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Adverse effects of bisphenol A on the testicular parenchyma of zebrafish revealed using histomorphological methods

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ABSTRACT: The purpose of this study was to evaluate the consequences of bisphenol A (BPA) exposure in zebrafish (*Danio rerio*). Short-term effects at a testes level were assessed by means of different biomarkers in 60 male zebrafish exposed for 14 days in aquariums to different concentrations of BPA (1, 10, 100 and 1000 µg/l) in addition to a control batch. The LC-MS/MS method was used for the quantification of BPA. The BPA levels in the zebrafish tissues increased together with increasing exposure of fish to BPA. Several alterations were observed in the zebrafish testes. Particularly notable morphological changes included, a pronounced degeneration of all cell components, an increase in the percentage of the sustentacular (Sertoli) cells, and a marked decrease in the percentage of germ cells starting from concentrations of 100 µg/l of BPA. Our results indicate that histopathology and testes morphometry are sensitive biomarkers for the analysis of early effects of an environmental concentration of BPA in zebrafish experimental models. Our observations reveal that the nature and intensity of the morphological changes in the testes are concentration-dependent in the case of BPA, and manifest as adverse effects on the fish's reproductive health.

Keywords: endocrine disruption; BPA; *Danio rerio*; sustentacular cells; spermatogonia; spermatocytes; spermatids; spermatozoa

The production of large amounts of synthetic industrial, as well as unwanted, pollutants has destructive consequences for our ecosystem and triggers negative health effects. Exposure to environmental pollutants is suggested to be one of the causes of reproductive problems worldwide. Some of the more damaging chemical contaminants are defined as exogenous chemicals or chemical mixtures that impact the endocrine system structure or function. These cause adverse effects (Flint et al. 2012), and are classified as endocrine-disrupting chemicals since they can interfere with the synthesis, metabolism and action of endogenous hormones (Yeung et al. 2011). There is growing evidence in support of the claim that contamination of a wildlife population with oestrogenic chemicals can disturb the reproductive function of vertebrates (Hassanin et al. 2002). This has long been established in wildlife and in laboratory animal studies, and has been shown to affect testis development and function although the mechanisms of action are not clear (Lopez-Casas et al. 2012).

Chemicals implicated in endocrine disruption include biocides, industrial compounds, surfactants, and plasticizers including bisphenol A (BPA) (Flint et al. 2012). BPA is one of the most highly produced chemicals worldwide. It is widely used in the manufacture of various polycarbonate plastics, which are employed in lacquer coatings in food cans, food and beverage containers and dental sealants. It is also present as an environmental contaminant in rivers and drinking water, probably due to the mi-

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gration of plastic containers from industrial rubbish heaps. BPA levels of 0.02–21 µg/l (Crain et al. 2007) and of 0.01–8 µg/l (Kang et al. 2007) were reported in river water. Higher concentrations were also reported near wastewater treatment plants or landfills (Kang et al. 2007). The importance of BPA as an environmental contaminant and the risk it entails, due to its low biodegradability rate and its bio-accumulation in the trophic chain, have caused the European Parliament to recently include it as a substance whose toxicity should be evaluated. The European Union has agreed to ban the presence of BPA in plastic feeding bottles because of its possible harmful effects on child health (Comission Directive 2011/8/EU). However, for the moment, the European Commission has no plans to further restrict the use of BPA and has agreed to maintain the admissible daily intake of BPA for humans at 0.05 mg/kg/day. However, the considerable amount of research on the action of BPA on health reflects certain doubts (EFSA 2008).

Much research has been focused on fish, since, as they are aquatic inhabitants, they receive sewage or industrial effluent and agricultural runoff containing oestrogenic chemicals (Hassanin et al. 2002). Nevertheless, data about the effects of BPA on fish reproduction are rare. Zebrafish are suitable for assessing the toxic effects of chemicals on development and reproduction. This is because test protocols, including OECD guidelines (OECD 204; OECD 210; OECD 212) that recommend zebrafish for chemical toxicity assessments, as well Annex 1 of Directive 2010/63/EU, relating to the protection of animals used for scientific purposes, have already been established.

At the fourth meeting of the OECD Task Force on Endocrine Disrupter Testing and Assessment, it was generally agreed that histopathology should be adopted as a core endpoint in the assessment of oestrogen-active compounds (Segner et al. 2003).

The purpose of this study was to evaluate the reproductive effects of environmental BPA concentrations on zebrafish testes using histological and histomorphometrical methods.

MATERIAL AND METHODS

Experimental exposure. Six-week-old male zebrafish (*Danio rerio*; n = 60; standard length; 4.22 ± 0.211 cm; 0.553 ± 0.118 g wet weight) were used, these being distributed randomly n = 12/aquarium. The experimental procedure was carried out in the Experimental Animal Service of the University of Cordoba. Treated groups were exposed for 14 days (OECD 204) to graded concentrations (1, 10, 100 and 1000 µg/l) of BPA (Sigma Aldrich®, St Louis, MO, USA) under flow-through conditions (10 water renewals/day) and a photoperiod of 16-h light : 8-h dark. Water temperature was 26 ± 1 °C and dissolved oxygen was maintained above 60% of saturation level by continuously aeration of the test solution. Zebrafish were fed twice a day with a non-oestrogenic granulated diet (Supervit® minigranulated, Tropical, Chorzow, Poland). A control group (unchlorinated tap water) completed the experimental design.

The research procedure was carried out after approval by the animal care committee of the University of Cordoba (Spain) and in concordance with the European Regulations for the Protection of Experimental Animals (Directive 2010/63/EU).

After two weeks of exposure, zebrafish were sacrificed with an overdose of anaesthetic solution of tricaine methanesulfonate (MS-222® 500 mg/l; Sigma Aldrich®, St Louis, MO, USA) buffered with sodium bicarbonate (300 mg/l; Sigma-Aldrich®, St Louis, MO, USA). Standard length and body weight were then measured immediately.

Testes from animals assigned to the histological study (n = 6) were dissected and fixed for qualitative and quantitative histological analysis. Each fish was necropsied by placing it in right lateral recumbency on the stage of a dissecting microscope. The left body wall was removed to excise the testes, dissecting the fish in a caudal-to-cranial direction, while applying very gentle traction to their testes (Wolf et al. 2004). Fish for toxicological analysis (n = 6) were dried with a sterile gauze and then frozen and stored at –80 °C for analytical BPA determinations.

Analysis of BPA content in water and fish. Three times a week, water from each tank was sampled and frozen pending the analytical verification of BPA exposure concentrations. Prior to analysis, the samples were thawed and subsequently processed by conditioning with ammonium hydroxide, followed by filtration through 0.22 micra and injection of a 20 µl sample into the LC-MS/MS system, with a detection limit of 0.2–0.3 µg/l.

Whole body homogenates were obtained for BPA quantification. Samples were processed for their

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extraction and purification, and finally transferred into vials, using a modification of the technique employed by Schmidt et al. (2008). For BPA determination, 20 µl were injected into the LC-MS/MS system with a detection limit of 5 µg/g. The HPLC system consisted of two Varian 210 series pumps, a Varian 410 series autosampler and a Metahcem Tecn degasificator with four channels in line. A Synergi Hydro RP 80 A, 150 × 2 mm 4 µ Phenomenex column was used for the chromatographic separation with an AQ C18 4 × 2 mm Phenomenex precolumn. For the HPLC-MS/MS experiments, a 1200 L Varian triple quadruple mass spectrometer was employed. The IS voltage was 1400 V, temperature of gas drying 350 °C, and pressure of gas drying 30 psi. Mass spectrometry analyses were performed in the MRM mode (multiple reaction monitoring). For the MRM experiments, the collision gas was argon. The mobile phases for negative ionisation were 0.05% TEA (triethylamine) in Milli Q water and acetonitrile with a gradient from 100% Milli Q water to 100% acetonitrile in 21 min at 200 µl/min.

Light and electron microscopy. For the structural evaluation, the fixed testes were routinely processed for paraffin sections by fixing in 10% formaldehyde, dehydrating in graded series of ethanol, immersing in xylol and embedding in paraffin wax. Every tenth section (4 µm thick) of each block was stained with haematoxylin and eosin and used for the morphological study.

For the ultrastructural study, small randomly selected samples of gonads were primarily fixed in a 2% glutaldehyde solution in 0.1M phosphate buffer (pH 7.4) overnight at 4 °C and then refixed in 1% osmium tetroxide in 0.1M phosphate buffer (pH 7.4) for 30 min. After dehydration in graded ethanol series and embedding in araldite, semithin and ultra-thin sections were cut on an ultramicrotome (Leica ultracut R). Semi-thin sections were stained with toluidine blue, whereas ultra-thin sections were double-stained with uranyl acetate and lead citrate. Ultra-thin sections were viewed and photographed using a CM 10 transmission electron microscope (Philips Export B.V.).

Morphometric study. For the morphological study, the fixed gonads were cut into three sections. Each portion was then processed and embedded in paraffin as for routine histology. The first section (4 µm thick) of each block was stained with haematoxylin and eosin and used for the stereological study.

The quantitative study was performed using an image analysis system consisting of a Leitz Ortholux triocular microscope connected by means of a SONY SSC-C370P® colour video camera to an IBM-compatible personal computer equipped with a frame grabber board. Each specimen was sampled in a systematic manner for the selection of microscopic images that were then digitised; a 40 × lens (N.A. 1.25) was used for this procedure. An average of 20 microscopic fields per slab was chosen in each specimen.

In each microscopic image the cellular composition of the testes parenchyma was estimated by calculating the percentage of sustentacular cells (Sertoli cells), spermatogonia, spermatocytes, spermatids and spermatozoa.

Statistics. Data were analysed using the statistical program Statgraphic (Centurion XVI®) to determine BPA effects on every exposed group. ANOVA (*F*-test) was used to determine if significant differences existed between the averages. The Fisher LSD post hoc test was used to perform multiple comparisons between groups. Results are expressed as mean values ± SD and $P < 0.05$ was considered to be significant.

Data of the cell population of the seminiferous tubule were analysed by applying bivariate comparisons considering non-parametric procedures (Kruskal Wallis). $P < 0.05$ was regarded as significant.

RESULTS

No fish mortality was observed during the experiment. No gross pathologic changes were found, and no significant differences in mean body weight and standard length were detected between the control and the BPA-exposed animals.

BPA levels

The concentrations of BPA in water coincided with the nominal ones, in agreement with prior studies reported by different authors (Dorn et al. 1987).

BPA concentrations in the zebrafish at Day 14 after 1, 10, 100 and 1000 µg/l of BPA exposure are reported in Table 1. An increase in the BPA concentration was observed in the zebrafish exposed to

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Table 1. Zebrafish bisphenol A levels (μg/g) expressed as mean ± SD

	Bisphenol A concentration (μg/l)				
	Control	1	10	100	1000
Bisphenol A concentration (μg/g)	nd	0.045 ± 0.03*	0.072 ± 0.014*	0.55 ± 0.009*	40.643 ± 1.404*

*Significantly different from the control at $P < 0.05$
nd = non-detected

graded concentrations of BPA, whereas BPA levels in the control group were non-detectable. In the treated groups, the data showed significant differences ($P < 0.05$) from the control group. There were significant differences between groups treated with 1 and 10 μg/l, and the groups exposed to higher BPA concentrations (100 and 1000 μg/l), while no differences were detected between groups at lower concentrations (1 and 10 μg/l).

Light and electron microscopy

Under the light microscope, in the testes of the fish belonging to the control group, a testicular parenchyma organised by numerous highly developed spermatocysts, exhibiting all the cell components and with an apparently normal distribution and abundant spermatozoa (Figures 1A and 1B), was observed. The interstitium showed the presence of abundant connective cells and blood vessels.

Under the electron microscope we observed cell components with an apparently normal morphology. The sustentacular cells formed a continuous lining of the spermatocysts, with no apparent alteration (Figure 1C). The spermatogonia were of a non-specific morphology (Figure 1D), while the spermatocytes exhibited chromatin condensation and were defined by the presence of meiotic divisions (Figure 1E). The spermatids were of different shapes but, in the last phase, a nuclear invagination was observed where the centriole was localised (Figure 1F).

Finally, the spermatozoa displayed fundamentally spherical heads, although some could be oval-shaped, in which a very dense nucleus stood out with an invagination in which a centriole was located in the basal body of an extremely long type 9 + 2 cilium (Figure 1G) was integrated. There was hardly any cytoplasm and only a protuberance in which some mitochondria related to the cilium and a thin laminar projection starting from the ciliary base were grouped (Figure 1H).

The morphology of the testes of the fish group treated with 1 μg/l of BPA was very similar to that of the control group fish. All the cell components forming the spermatogenesis could be observed, as well as the sustentacular cells, with numerous spermatozoa in the lumens of the collecting ducts being noted. The interstitial tissue was slack and no signs of oedema were detected.

In the fish group treated with 10 μg/l of BPA, certain signs of degeneration of the germinal epithelium and of the spermatozoa were observed (Figures 2A and 2B) under the light microscope. Under the electron microscope, clear signs of cell component degeneration were seen. The sustentacular cells presented tumefaction processes (Figure 2C), and a partial loss of spermatagonia cells was observed, with the presence of degeneration in the nuclear envelope (Figure 2D). The spermatocytes exhibited alterations to their chromatin (Figure 2E) as well as spermatid alterations in their invaginations (Figure 2F). The spermatozoa not only diminished in number; we also found vacuole alterations in their cilium and the presence of immature shapes in the lumen of their cysts and collecting ducts (Figures 2G and 2H).

The fish treated with 100 μg/l of BPA, showed clear signs of degeneration in their testicular parenchyma, with a clear decline in their germinal epithelium cells and in their spermatozoa, and an accentuated increase in sustentacular cells (Figures 3A and 3B) observed under the light microscope. Under the electron microscope it was seen that all the cell components were altered. The degenerated sustentacular cells exhibited a vacuolisation of the cytoplasm (Figure 3C). Degeneration and tumefaction of the spermatogonia (Figure 3D), degradation of the chromatin of the spermatocytes (Figure 3E) and nuclei and implantation fossae of the spermatids (Figure 3F) were also observed. The spermatozoa exhibited immature and degenerated shapes (Figures 3G and 3H).

The testicular morphology of the fish treated with 1000 μg/l of BPA showed a highly transformed parenchyma under the light microscope. The cysts

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presented themselves as areas which had lost most of their immature germ cells and spermatozoa, and, in their place, very numerous deposits of sustentacular cells remained (Figures 4A and 4B). Generally, cell degeneration was observed under the electron microscope. A highly significant increase in sustentacular cells was observed and morphological characteristics of active cells were evident (Figure 4C). The spermatogonia decreased in number and were degenerated (Figure 4D). Although they were altered, the spermatocytes maintained their meiotic divisions (Figure 4E). Vacuolisation processes in the spermatids were observed both in the cytoplasm and in the nuclear envelopes (Figure 4F). The number of spermatozoa was highly reduced; some showed a normal morphology, while others exhibited degenerative processes and highly altered shapes (Figures 4G and 4H).

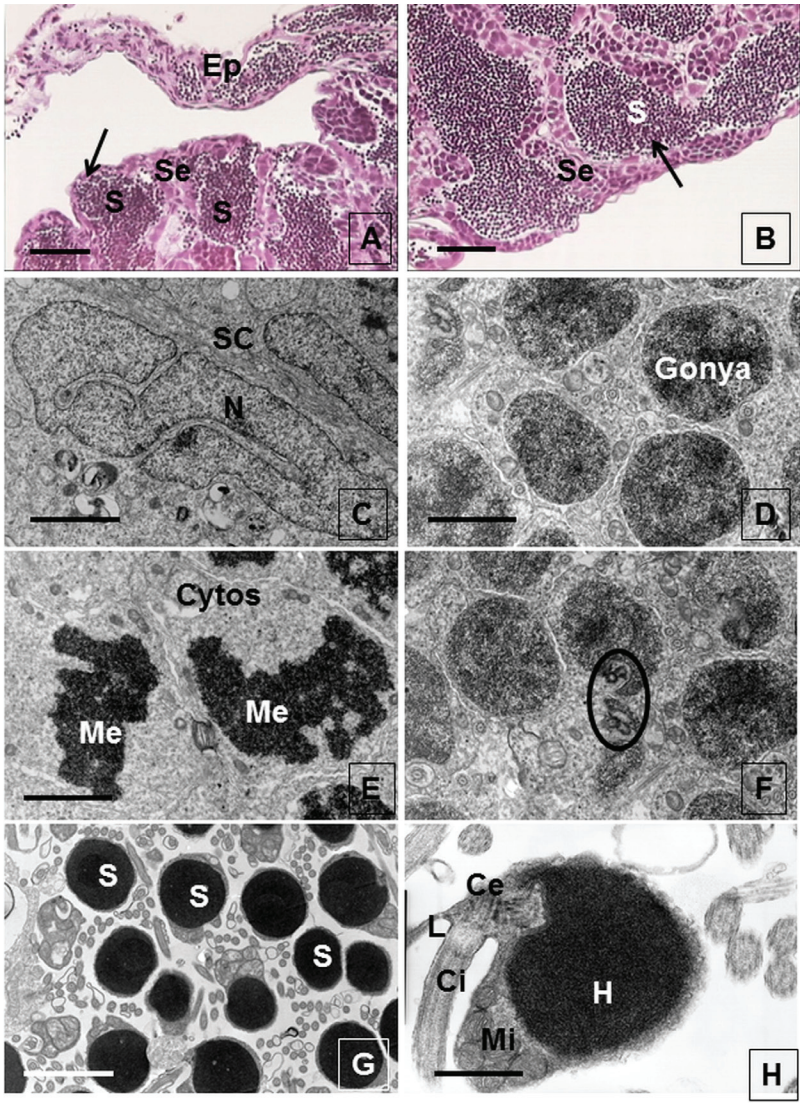

Figure 1. Histological changes in zebrafish testes control group. (**A**, **B**) Light microscope, bars = 100 µm, (**C**, **D**, **E**, **F**, **G**, **H**) ultrastructural observations, bars = 10 µm; (**A**) detail of the testicular parenchyma with spermatocysts (arrow) with seminal epithelium (Se) and accumulations of spermatozoa (S) and a normal epididymis (Ep); (**B**) detail of the testicular epithelium with spermatocysts (arrow) with seminal epithelium (Se) and spermatozoid accumulation (S); (**C**) detail of the sustentacular (Sertoli) cell (SC) under electronic microscope with a pleomorphic nucleus (N) and with highly signalled invaginations; (**D**) detail of spermatogonia (Gonya) with a mosaic arrangement; (**E**) spermatocytes (Cytos) which show meiotic divisions (Me); (**F**) detail of spermatid with implantation fossae (circle), (**G**) abundant spermatozoa (S); (**H**) detail of spermatozoid with electron-dense head (H), the centriole (Ce), the cytoplasmic lamella (L) with mitochondria (Mi) and a single cilium (Ci)

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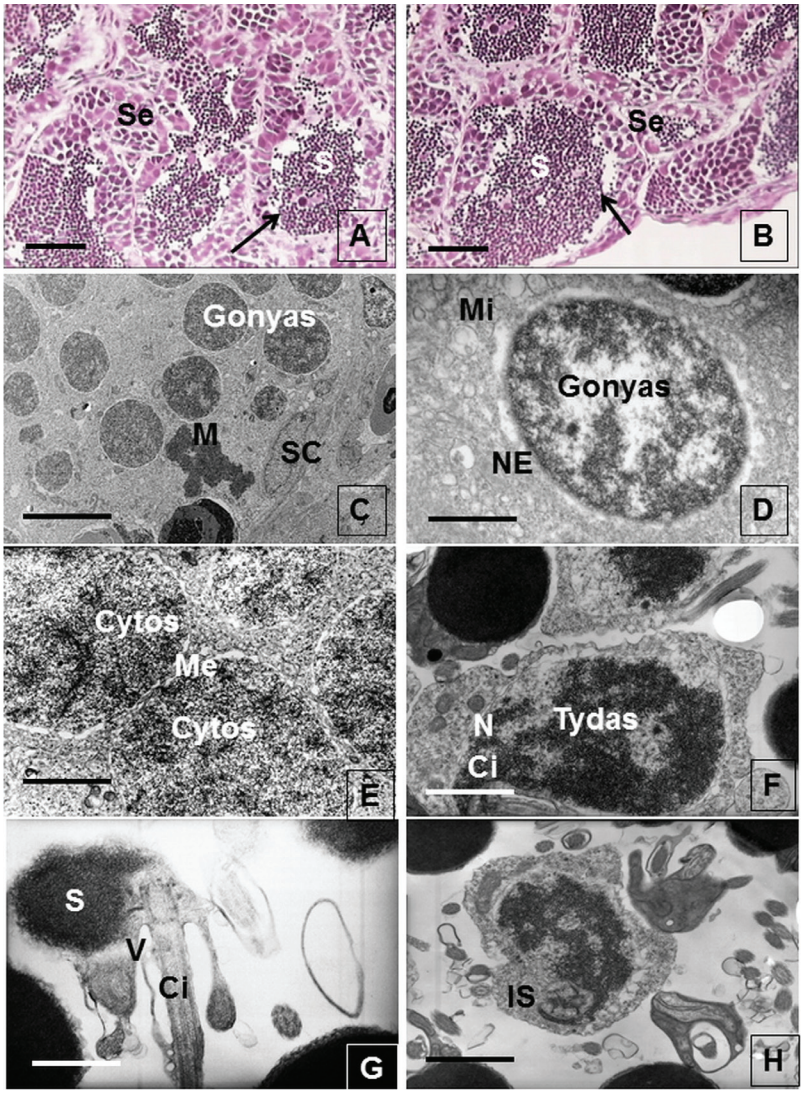

Figure 2. Histological changes in zebrafish testes exposed to 10 µg/l of bisphenol A. (**A**, **B**) Light microscope, bars = 100 µm; (**C**, **D**, **E**, **F**, **G**, **H**) ultrastructural observations, bars = 10 µm; (**A**) detail of testicular parenchyma with spermatocysts (arrow), with a vacuolised seminal epithelium (Se); the same happens in areas containing the spermatozoa (S); (**B**) Detail of the testicular epithelium with spermatocysts (arrow) with germinal epithelium (Se) and spermatozoid accumulation (S); (**C**) detail of Sertoli cell (SC) which is related to the spermatogonia (Gonyas), some of them in mitosis (M); (**D**) spermatogonia (Gonyas) with dilated nuclear envelopes (NE) and tumefact mitochondria (Mi); (**E**) spermatocytes (Cytos) in an initial meiosis phase (Me); (**F**) spermatids (Tydas) which develop the neck (N) and the cilium (Ci); (**G**) detail of spermatozoid (S) with vacuolisation (V) of the lamellae and cilia (Ci); (**H**) detail of immature shapes (IS) in the lumen of the spermatocysts

Morphometric study

Sustentacular and germ cell percentages in the sperm ducts from each study group are reported in Table 2. Significant differences were observed between the different groups.

The data reported in the table indicate that there were no significant differences between the percentage of sustentacular cells in the control group and the groups with the lower exposure doses (1 and 10 µg/l), nor were there any significant differences between the groups exposed to the higher concentrations (100 and 1000 µg/l), although, as indicated by the data, there was a marked increase in the sustentacular cell percentage in those two exposure groups, with significant differences ($P < 0.05$) between them, the control, and the 1 and 10 µg/l groups.

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Table 2. Germ cell percentages in each group expressed as mean ± SD

Study groups	Sustentacular (Sertoli) cells	Germ cells			
		spermatogonia	spermatocytes	spermatids	spermatozoa
Control	7.05 ± 2.61	10.9 ± 4.05	14.2 ± 4.49	14.75 ± 5.96	53.1 ± 9.61
1 µg/l	11.15 ± 4.25	10.9 ± 3.82	12.15 ± 4.49	14.35 ± 3.69	51.45 ± 6.42
10 µg/l	11.9 ± 4.54	9.15 ± 2.96	11.15 ± 5.58	16.6 ± 6.87	51.2 ± 6.35
100 µg/l	36.95 ± 3.38	7 ± 2.70	10.75 ± 2.81	6.15 ± 2.83	39.15 ± 6.06
1000 µg/l	41.5 ± 2.84	6.85 ± 2.23	8.2 ± 3.29	7.45 ± 2.67	36 ± 3.87

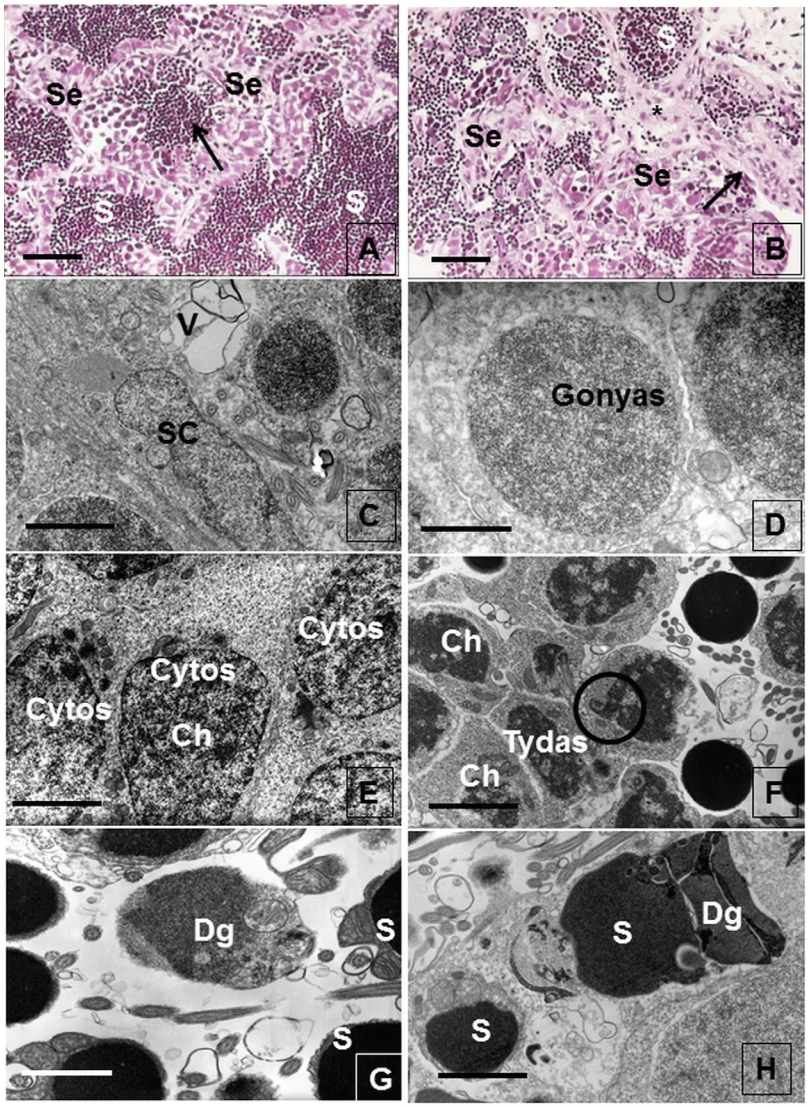

Figure 3. Histological changes in zebrafish testes exposed to 100 µg/l of bisphenol A. (**A**, **B**) Light microscope, bars = 100 µm; (**C**, **D**, **E**, **F**, **G**, **H**) ultrastructural observations, bars = 10 µm; (**A)** detail of the seminiferous epithelium (Se), tumefact and apparently oedematous; the lumens of the cysts (arrow) are irregular with spermatozoa (S); (**B**) detail of the testicular parenchyma in which the seminiferous epithelium (SE) cell concentration has diminished and there is oedema (*) and irregular cysts (arrow) with spermatozoa (S); (**C**) detail of the sustentacular (Sertoli) cell (SC) with vacuolisation (V) of the cytoplasm; (**D**) spermatogonia (Gonyas) with cytoplasmic tumefaction and mitochondria; (**E**) spermatocytes (Cytos) with degradation of the chromatin (Ch); (**F**) detail of the spermatid (Tydas) with implantation fossae (circle) and alterations in the chromatin (Ch); (**G**, **H**) detail of the light with normal spermatozoa (S) among which immature and degenerated (Dg) spermatozoa are found

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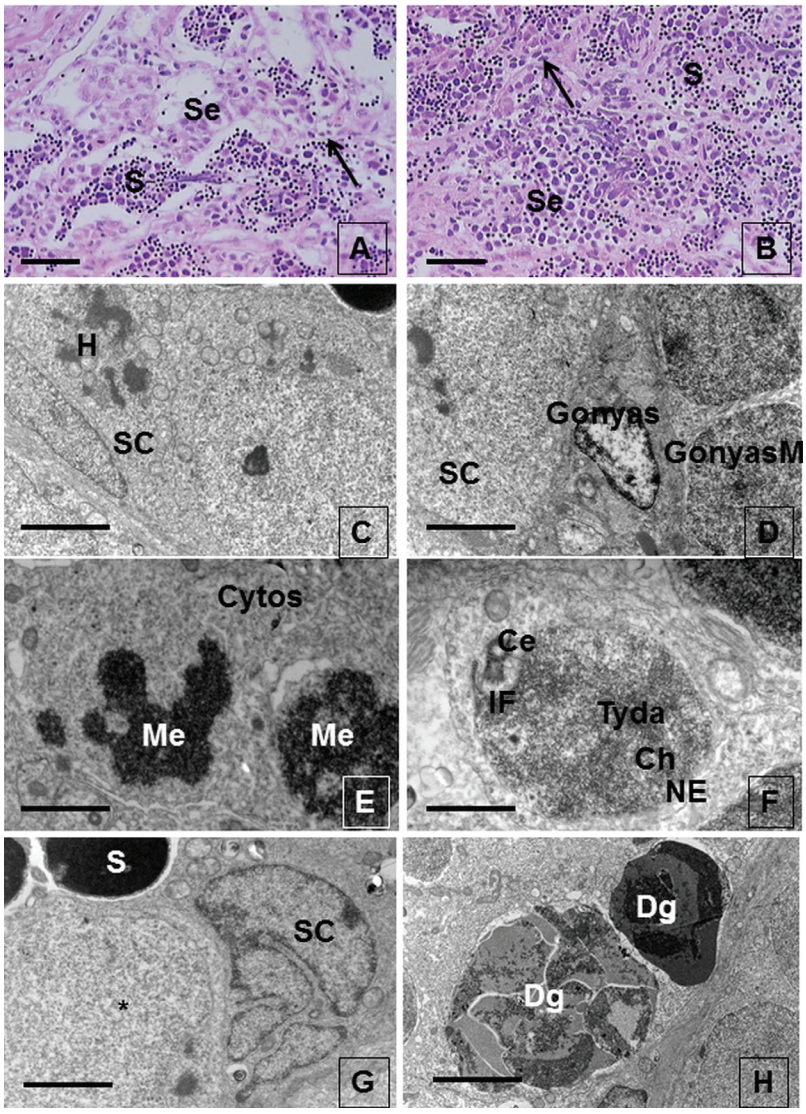

Figure 4. Histological changes in zebrafish testes exposed to 1000 µg/l of bisphenol A. (**A**, **B**) Light microscope, bars = 100 µm; (**C**, **D**, **E**, **F**, **G**, **H**) ultrastructural observations, bars = 10 µm; (**A**, **B**) detail of the testicular parenchyma with disintegration of the seminiferous cysts (arrow) with few cells in the seminiferous epithelium (Se) and a diminution in the number of spermatozoa (S); (**C**) sustentacular cell (SC) with hyaline accumulations (H); (**D**) sustentacular cell (SC) relating itself to very immature spermatogonia (Gonyas) together with other mature ones (GonyasM); (**E**) detail of spermatocytes (Cytos) with meiosis processes (Me); (**F**) detail of implantation fossa (IF) with centrioles (Ce) of spermatid (Tyda), degraded chromatin (Ch) and altered nuclear envelope (NE); (**G**) detail of tumefaction (*) in non-organised spermatozoa (S), highly active sustentacular cells (SC); (**H**) presence of degenerated (Dg) spermatozoa

With regard to the germ cells, in the case of the **spermatogonia**, there were significant differences ($P < 0.05$) between the control group and the 1 µg/l group compared to the groups exposed to higher concentrations of BPA. However, in the case of the tubular population of the **spermatocytes**, the results showed significant differences between the groups with the highest concentrations and the control and 1 µg/l groups. In the cases of the populations of **spermatids** and **spermatozoa**, there were no significant differences between the control, and 1 and 10 µg/l groups, or between the groups exposed to the higher concentrations, although there were differences ($P < 0.05$) between those groups (100 and 1000 µg/l), and the rest of the study groups.

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DISCUSSION

The presence of BPA in consumer products has raised concerns about its potentially adverse effects on reproductive health, its biological effects at environmentally relevant exposure levels, and its presence in consumer products, which has potential implications for public health.

Due to cost effectiveness, ease of maintaining broodstock for studies, and likelihood of exposure, several fish species are currently utilised as model systems for endocrine disruption assays (Flint et al. 2012). Zebrafish are one of a group of small fish species that can be kept in the laboratory as they are easily exposed to endocrine-disrupting chemicals in tank water at different stages in their life cycle, and exhibit a measurable sensitivity to such agents (Van den Belt et al. 2001; Orn et al. 2003; McGonnell and Fowkes 2006; Molina et al. 2013).

This study was focused on the action of BPA on zebrafish sperm ducts, and in particular, on the alterations in their structure and the modifications in the relative proportion of germ cells.

In the 2010 OECD Directive, there is a series of diagnosis criteria for testes histopathology in relation to the analysis of the action of potentially oestrogenic compounds. Among the primary and secondary diagnosis objectives, an increase in testes degeneration and gonadal staging was found to be a marker of damage to gonadal histopathology owing to the action of these compounds (OECD 2010).

Exposure to different concentrations of BPA, i.e. 1, 10, 100 and 1000 μg/l, was achieved by means of a continuous flow system in the water of the aquariums. These doses are very similar to those previously employed by different authors in diverse fish species (Ishibashi et al. 2005; Mandich et al. 2007; Villaneuve et al. 2012; Molina et al. 2013). In agreement with previously reported data, no mortalities occurred during the BPA exposure period (Hatef et al. 2012; Villaneuve et al. 2012), nor were there any differences with regard to the weight and length of the animals after the study period. These data are in accordance with those previously reported by Mandich et al. (2007) and with our own earlier study (Molina et al. 2013). However, the macroscopic aspect of the gonads remained apparently normal in our study, whereas other authors, after a period of exposure to 17-β-oestradiol, noted an attenuated appearance in sperm ducts (Wolf et al. 2004).

In our study, a bio-accumulation of BPA was observed in the fish tissues, which increased together with increasing exposure to BPA. This observation is in agreement with our previous study (Molina et al. 2013).

In the histological analysis, the fish group exposed to 1 μg/l of BPA showed a very similar testes structure to that of the control group, with no abnormalities observed, and it was possible to see all the cell components involved in spermatogenesis. These observations are in contrast to those made in the study of Mandich et al. (2007), who, after exposure of carp to the same concentration of BPA, described a reduction in the sperm ducts and the lobule diameter. Similar observations were made in a study on fathead minnows (*Pimephales promelas*), in which a diminution was observed in the proportion of the different types of cells (an increase in the proportion of spermatocytes) after exposure to 1 μg/l of BPA (Sohoni et al. 2001). In contrast, other authors observed cell debris in efferent ducts, germ cells syncytia, granulomas, mineralisation in efferent ducts and sperm necrosis in response to 17-β-oestradiol exposure at a concentration of 2.7 μg/l over the course of 10 days (Wolf et al. 2004). Our histological results do not coincide with what was indicated by those authors. Moreover, morphometrically, the data do not reflect significant differences in this exposed group with respect to the control in any of the cell populations quantified. However, there are significant differences ($P < 0.05$) with respect to the two groups with the higher concentrations (100 and 1000 μg/l) in all the percentages reported. We could therefore suggest that BPA exerts a weak oestrogenic action since, at those doses, lesions were observed after exposure to other endocrine disruptors but not to BPA. It could therefore be assumed that initially BPA has a less powerful endocrine action than 17-β-oestradiol, possibly because its binding to oestrogenic receptors is weaker (Weber et al. 2003).

When increasing the exposure dose to 10 μg/l of BPA, we began to observe signs of degeneration in the testes, with a tumefaction of the sustentacular cells and a decrease in the number of spermatozoa. This is in agreement with other studies in which, at this same exposure dose, lesions were observed, with the diameter of some lobules being seen to be reduced and spermatogenesis inhibited (Mandich et al. 2007). Also, at 16 μg/l of exposure in fathead minnows, a decrease in the number of mature

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spermatozoa was elicited (Sohoni et al. 2001). This differs from our study since, when carrying out the morphometric analysis of this group (10 µg/l), the only case in which we did observe significant differences ($P < 0.05$) was in the percentage of spermatids and spermatozoa compared to the groups with the higher concentrations. Also, there were no significant differences in the percentage of the rest of the germ cells compared to the control. In a study performed in rodents exposed to BPA concentrations of 20 or 200 µg/kg, no differences were noted between the results observed at the two doses. After seven days of treatment and under a light microscope, multinucleated cells in the seminiferous epithelium and deformed heads of mature spermatids could be seen. Under the electron microscope, abnormal acrosomal caps and invagination and/or vacuole formation in the nuclei were observed. A severe deformation of the acrosome and nucleus in older spermatids (Toyama et al. 2004), much more accentuated lesions, therefore, than those observed by us at the concentration of 10 µg/l, could indicate a differential sensitivity to BPA exposure between rodents and zebrafish. The OECD has identified the latter as one of the alternatives to mammals for use as a model organism for the evaluation of neuroendocrine disruption (OECD 2006).

At concentrations of 100 and 1000 µg/l of BPA, we detected the greatest testicular modifications. At these doses, the testis showed clear signs of degeneration especially in the zebrafish exposed to the highest concentration, with a totally transformed parenchyma, degeneration of germ cells and a large increase in sustentacular cells. This is in agreement with what was observed in the testes of carp exposed to 100 and 1000 µg/l of BPA (Mandich et al. 2007). Most of the samples exhibited an alteration in their testicular structure with an extensive degeneration in the testes, a major reduction in the tubules of the spermatogenic lines, and with a local atrophy of the germinal epithelium, and remains of germinal cells and inflammatory processes inside the tubules (Mandich et al. 2007; Al-Sakran et al. 2016; Li et al. 2016). There are other studies in which it was observed that the alteration in the blood-testis barrier at 200µM perturbed the tight junction of sustentacular cells (Cheng et al. 2011). This differs from what was observed in rats after exposure to 20 and 100 mg/kg of BPA, where there were no adverse effects on the testicular morphology compared to the control (Nakamura et al. 2010).

The greatest testicular degeneration occurred at the higher concentrations of exposure (100 and 1000 µg/l), and the most important modifications in the cell percentages of the sperm ducts were also evident at these concentrations, in agreement with other authors (Al-Sakran et al. 2016). Germ cell percentages dropped as the BPA dose increased. In the control group, the total percentage of germ cells exceeded 90%, whereas at the dose of 1000 µg/l this percentage did not reach 60%. The lower doses of BPA (1 and 10 µg/l) did not significantly affect the cell population percentages, and only when increasing the exposure dose did the numbers of mature shapes drastically decrease. The decline in the proportion of germinal cells was more gradual in the case of the spermatocytes and spermatogonia, with greater differences existing in the more mature shapes (spermatids and spermatozoa). This suggests that BPA causes modulations in sperm maturation, and these alterations in the final sperm maturation in the sperm ducts could result in adverse effects on sperm quality (Al-Sakran et al. 2016). Hatef et al. (2012) reported that BPA exposure leads to lower production of sex steroids as well as causing a diminution in sperm quality.

The exposure of male fish to oestrogenically active compounds has given rise in various studies to a hypertrophy of sustentacular cells, with or without an increase in their number (Miles-Richardson et al. 1999; Kinnberg et al. 2000; Kinnberg and Toft 2003; Van der Ven et al. 2003). This differs from what was observed in rodents when, at seven days after exposure to 20–200 µg/kg, the sustentacular cells were not affected except for the ectoplasmia specialisation around the spermatids (Toyama et al. 2004). In contrast, in our study, the tumefaction of these cells was not observed below the concentration of 10 µg/l. In another study, when exposing fish to other oestrogenic compounds, hypertrophy of the sustentacular cells was observed in guppy fish (*Poecilia reticulata*) exposed to concentrations of 1 µg/l of 17-β-oestradiol (Kinnberg and Toft 2003). In another study, hypertrophy of the sustentacular cells was observed in the testes of male adult zebrafish after exposure to the action of the synthetic oestrogen methyldihydrotesterone (Van der Ven et al. 2003). This differs from our histological data, where at 1 µg/l there were no differences with respect to the control, with the oestrogenic action of BPA again being manifested

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but much more intensely than that elicited by other oestrogenic compounds at the testes level.

Morphometrically, our results reflect how increasing concentrations of BPA led to incremental increases in the percentages of sustentacular cells. There were no significant differences between the control group and those exposed to the lower concentrations (1 and 10 μg/l), or between the two groups exposed to the higher concentrations. However, there were significant differences ($P < 0.05$) between those groups (100 and 1000 μg/l) and the rest of the study groups. The sustentacular cells in the control group reached 7% of the cell population of the sperm ducts, whereas in the groups exposed to the higher doses these values reached 36.95% and 41.5%, respectively. The action of BPA was therefore very pronounced at this level, with a radical increase observed in the two highest exposure concentrations. This implied that when the percentage of sustentacular cells increased, a diminution in the germ cells occurred.

Taken together, our results show that the nature and intensity of the morphological changes in the testes are dependent on the concentration of BPA that the zebrafish are exposed to, and that BPA elicits adverse effects on fish reproductive health. These data underline the fact that histopathology and the morphometry of spermatic ducts are sensitive biomarkers for the analysis of the early effects of exposure to environmental concentrations of BPA on the testes of zebrafish.

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REFERENCES

Al-Sakran AAM, Virk P, Elobeid M, Hamed SS, Siddiqui MI, Omer S, Mirghani NM (2016): Histopathological effects on testis of adult male carp, Cyprinus carpio carpio, following exposure to graded concentrations of waterborne bisphenol A. Tropical Journal of Pharmaceutical Research 15, 73–80.

Cheng CY, Wong EWP, Lie PPY, Li MWM, Su L, Siu ER, Yan HHN, Mannu J, Mathur PP, Bonanomi M, Silvestrini B, Mruk DD (2011): Environmental toxicants and male reproductive function. Spermatogenesis 1, 2–13.

Commission Directive 2011/8/EU of 28 January 2011 amending Directive 2002/72/EC as regards the restriction of use of Bisphenol A in plastic infant feeding bottles. Available: http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2011:026:0011:0014:EN:PDF.

Crain DA, Eriksen M, Iguchi T, Jobling S, Laufer H, LeBlanc GA, Guilette Jr LJ (2007): An ecological asessment of bisphenol-A: evidence from comparative biology. Reproductive Toxicology 24, 225–239.

Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes. Available: http://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:32010L0063&from=EN.

Dorn PB, Chou CS, Gentempo JJ (1987): Degradation of bisphenol A in natural waters. Chemosphere 16, 1501–1507.

EFSA – European Food Safety Authority (2008): Statement of EFSA on a study associating bisphenol A with medical disorders. The EFSA Journal 838, 1–3.

Flint S, Markle T, Thompson S, Wallace E (2012): Bisphenol A exposure, effects, and policy: A wildlife perspective. Journal of Environmental Management 104, 19–34.

Hassanin A, Kuwahara S, Tsukamoto Y, Ogawa K, Hiramatsu K, Sasaki F (2002): Gonadosomatic index and testis morphology of common carp (Cyprinus carpio) in rivers contaminated with estrogenic chemicals. Journal of Veterinary Medical Science 64, 921–926.

Hatef A, Alavi SMH, Abdulfatah A, Fontaine P, Rodina M, Linhert O (2012): Adverse effects of bisphenol A on reproductive physiology in male goldfish at environmentally relevant concentrations. Ecotoxicology and Environmental Safety 76, 56–62.

Ishibashi H, Watanabe N, Matsumura N, Hirano M, Nagao Y, Shiratsuchi H, Kohra S, Yoshihara S, Arizono K (2005): Toxicity to early life stages and an estrogenic effect of a bisphenol A metabolite, 4-methyl-2,4-bis(4-hydroxyphenyl)pent-1-ene on the medaka (Oryzias latipes). Life Sciences 77, 2643–2655.

Kang JH, Aasi D, Katayama Y (2007): Bisphenol A in the aquatic environment and its endocrine-disruptive effects on aquatic organisms. Critical Reviews in Toxicology 37, 670–625.

Kinnberg K, Toft G (2003): Effects of estrogenic and antiandrogenic compounds on the testis structure of the adult guppy (Poecilia reticulata). Ecotoxicology and Environmental Safety 54, 16–24.

doi: 10.17221/212/2015-VETMED

Kinnberg K, Korsgaard B, Bjerregaard P, Jespersen A (2000): Effects of nonhylphenol and 17β-estradiol on vitellogenin synthesis and testis morphology in male platyfish Xiphophorus maculatus. Journal of Experimental Biology 203, 171–181.

Li D, Chen Q, Cao J, Chen H, Li L, Cedergreen N, Xie H, Xie L (2016): The chronic effects of ligning-derived bisphenol and bisphenol A in Japanese medaka Oryzias latipes. Aquatic Toxicology 170, 199–207.

Lopez-Casas P, Mizrak SC, Lopez-Fernandez LA, Paz M, de Rooij DG, del Mazo J (2012): The effects of different endocrine disruptors defining compound-specific alterations of gene expression profiles in the developing testis. Reproductive Toxicolology 33, 106–115.

Mandich A, Bottero S, Benfenati E, Cevasco A, Erratico C, Maggioni S, Massari A, Pedemonte F, Vigano L (2007): In vivo exposure of carps to graded concentrations of bisphenol A. General and Comparative Endocrinology 153, 15–24.

McGonnell IM, Fowkes RC (2006): Fishing for gene function-endocrine modelling in the zebrafish. Journal of Endocrinology 189, 425–439.

Miles-Richardson SR, Pierens S, Nichols KM, Kramer VJ, Snyder EM, Snyder SA, Render JA, Fitzgerald SD, Giesy JP (1999): Effects of waterborne exposure to 4-nonylphenol and nonylphenol ethoxylate on secondary sex characteristics and gonads of fathead minnows (Pimephales promelas). Environmental Research 80, 122–137.

Molina AM, Lora AJ, Blanco A, Monterde JG, Ayala N, Moyano R (2013): Endocrine-active compound evaluation: qualitative and quantitative histomorphological assessment of zebrafish gonads after bisphenol-A exposure. Ecotoxicology and Environmental Safety 88, 155–162.

Nakamura D, Yanagiba Y, Duan Z, Ito Y, Okamura A, Asaeda N, Tagawa Y, Li C, Taya K, Zhang S, Naito H, Ramdhan DH, Kamijima M, Nakajima T (2010): Bisphenol A may cause testosterone reduction by adversely affecting both testis and pituitary systems similar to estradiol. Toxicology Letters 194, 16–25.

OECD (1992): Guideline 210: Fish, Early Life Stage Toxicity Test. In: OECD Guidelines for Testing of Chemicals, Section 2. Available: http://www.oecd-ilibrary.org/docserver/download/9713191e.pdf?expires=1470224702&id=id&accname=guest&checksum=BA62DB6A22DE49D5BF21971F9549CD9E.

OECD (1993): Guideline 204: Fish, Prolonged Toxicity Test: 14-Day-Study. In: OECD Guidelines for Testing of Chemicals, Section 2. Available: http://www.oecd-ilibrary.org/docserver/download/9720401e.pdf?expires=1470226596&id=id&accname=guest&checksum=FF3EF01FEADF44CC7129552FFBF2CEB3.

OECD (1998): Guideline 212: Fish, Short-term Toxicity Test on Embryo and Sac-fry Stages. In: OECD Guidelines for Testing of Chemicals, Section 2. Available: http://www.oecd-ilibrary.org/docserver/download/9721201e.pdf?expires=1470228946&id=id&accname=guest&checksum=4A44381E3F5388FEA64F3BF10CA72776.

OECD (2006): OECD Report of the validation of the 21-day fish screening asay for the detection of endocrine substances (Phase 1B). In: OECD Series on Testing and Assessment: Testing for Endocrine Disrupters, No. 61. Available: http://www.oecd.org/officialdocuments/publicdisplaydocumentpdf/?cote=env/jm/mono(2006)29&doclanguage=en.

OECD (2010): OECD Guidance document for the diagnosis of endocrine-related histopathology of fish gonads. In: OECD Series on Testing and Assessment: Testing for Endocrine Disrupters, No. 123. Available: http://www.oecd.org/officialdocuments/publicdisplaydocumentpdf/?cote=env/jm/mono(2010)14&doclanguage=en.

Orn S, Holbech H, Madsen TH, Norrgren L, Petersen GI (2003): Gonad development and vitellogenin production in zebrafish (Danio rerio) exposed to ethinylestradiol and methyltestosterone. Aquatic Toxicology 65, 397–411.

Schmidt K, Stachel C, Gowik P (2008): Development and in-house validation of an LC-MS/MS method for the determination of stilbenes and resorcylic acid lactones in bovine urine. Analytical and Bioanalytical Chemistry 391, 1199–1210.

Segner H, Caroll K, Fenske M, Janssen CR, Maack G, Pascoe D, Schafers C, Vandenbergh GF, Watts M, Wenzel A (2003): Identification of endocrine-disrupting effects in aquatic vertebrates and invertebrates: report from the European IDEA project. Ecotoxicology and Environmental Safety 54, 302–314.

Sohoni P, Tyler CR, Hurd K, Caunter J, Hetheridge M, Williams T, Woods C, Evans M, Toy R, Gargas M, Sumpter JP (2001): Reproductive effects of long-term exposure to bisphenol A in the fathead minnow (Pimephales promelas). Environmental Science and Technology 35, 2917–2925.

Toyama Y, Suzuki-Toyota F, Maekawa M, Ito C, Toshimori K (2004): Adverse effects of bisphenol A to spermiogenesis in mice and rats. Archives of Histology and Cytology 67, 373–381.

Van den Belt K, Verheyen R, Witters H (2001): Reproductive effects of ethynylestradiol and 4t-octylphenol on the zebrafish (Danio rerio). Archives of Environmental Contamination and Toxicology 41, 458–467.

Van der Ven LT, Wester PW, Vos JG (2003): Histopathology as a tool for the evaluation of endocrine disruption in zebrafish (Danio rerio). Environmental Toxicology and Chemistry 22, 908–913.

doi: 10.17221/212/2015-VETMED

Villaneuve DL, Garcia-Reyero N, Escalon BL, Jensen KM, Thomas LM, Perkins EJ, Ankley GT (2012): Ecotoxicogenomics to support ecological risk assessment: A case study with bisphenol A in fish. Environmental Science and Technology 46, 51–59.

Weber LP, Hill RL, Janz DM (2003): Developmental estrogenic exposure in zebrafish (Danio rerio): II. Histological evaluation of gametogenesis and organ toxicity. Aquatic Toxicology 63, 431–446.

Wolf JC, Dietrich DR, Friederich U, Caunter J, Brown A (2004): Qualitative and quantitative histomorphologic assessment of fathead minnow Pimephales promelas gonads as an endpoint for evaluating endocrine-active compounds: a pilot methodology study. Toxicologic Pathology 32, 600–612.

Yeung BH, Wan HT, Law AYS, Wong CKC (2011): Endocrine disrupting chemicals. Multiple effects on testicular signalling and spermatogenesis. Spermatogenesis 1, 231–239.

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