Effects of bisphenol A on adult fathead minnow (*P. promelas*) gonadal histopathology: a 42-day exposure study

DR Dietrich[1], J Wolf[2], AR Brown[3], JE Caunter[3], N van der Hoeven[4] and U Friederich[5]

[1]University of Konstanz, Germany; [2]Experimental Pathology Laboratories, Virginia, USA; [3]Brixham Environmental Laboratories, UK; [4]Ecostat, Leiden, NL; [5]Dow Chemical Europe, Horgen Switzerland.

Background:

In a previous study [1], the frequency of spermatogonia (G), spermatocytes (C), spermatids (T), spermatozoa (Z) and other cells (O) in testis sections of male fathead minnows (FHM) exposed to various concentrations of bisphenol A (BPA) were assessed. The histopathological assessment of this study appeared compromised with regard to histopathological preparation of the slides, cell type identification and counting, and statistical evaluation of gonadal cell distribution. In particular, the use of linear regression analysis based on single cell type frequencies to identify dose-response effects raised concern, as well as the number of replicates used in each dose group was questioned as to whether these would have provided enough statistical power to allow observation of statistically significant dose-related effects.

Objectives:

Determination of the effective dose of BPA on gonadal histology

Determination of the level of biologically inherent variation in the distribution of spermatogenic and oogenic cell types

Determination of the optimal number of replicates/ concentration and fish/ exposure group in order to determine significant effects of BPA on gonadal cell type distribution with 0.2.

Materials and Methods:

In-Life-Phase:
4 male and female FHM 150 dph/ replicate (5 replicates/ dose, Table 1), were exposed for max. 42 days to nominally 0, 1, 160, 640 and 1280 µg l-1 BPA using a flow-through regimen including analytical verification of the test concentrations. Exposure condition parameters followed EPA guideline [2].

Parameters:
Mortality, body weight, fork length, gonadosomatic index (GSI), and plasma vitellogenin concentration (Vtg-ELISA), pathology of somatic tissues (gill (2nd gill arch), liver, kidney (mid-section) and spleen) and gonads (testis and ova), and gonadal cell type distribution.

Gonadal cell type distribution (see [3]):

- Testes (5% (v/v) glutaraldehyde fixative, GMA embedding): manual tagging of cell types was carried out on 4 digital images (0.22 x 0.29 mm subject area) from each of the left and right testis sections (8 images/ fish (40x objective)). A virtual grid consisting of 400 (20 x 20) individual intersection points (GIP) was applied to each image. Each GIP was tagged with colored dots for: spermatozoa, spermatid, spermatocyte, spermatogonia, vacuolated cell (VC), or apoptotic body cell (ABC), or oocytes (Fig. 1).
- Ovaries (Bouin's fixative, paraffin embedding): manual tagging of ovary cell types was carried out on 2 digital images (2.2 x 2.9 mm subject area) obtained from each of the left and right ovary sections (4 images/ fish (4x objective)). Each ovarian follicle was tagged with colored squares for: perinucleolar, cortical alveolar, early vitellogenic, late vitellogenic, mature/spawning, and atretic follicles (Fig. 2).

Statistics:

- Jonckheere-Terpstra test (non-parametric): significant effect between ordered treatment and effect.
- Dunnett's Test (parametric test): comparison of exposed group to control group.
- Williams Test (parametric test): testing for a dose-effect relationship
- Redundancy Analysis (RDA): description and test of the relationship between the dosage and the measured biological parameters

References:

[1] Sohoni, P. et al., ES&T 35: 2917-2925 (2001).
[2] EPA/600/4-90/027F (1993): Methods for measuring the acute toxicity of effluents and receiving waters to freshwater and marine organisms. EPA/600/4-90/027F, Fourth Edition, Appendix A: Distribution, Life Cycle, Taxonomy, and Culture Methods A.5. Fathead Minnows (Pimephales promelas). 200-218.
[3] Wolf, J. et al., Tox. Path., 2004 in press.

Results and Discussion:

Table 1: Experimental design and geometric time-weighted mean measured concentrations of BPA in the 42-day study

Dose Groups	Measured BPA µg l^{-1}	Replicates 1-5 Male	Female
No-Treatment Control	0	4	4
BPA 1 µg/l	0,94	4	4
BPA 160 µg/l	88	4	4
BPA 640 µg/l	360	4	4
BPA 1280 µg/l	760	4	4

Table 2: No-observable and lowest-observable-effect concentrations of BPA (geometric time-weighted mean measured concentrations), determined via Dunnett's test statistics for the comparison of all four treatments with the control.

Parameters	BPA concentrations (geometric time-weighted mean measured concentrations in µg l^{-1}) Males NOEC	Males LOEC	Females NOEC	Females LOEC
Mortality	360	760	760	>760
Weight	760	>760	760	>760
Fork-Length	760	>760	760	>760
Gonadosomatic index	360	760	760	>760
VTG concentration	0,94	88	0,94	88

Table 3: Type and incidence of histopathological lesions observed

BPA µg l^{-1}	0	0.94	88	360	760
MALE KIDNEY	No.(%) affected:				
No. examined:	**20**	**20**	**20**	**19**	**12**
Proteinaceous Fluid, Intravascular	0 (0)	1 (5)	5 (25)	19 (100)	12 (100)
Glomerulus, Hyaline Deposits	0 (0)	0 (0)	2 (10)	14 (74)	11 (92)
Glomerulus, Epithelial Cells, Hyperplasia	1 (5)	2 (10)	5 (25)	15 (79)	12 (100)
Glomerulus, Mesangial Membranes, Thickened	0 (0)	0 (0)	3 (15)	14 (74)	11 (92)
Tubule, Epithelial Cells, Vacuolar Hypertrophy	0 (0)	0 (0)	0 (0)	6 (32)	9 (75)
Tubule, Epithelial Cells, Hyaline Droplets	1 (5)	2 (10)	2 (10)	9 (47)	10 (83)
Glomerulus, Periglomerular Fibrosis	0 (0)	0 (0)	0 (0)	8 (42)	8 (67)
MALE LIVER	No.(%) affected:				
No. examined:	**20**	**20**	**20**	**19**	**12**
Proteinaceous Fluid, Intravascular	4 (20)	4 (20)	8 (40)	16 (84)	12 (100)
Hepatocyte, Basophilia, Diffuse	0 (0)	0 (0)	1 (5)	18 (95)	12 (100)
Hepatocyte, Cytoplasmic Vacuolization, Diffuse	20 (100)	20 (100)	20 (100)	10 (53)	6 (50)
MALE GILL	No.(%) affected:				
No. examined:	**20**	**20**	**20**	**19**	**12**
Proteinaceous Fluid, Intravascular	0 (0)	0 (0)	0 (0)	18 (95)	12 (100)
Lamellae, Individual Cell Necrosis	2 (10)	2 (10)	3 (15)	9 (47)	8 (67)
FEMALE KIDNEY	No.(%) affected:				
No. examined:	**17**	**19**	**18**	**18**	**20**
Glomerulus, Epithelial Cells, Hyperplasia	4 (24)	7 (37)	0 (0)	7 (39)	11 (55)
Proteinaceous Fluid, Intravascular	2 (12)	5 (26)	4 (22)	4 (22)	11 (55)
FEMALE LIVER	No.(%) affected:				
No. examined:	**17**	**19**	**18**	**18**	**20**
Proteinaceous Fluid, Intravascular	6 (35)	3 (16)	3 (17)	10 (56)	11 (55)
Hepatocyte, Basophilia, Diffuse	17 (100)	19 (100)	18 (100)	18 (100)	20 (100)

Table 4: NOEC and LOEC of histopathological lesions assessed via Dunnett's and Williams test for the comparison of all 4 treatments with the control.

Organs	BPA µg l^{-1} Males NOEC	Males LOEC	Females NOEC	Females LOEC
Gill	88	360	> 760	> 760
Kidney	88	360	> 760	> 760
Liver	88	88	> 760	> 760
Spleen	360	> 760	> 760	> 760

Table 5: Concentration-dependent lesions identified via RDA analysis (listed according to their strongest concentration dependency)

Males	Females
Arbitrary cut-off 20 %	Arbitrary cut-off 9 %
Kidney: proteinaceous fluid intravascular (77.6%)	Liver: proteinaceous fluid intravascular (18.4%)
Liver: hepatocyte basophilia (71.9%)	Liver: hepatocyte basophilia (14.2%)*)
Gill: proteinaceous fluid intravascular (70.6%)	Kidney: glomerular, epithelial hyperplasia (10.5%)
	Kidney: proteinaceous fluid intravascular (9.7%)

*) negative trend

Figure 1: tagged testis germ cell types

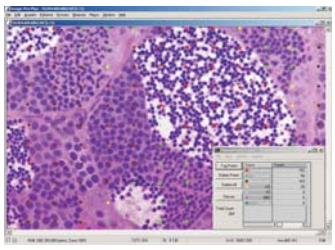

Figure 2: tagged ovarian germ cell types

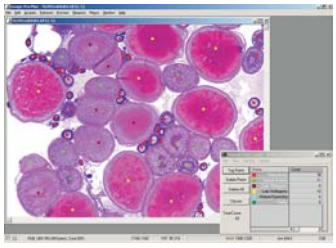

Figure 3: box plot of male VTG values (median, the first and third quartile, interval given with whiskers and outliers individually)

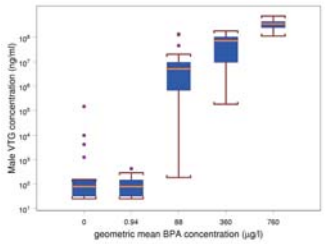

Figure 4: box plot of female VTG values (median, the first and third quartile, interval given with whiskers and outliers individually)

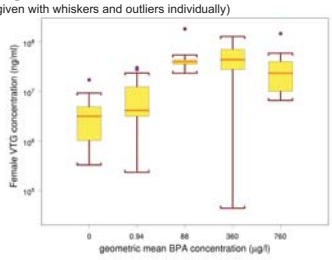

Table 6: Summary table: Range of relative occurrence of testicular cell types in individual male fish. On average 1492 spermatogenic cells (incl. possibly occuring oocytes) were counted per testis.

BPA µg l^{-1}	ABC	VC	Spermatogonia	Spermatocytes	Spermatids	Spermatozoa
0	0 - 23	0 - 11	18 - 152	129 - 916	26 - 292	129 - 1194
0.94	0 - 8	0 - 17	35 - 181	344 - 906	90 - 308	78 - 907
88	0 - 152	0 - 18	36 - 136	386 - 976	29 - 260	47 - 922
360	0 - 78	0 - 26	46 - 261	181 - 1045	32 - 309	77 - 1137
760	0 - 123	0 - 73	56 - 464	338 - 929	0 - 329	58 - 899

Table 7: Summary table: Range of relative occurrence of follicular types in individual female fish. On average 146 follicle types were counted per ovary.

BPA µg l^{-1}	Perinu-cleolar	Cortical alveolar	Early vitellogenic	Late vitellogenic	Mature	Atretic
0	39– 212	5 – 41	5 – 32	0 – 25	0	0 – 11
0.94	38 – 149	13 – 45	8 – 28	0 – 27	0 - 1	0 - 17
88	31 – 271	5 – 45	2 – 26	2 – 24	0 -2	0 – 4
360	33 – 211	6 – 38	2 – 26	0 – 37	0 – 4	0 – 32
760	39– 278	14 – 48	5 – 21	0 – 27	0 – 3	0 – 20

Flow-through application of BPA resulted in approximately 60% of the nominal concentrations (Table 1). The highest BPA concentration induced significantly increased male mortality, while weight and length were not affected (Table 2). BPA induced an increased induction of VTG in males and females (Table 2, Fig. 3 & 4), although the variability of values in both sexes was extraordinary. This increased VTG appeared to be causally related to the most prominent histopathological lesions, primarily observed in the male fish (e.g. proteinaceous fluid intravascular, hepatocyte basophilia, Tables 3 - 5). It is suggested that the mortality observed in the highest dose group male fish, may be associated with the excessive VTG induction in the liver (hepatocyte basophilia) and protein accumulation in all tissues, including the gill. Overall histopathological lesions in male fish prominently appeared in conjunction with high VTG concentrations (Table 2 and 4), i.e. at concentrations = 88 µg l^{-1}. It is of interest to note that in female fish hepatocyte basophilia presented with a negative trend (Table 5), suggesting that the highest concentrations of BPA reduced the amount of mRNA present in the hepatocytes possibly via a competitive inhibitory action at the estrogen receptor. The analysis of the germ cell type distribution (Tables 6 & 7) demonstrated the huge inherent biological variability within a group of fish (control or exposure groups). Variability amongst fish was much greater than variability amongst replicates, supporting the use of the pseudo-replicate design used. Preliminary analysis of the cell type distribution did not demonstrate a change of the cell types counted, irrespective of the BPA dose used. Furthermore, preliminary calculations using observed cell type variabilities suggest that an extremely high number of replicates (incl. pseudo-replicates) will be necessary to allow detection of a significant change in cell type distribution (e.g. = 20%) with a power of 80% (= 0.2), and thus support the reservations brought forth regarding the cell type evaluation of the Sohoni et al. study [1].