathogenic pathways for neuro-behavioural toxicity were not investigated by our present work and ad hoc experiments to explain this interesting point are needed. These data must be considered only preliminar and need an ad hoc refined evaluation including increasing the sample size and investigating eventual involved pathogenic pathways.

In conclusion, R-FETAX methodology resulted sensitive to detect teratogenic and neuro-behavioural effects related to BP-exposure. We suggest R-FETAX as a rapid, unexpensive and sensitive method as elective for screening known or suspected endocrine-disrupting chemicals or their mixtures, potentially detrimental to aquatic and terrestrial systems and to human development.

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Ethical approval

The study was conducted according to the relevant European (EU Directive 2010/63/EU for animal experiments) and Italian (Legislative Decree No. 26/2014) laws, rules, and regulations. All procedures were examined and approved by the Animal Welfare Organization of the Università degli Studi di Milano. Facility authorization number: 198283; date: 19/12/2019.

Author contributions

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by all authors. The first draft of the manuscript was written by Elena Menegola and all authors commented on previous versions of the manuscript. All authors

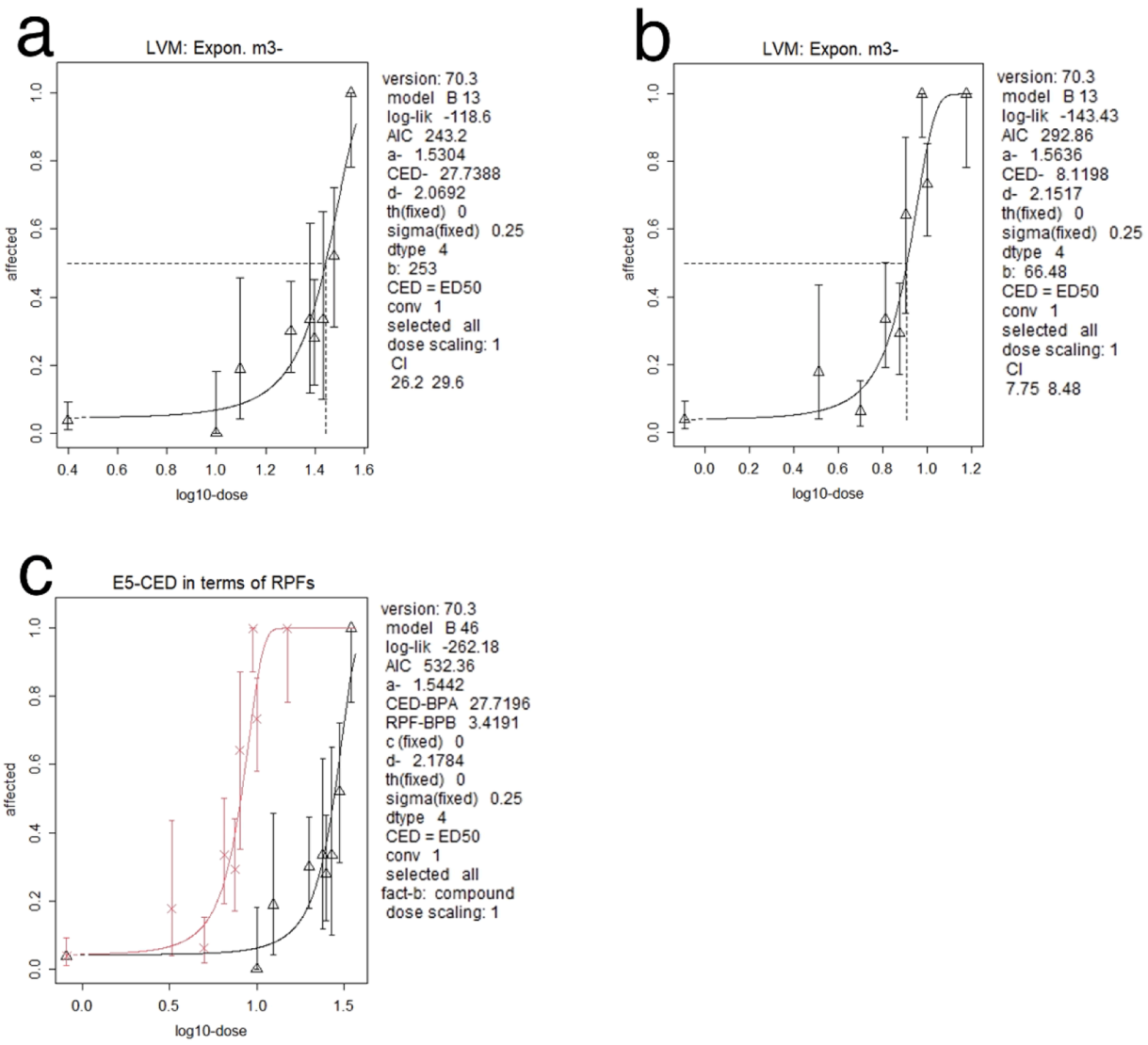

Fig. 6. Exponential models showing teratogenicity dose-response curves of BPA (a) and BPB (b) and curves modelled fixing c= 0 to derive the relative potency factor (RPF, c), indicating BPB (red line, cross) nearly three times more potent than BPA (black line, triangles). X axis= $\log_{10}$ dose; Y axis= % total effect (dead + malformed).

Table 3
Main test: lethal, teratogenic (abnormal) and total (dead + abnormal) effects observed in groups exposed to BPA or BPB during neuro-behavioural toxicity window (tadpole period, NF 38–46). Statistics (Chi-square for trend, calculated on frequencies) are shown.

Concentration (μM)	Dead (% of exposed)	Abnormal (% of living tadpoles)	Total (% of exposed)	Concentration (μM)	Dead (% of exposed)	Abnormal (% of living tadpoles)	Total (% of exposed)
DMSO (BPA 0)	4.2	0.0	4.2	DMSO (BPB 0)	4.2	0.0	4.2
(N = 24)				*(N = 24)*			
BPA 10	0.0	0.0	0.0	BPB 5	0.0	0.0	0.0
(N = 15)				*(N = 9)*			
BPA 20	0.0	0.0	0.0	BPB 7.5	11.1	0.0	11.1
(N = 15)				*(N = 9)*			
BPA 25	0.0	0.0	0.0	BPB 10	11.1	0.0	11.1
(N = 18)				*(N = 9)*			
p=	**0.2032**		**0.2031**	**p=**	**0.4566**		**0.4566**

Table 4
Main test: swimming parameters in groups exposed to BPA or BPB during neuro-behavioural toxicity window (tadpole period, NF 38-46). Statistics (ANOVA followed by Tukey's test) are shown: *= p<0.05, **= p<0.01 vs DMSO.

	N	Immobility Time (s)			Total distance (mm)			Outer ring distance (mm)			Inner circle distance (mm)			Speed (mm/s)		
DMSO (BPA 0 μM)	19	31.04	±	17.45	135.22	±	154.91	127.64	±	152.02	7.58	±	15.01	8.18	±	5.91
BPA 10 μM	14	18.12	±	9.72	131.61 *	±	117.12	111.89	±	99.94	19.72	±	32.54	7.87	±	5.80
BPA 20 μM	15	16.14 *	±	10.38	171.97	±	159.63	156.33	±	147.03	15.65	±	19.58	9.31	±	6.19
BPA 25 μM	18	21.59	±	20.98	310.38 *	±	229.89	253.46	±	217.69	56.92 **	±	68.09	12.02	±	6.12
p (AVOVA)		**0.0375**			**0.0101**			**0.0601**			**0.0028**			**0.1752**		
DMSO (BPB 0 μM)	19	31.04	±	17.45	135.22	±	154.91	127.64	±	152.02	7.58	±	15.01	8.18	±	5.91
BPB 5 μM	9	40.82	±	18.10	196.15	±	198.48	165.01	±	169.77	31.46	±	31.89	8.35	±	5.26
BPB 7.5 μM	8	40.02	±	13.10	144.73	±	73.29	123.55	±	57.09	22.62	±	30.12	8.46	±	2.88
BPB 10 μM	8	34.32	±	19.28	322.36	±	236.17	244.41	±	199.39	83.23 **	±	82.70	10.29	±	6.33
p (AVOVA)		**0.4489**			**0.0815**			**0.3137**			**0.0010**			**0.8246**		

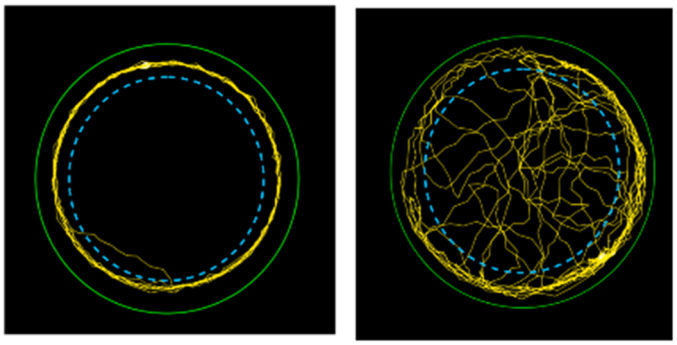

Fig. 7. Exemplificative swimming profiles of tadpoles exposed during the neuro-behavioural toxicity window (tailbud stages, NF 38–46) to DMSO alone (left) or to BPA 10 μM (right). Green circle represents the arena, light blue dotted circle represents the 0.75 inner circle area, set for data analysis. Yellow lines: swimming tracking elaboration. Note, in the left image, the normal behaviour with the tracking profile mostly limited to the outer ring and, in the right image, the disorganised swimming profile typical of BPA 10 μM tadpoles.

Table 5
Skeletal derivatives of branchial arches in amphibians and mammals.

Embryonic pharyngeal arch	*X. laevis* tadpole structures	Mammalian structures
I	Maxilla Mandible	Maxilla Mandible Secondary palate Temporal bone Incus Malleus
II	Ceratohyal cartilage	Styloid process of temporal bone Hyoid bone (upper part) Stapes
III-VI	Branchial basket cartilages	Hyoid bone (lower part and greater horns)

read and approved the final manuscript.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Elena Menegola reports financial support was provided by Università degli Studi di Milano. Elena Menegola, Francesca Di Renzo, Renato Bacchetta reports a relationship with Università degli Studi di Milano that includes: employment.

Data availability

Data will be made available on request.

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