Effects of BPA on zebrafish gonads: Focus on the endocannabinoid system

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Abstract

Bisphenol A (BPA), a monomer used for polycarbonate manufacture, has been widely reported as an endocrine-disrupting chemical (EDC). Among other alterations, BPA induces reproductive dysfunctionalities. Changes in the endocannabinoid system (ECS) has been recently shown to be associated with reproductive disorders. ECS is a lipid-based signaling system (cannabinoid receptors, endocannabinoids and enzymatic machinery) involved in several physiological functions. The main goal of the present study was to assess the effects of two environmental concentrations of BPA (10 and 20 µg/L) on the ECS in 1-year zebrafish gonads. In the males, BPA increased the gonadosomatic index (GSI) and altered testicular levels of endocannabinoids as well as reduced the testicular area occupied by spermatogonia. In the male liver, exposure to 20 µg/L BPA significantly increased vitellogenin (vtg) transcript levels. In female zebrafish, BPA altered ovarian endocannabinoid levels, elevated hepatic vtg mRNA levels as well as increased the percentage of vitellogenic oocytes in the ovaries. In conclusion, exposure to two environmentally relevant concentrations of BPA altered ECS and consequently, gonadal function in both male and female zebrafish.

Keywords	Danio rerio; endocannabinoid; Endocrine disruptors; reproduction; vitellogenin

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Data will be made available on request

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Dipartimento
di Scienze
della Vita
e dell'Ambiente
DISVA

January 30th 2020

Dear Editor,

I enclose an original article entitled "**Effects of BPA on zebrafish gonads: focus on the endocannabinoid system**" co-authored by Isabel Forner-Piquer, Silvia Beato, Fabiana Piscitelli, Stefania Santangeli, Vincenzo di Marzo, Hamid R. Habibi, Francesca Maradonna and myself, Oliana Carnevali, for consideration as an article in Environmental Pollution.

In the present manuscript, we reported the results obtained after 21 days exposure to two different concentrations of Bisphenol A (BPA), 10 and 20 µg/L (based on BPA environmental concentrations), on the endocannabinoid system in both ovary and testis. The endocannabinoid system was used as a novel target due to its role on reproductive physiology.

Thus, this study can be considered as continuum of the recent effort on investigating the effects of Bisphenol A. Indeed, our results reported that BPA induced alterations on the levels of endocannabinoids and transcriptomic changes in genes coding for the endocannainoid system and reproductive markers. In addition, the histological analyses on gonads evidenced alterations of gametogenesis in both ovary and testis.

Finally, we declare no conflict of interest associated with this publication, and we state that none of the material contained in the present manuscript has been published or is under consideration in any other place.

We believe that our findings will be of interest to the readers of your journal.

Thank you for considering this article and please, let us know if there is further information we can provide.

Sincerely,

Prof Oliana Carnevali

Department of Life and Environmental sciences
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ENVPOL_2020_788

Reviewer 4

Minor points:

1) GSI should be defined in the main text the first time it is used (not just the abstract).

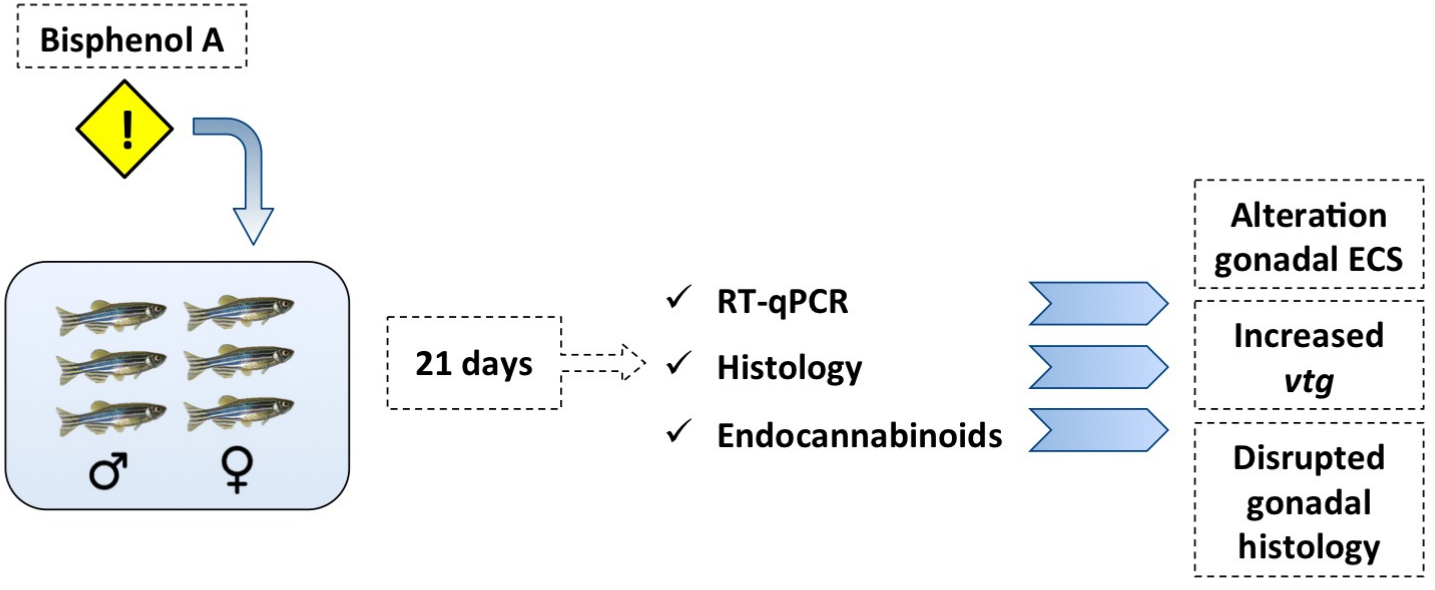
GSI has been defined in line 106 (materials and methods) and 176 (results).

2) The authors are expressing their data in deltaCt in table 3 and 4 and figure 3, and this must be stated. Note that these are not 'arbitrary units' as indicated in the methods. The authors must explicitly state this and indicate where this is different from the fold change analysis 2^-ddCt. Therefore, I suggest that the legend for table 3 and 4 and figure 3 be changed from "Data are expressed as means ± SEM" to "Data are expressed as mean dCt ± SEM".

As the reviewer suggested, the image legends have been modified.

HIGHLIGHTS

1. BPA altered the endocannabinoid system in zebrafish gonads.
2. BPA (20 µg/L) decreased the percentage of the area occupied by spermatogonia in the testis.
3. BPA (20 µg/L) up-regulated the hepatic *vtg* expression in male and females.
4. BPA increased male GSI.


Bisphenol A
!
♂
♀
21 days
✓ RT-qPCR
✓ Histology
✓ Endocannabinoids
Alteration gonadal ECS
Increased *vtg*
Disrupted gonadal histology

Effects of BPA on zebrafish gonads: focus on the endocannabinoid system

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HIGHLIGHTS

1. BPA altered the endocannabinoid system in zebrafish gonads.
2. BPA (20 µg/L) decreased the percentage of the area occupied by spermatogonia.
3. BPA (20 µg/L) up-regulated the hepatic vitellogenin (*vtg*) expression.
4. BPA increased male gonadosomatic index (GSI).

ABSTRACT

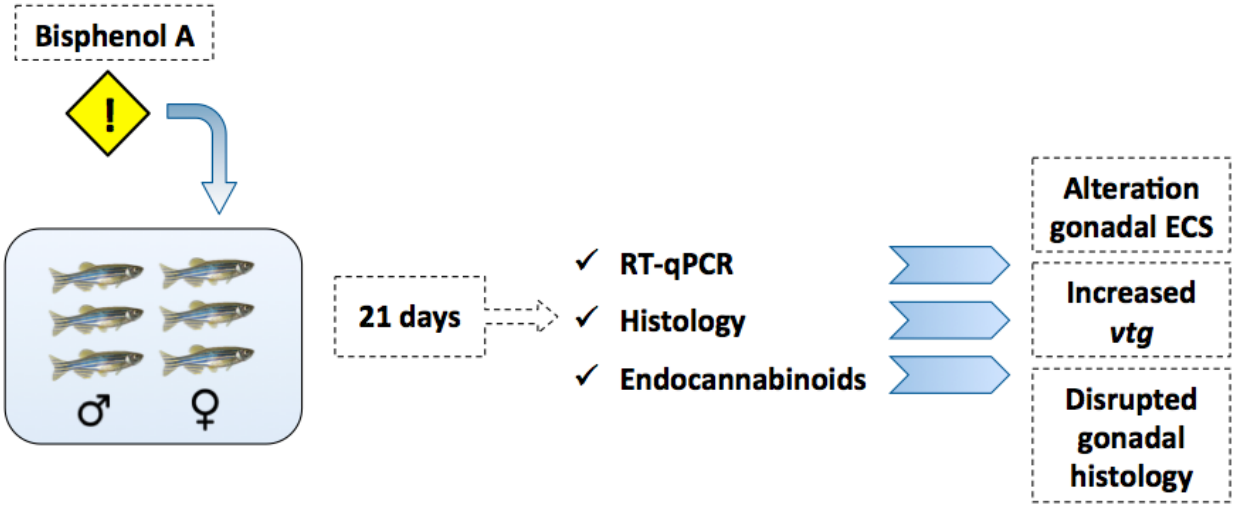
Bisphenol A (BPA), a monomer used for polycarbonate manufacture, has been widely reported as an endocrine-disrupting chemical (EDC). Among other alterations, BPA induces reproductive dysfunctionalities. Changes in the endocannabinoid system (ECS) has been recently shown to be associated with reproductive disorders. ECS is a lipid-based signalling system (cannabinoid receptors, endocannabinoids and enzymatic machinery) involved in several physiological functions. The main goal of the present study was to assess the effects of two environmental concentrations of BPA (10 and 20 µg/L) on the ECS in 1-year zebrafish gonads. In the males, BPA increased the gonadosomatic

index (GSI) and altered testicular levels of endocannabinoids as well as reduced the testicular area occupied by spermatogonia. In the male liver, exposure to 20 µg/L BPA significantly increased vitellogenin (*vtg*) transcript levels. In female zebrafish, BPA altered ovarian endocannabinoid levels, elevated hepatic *vtg* mRNA levels as well as increased the percentage of vitellogenic oocytes in the ovaries. In conclusion, exposure to two environmentally relevant concentrations of BPA altered ECS and consequently, gonadal function in both male and female zebrafish.

KEY WORDS: *Danio rerio*; endocannabinoid; endocrine disruptors; reproduction; vitellogenin.

Capsule: 21-day BPA exposure altered the zebrafish endocannabinoid system and changed the gonad morphology.

GRAPHICAL ABSTRACT



ABBREVIATIONS: AEA: anandamide; 2-AG: 2-arachidonoylglycerol; OEA: *N*-oleyl ethanolamine; PEA: *N*-palmityl ethanolamine; CB1/cnr1: endocannabinoid receptor type 1; CB2/cnr2: endocannabinoid receptor type 2; TRPV1: transient receptor potential vanilloid type I ion channel; GPR55: G protein-coupled receptor 55; NAPE-PLD: N-acyl phosphatidylethanolamine phospholipase D, FAAH: Fatty acid amide hydrolase, DAGLα: Diacylglycerol lipase alpha, MGLL: monoglyceride lipase, ABDH4: abhydrolase domain containing 4, VTG: vitellogenin.

1. INTRODUCTION

Since 1940, BPA has been used for the manufacture of polycarbonate products and epoxy resins in the plastic industry because of its capacity to increase heat resistance and elasticity [1]. Due to its extensive use, BPA can be globally found in the aquatic environment and therefore, in humans and wildlife [2]. BPA is known to enter the aquatic ecosystem by diffusion from wastewater treatment plants and landfill sites [3]. Overall, BPA concentrations in the aquatic environment are variable, depending on the location, reaching on average 21 µg/L in surface waters [4,5]. BPA can interact with nuclear estrogen receptors (ER) alpha and its membrane-bound form (mER) [6,7] , ER beta [8] and the G protein-coupled receptor 30 (GPR30) [9]. Additionally, BPA exerts estrogenic activity at low concentrations [10] and can interfere with androgen mediated pathways [11–14]. Consequently, BPA has been linked with alterations in male and female reproduction and epigenetic markers [15–18], gonadotrophic cell hypertrophy and hyperplasia [19], follicular atresia [20], transcriptomic profile of genes coding for reproductive signals [15,19,21–23], sperm physiology and motility [21,24,25], hormone levels [23,26,27], sexual differentiation [28], abnormal neuronal development [11], morphological development [29], and metabolic dysfunction [30].

It is now well established that the ECS is involved in different reproductive processes [31–33]. The ECS is a lipid signaling system based on lipid mediators, the endocannabinoids (Anandamide: AEA, 2-arachidonoylglycerol: 2-AG). It can be expanded to include endocannabinoid-like mediators such as *N*-oleoyl ethanolamine (OEA) and *N*-palmityl ethanolamine (PEA). AEA and 2-AG exert their activity through G protein-coupled receptors, i.e. the cannabinoid receptor type I (CB1 or *CNR1*) and type II (CB2 or *CNR2*), as well as other molecular targets. The endocannabinoids are synthetized on demand, mainly through the N-acyl phosphatidylethanolamine-specific phospholipase D (NAPE-PLD, for AEA) and Diacylglycerol Lipase Alpha (DAGLα, for 2-AG), from membrane phospholipid precursors after reuptake into the cytosol, facilitated by intracellular degrading enzymes such as fatty acid amide hydrolase (FAAH) and monoglyceride lipase (MGLL). NAPE-PLD and FAAH also regulate OEA and PEA tissue concentrations.

In humans, higher levels of AEA impaired sperm viability and motility [34], and in mice, lower levels of 2-AG promoted epididymal sperm cell start-up [35]. Inactivation of CB1 caused ineffective histone displacement, reduced chromatin condensation and DNA damage in mouse sperm [36]. In females, a correct tone of AEA was reported to be necessary during human oocyte maturation [37,38]; however, higher levels of AEA may also decrease the levels of progesterone, which is associated with

implantation failure and/or abnormal development of the mammalian fetus [39]. Indeed, a crosslink between endocannabinoids and sex steroids is reported elsewhere [40].

Finally, recent studies demonstrated the ability of BPA to deregulate the ECS in different species [22,26,41–43]. Thus, we here examined the adverse effects of BPA on the gonadal ECS in adult zebrafish, following 21-day exposure to two different environmental concentrations (10 and 20 µg BPA/L).

2. MATERIALS AND METHODS

2.1. BPA treatment

BPA exposure was conducted in one-year zebrafish (AB strain) following previous publications [4,5,15,22,44]. Two nominal concentrations of BPA were tested: 10 and 20 µg/L. Absolute ethanol (100% EtOH) was used as a solvent carrier with a final concentration of 10 µL EtOH / L water (0.001% v/v), which was well below the activity threshold (Hutchinson et al., 2006). After three weeks, fish were euthanized with MS-222 (3-aminobenzoic acid ethyl ester) (Sigma-Aldrich) according to University of Calgary Animal Care protocol (AC15-0183). Ovaries and testes were immediately frozen in dry ice and stored at -80 °C until use for subsequent RNA extraction and endocannabinoid assays. For histological procedures, the gonads were fixed in Bouin solution overnight, washed and stored within EtOH 70%.

Gonadosomaic Index (GSI) was calculated: [(gonad weight/ total zebrafish weight) * 100]

2.2 Quantitative real-time PCR (RT-qPCR).

RNA was extracted from the gonads after gently homogenisation with TRIzol reagent (Invitrogen), followed by chloroform disaggregation into a light aqueous layer in the upper part containing RNAs and a lower one with proteins and DNAs. Then, the upper layer was transferred to a new Eppendorf and the RNA was precipitated with isopropanol and washed twice with absolute ethanol. Finally, the samples were treated with DNAse following [15]. Quantification of the RNA was determined by spectrophotometry using a Nanophotomether TM P-Class (Implen). Quality of the mRNA (integrity, presence of genomic-DNA) was assessed by electrophoresis in 1% agarose gel.

Reverse transcription was conducted from 1 µg mRNA with High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) following the manufacturer guidelines. The cDNA obtained with the retrotranscription (MyCycler Thermal Cycler System, Bio-Rad) was considered as the stock

(1:1), this stock was diluted with miliQ water (1:10) to obtain the working concentrations. All the cDNAs were kept at – 20 °C.

For the RT-qPCRs, SYBR Green method was used with an iQ5 multicolor Real-Time PCR detection system (Bio-Rad) with single transparent strips. All replicates (n = 5) were run in duplicate. For each reaction, the mix contained: 1 µL of cNDA (1:10) + 5 µL iQ SYBR Green Supermix (Bio-Rad) + 3.8 µL miliQ water + 0.1 µL forward primer + 0.1 µL reverse primer. Final concentration of primers was 10 pmol/µL. The thermal cycling was as follow: 95 °C for 3 minutes; 45 cycles of denaturation (10 seconds at 95 °C) followed by 20 seconds for annealing at 60 °C for *faah*, *abhd4*, *vtg*; 59 °C for *dagla*, *trpv1*; 57 °C for *napepld*; 55 °C for *cnr1*; 53 °C for *gpr55* and *mgll*; and finally, 20 seconds for elongation at 72 °C.

Two *no template control* (NTC) were added in each run to guarantee absence of contamination.

Annealing temperatures (Ta) for each primer were optimized with temperature gradient assays. Primer specificities were assessed with the absence of primer-dimer formation and dissociation curves. Additionally, for each pair of primers, the efficiencies were evaluated with a mix of cDNA (CTRL group) at different concentrations (1:1, 1:10, 1:100, 1:1000).

The reference genes were *rplp0* (ribosomal protein large P0) and *18s* (ribosomal subunit 18s) for ovary, and *rplp0* and *rpl13a* (ribosomal protein L13a) for testes and liver for being their expression stable and abundant along all the experimental groups. List of primers is stated in Table 1 and additional information can be found in Table S1 (Supplemental material).

RT-qPCR results have been analysed with the C_t (cycle threshold) values of both target and reference genes with the Delta Delta C_t method [45,46] using the spreadsheet provided by Bio-Rad (Gene Expression analysis for iCycler iQ Rela-time PCR detection system version 2.0).

Table 1. Primer list.

GENE	ABB	FORWARD (5' - 3')	REVERSE (5' - 3')	Accession number
Cannabinoid receptor 1	*cnr1*	TCTGTGGGAAGCCTGTTTC	ACCGAGTTGAGCCGTTTG	NM_212820.2
Transient receptor potential cation channel, subfamily V, member 1	*trpv1*	TGATCGTCGCTGGTGCTT	GACTGGGCTCTCTCTGAACG	NM_001126399.1
G protein-coupled receptor 55	*gpr55*	AACTGAAGGTGTGGATAC	ACGCCATAATGTTTAACG	XM_005163567.3
N-acyl phosphatidylethanolamine phospholipase D	*napepld*	CTCAAGGACATGCACTCA	GAGCACAATCTTCAAGACAAT	NM_001080613.2
Monoglyceride lipase	*mgll*	AAGTGAAGGTGAGAGGAT	AATGTCCAACGATGAAGA	NM_200297.2

Fatty acid amide hydrolase	*faah*	CATTCTCGGACCAGCCTTCA	CCTTCACTGCCTGTTGGAAGA	NM_001109825.1
Diacylglycerol lipase alpha	*dagla*	GAGGGTTTCCGTCGTCAC	TGTTCCTCCAGCAATGATCC	XM_692781.8
Abhydrolase domain containing 4	*abhd4*	GAAGAGCAGTTTGTTTCCTCCATAG	GACTCACTCTTTCTGGGTATTGGAT	NM_001017613.1
Vitellogenin 1	*vtg*	GCCAAAAAGCTGGGTAAACA	AGTTCCGTCTGGATTGATGG	NM_001044897.3
Ribosomal subunit 18s	*18s*	TCGGAAAACGGTGAACCTG	AAGGTCTTTGAACCCACGG	NR_195818.1
Ribosomal protein large P0	*rplp0*	CTGAACATCTCGCCCTTCTC	TAGCCGATCTGCAGACACAC	NM_131580.2
Ribosomal protein L13a	*rpl13a*	TCTGGAGGACTGTAAGAGGTATGC	AGACGCACAATCTTGAGAGCAG	NM_212784.1

2.3 Measurement of endocannabinoids (AEA, 2-AG) and endocannabinoid-like mediators (PEA, OEA) in testis and ovary.

Endocannabinoids and endocannabinoids-like mediators were extracted, purified and quantified from 5 testis and ovaries as previously described [47,48].

2.4 Gonad histology and image analysis

After being fixed overnight in Bouin, gonads were rinsed and stored in EtOH 70 % till processing. Tissues were dehydrated with an increasing series of EtOH (70-100 %) and embedded in paraffin overnight. Then, the paraffin blocs were sectioned with a microtome (Leica RM2125 RTS) at 4 µm and stained with Eosin - Mayer's haematoxylin (Bio-Optica). Microphotograpies were taken using a Zeiss Axio Imager.M2 microscope coupled with a high-resolution camera Zeiss Aixocam 105 color. One slide from the anterior, medium and posterior part of the testis and ovaries were chosen for the analysis. Three microphotographies (40x) were taken per slide. Using *Image J* (ImageJ, NIH, USA, https://imagej.nih.gov/ij/), the percentage of the area occupied by spermatogonia and spermatozoa was evaluated from nine microphotographs per gonad [18] and 5 gonads per experimental group. The area covered with spermatozoa and spermatogonia were manually delimited and evaluated against the total surface of the slide and expressed as percentage. For the oocytes, using Cell Counter plugging (Image J), three classes of oocytes were calculated: previtelogenic oocytes (oogonia and cortical alveoli), vitelogenic oocyte and mature oocyte.

2.5 Statistical analysis

All the data was analysed by one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test. When the collected data was expressed in percentage, arcsin transformation was conducted before ANOVA. When data did not meet the conditions for using ANOVA, Kruskall-Wallis (non-parametric test) was applied. Superscript asterisks (*) evidenced statistical differences respect

the control (CTRL): * ($p < 0.05$), ** ($p < 0.01$). Data are reported as mean ± SEM (Standard Error of the Mean). The statistical software: *GraphPad Prism 6* and *SigmaStat 3.5*. Expression heatmaps of genes were created by fold change means (BPA/CTRL) with *Genesis software v1.7.7*. When fold changes were < 1, values were transformed to the negative inverse following: [-1 / fold change] due to Genesis does not allow to enter values in the 0 - 1 interval (see Supplemental Material 2).

3. RESULTS

3.1 Gonadosomatic index (GSI). In females, the GSI was not affected by BPA (Fig. 1a). However, in males, the GSI was increased in a dose-dependent manner (Fig. 1b).

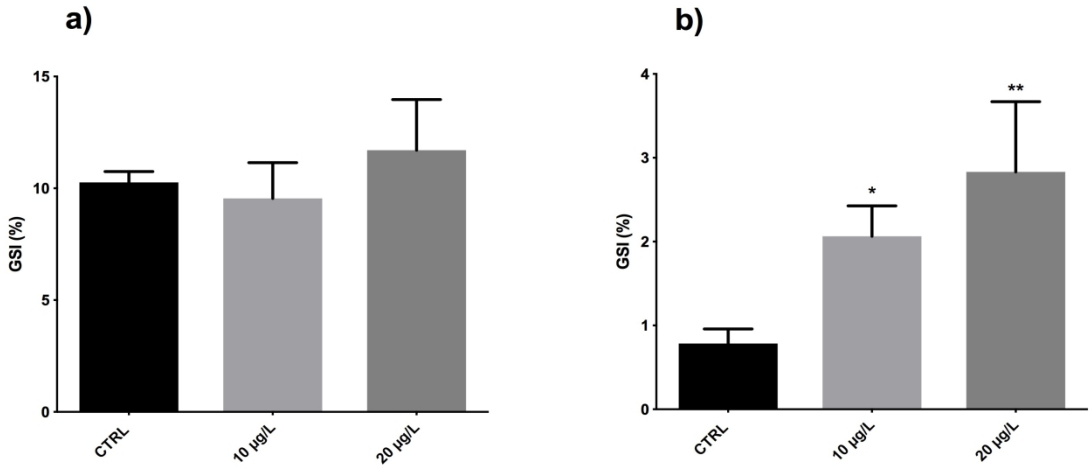


Figure 1. Gonadosomatic Index, female (a) and male (b) treated with 10 and 20 µg/L BPA. Data are reported as means ± SEM. Superscript asterisks (*) denote statistically significant differences with respect to the control (CTRL): * ($p < 0.05$), ** ($p < 0.01$) (one-way ANOVA, $p < 0.05$, Dunnett's multiple comparison test).

3.2 Measurement of endocannabinoids (AEA, 2-AG) and AEA-like mediators (PEA, OEA) in testes and ovaries.

In ovary, the levels of AEA were significantly increased following exposure to 10 µg BPA/L (Table 2). However, OEA was reduced in both BPA groups (10 and 20 µg/L). In testes, exposure to 20 µg BPA/L significantly reduced the levels of AEA and 2-AG, whereas the lowest concentration of BPA (10 µg/L) was without effect.

Table 2. Endocannabinoids and endocannabinoid-like levels in testes and ovaries.

	CTRL		10 µg/L		20 µg/L	
	FEMALE	**MALE**	**FEMALE**	**MALE**	**FEMALE**	**MALE**
AEA	15.92 ± 3.206	23. 18 ± 1.834	30.83 ± 4.961 *	31.97 ± 5.247	13.65 ± 1.936	7.85 ± 2.252 **
2-AG	3.08 ± 0.563	6.86 ± 1.411	2.96 ± 0.545	7.50 ± 1.266	2.75 ± 0.751	2.56 ± 0.549 *

OEA	0.21 ± 0.037	0.09 ± 0.032	0.03 ± 0.024**	0.06 ± 0.014	0.01 ± 0.004 **	0.08 ± 0.014
PEA	0.10 ± 0.014	0.10 ± 0.027	0.09 ± 0.013	0.09 ± 0.027	0.11 ± 0.009	0.10 ± 0.014

Table 2. Levels of endocannabinoids and endocannabinoid-like mediators in the testes and ovaries of zebrafish treated with 10 and 20 µg/L BPA. Data reported as means dCt ± SEM. Superscript asterisks (*) denote statistically significant differences with respect to the control (CTRL): * ($p<0.05$), ** ($p<0.01$) (one-way ANOVA, Dunnett's multiple comparison test, $p < 0.05$). AEA expressed as pmol/g tissue, while 2-AG, PEA and OEA as pmol/mg tissue.

3.3 ECS gene transcript levels in gonads.

Ovaries. As shown in Table 3, exposure to BPA did not alter the transcript levels of genes coding for the cannabinoid receptors. However, 20 µg BPA/L significantly increased mRNA expression of the gene coding for the catabolic enzyme *faah* (Table 3).

Table 3. Relative gene expression of ECS components in zebrafish ovary.

GENE	CTRL	10 µg/L	20 µg/L
cnr1	2.70 ± 0.552	3.41 ± 0.578	1.96 ± 0.359
trpv1	4.23 ± 1.183	3.69 ± 1.106	2.04 ± 0.492
gpr55	1.69 ± 0.208	2.10 ± 0.539	1.91 ± 0.268
napepld	3.14 ± 1.042	5.27 ± 0.608	4.79 ± 0.378
mgll	2.10 ± 0.541	2.35 ± 0.146	3.87 ± 1.011
faah	2.89 ± 0.988	4.62 ± 0.881	9.67 ± 2.549*
dagla	2.68 ± 0.701	3.28 ± 0.585	3.27 ± 0.559

Table 3. RT-qPCR results for the zebrafish ovaries after 21-day exposure to 10 and 20 µg/L BPA. Data are expressed as means dCt ± SEM. All data were normalized against the expression levels of *18s* and *rplp0*. Asterisks (*) denote statistically significant differences with respect to the control (CTRL): * $p < 0.05$; (one-way ANOVA, $p < 0.05$, Dunnett's multiple comparison test). Relative levels of mRNA are in arbitrary units (a.u.).

Testes. Exposure to BPA was without effect on the genes coding for the cannabinoid receptors. However, BPA had a biphasic effect on *mgll* mRNA levels; 10 µg BPA/L increased *mgll* transcript levels, whereas the highest concentration of BPA (20 µg/L) decreased them (Table 4).

Table 4. Relative gene expression of ECS components in zebrafish testes.

GENE	CTRL	10 µg/L	20 µg/L
cnr1	2.51 ± 0.491	2.32 ± 0.429	2.68 ± 0.661
gpr55	3.35 ± 0.801	5.57 ± 1.214	2.34 ± 0.591

trpv1	3.41 ± 0.633	3.44 ± 0.778	2.93 ± 0.798
napepld	1.84 ± 0.393	1.80 ± 0.405	1.64 ± 0.200
mgll	3.86 ± 0.319	6.24 ± 0.694*	1.32 ± 0.15*
faah	3.56 ± 0.587	3.70 ± 0.989	3.03 ± 1.009
dagla	3.18 ± 0.223	2.73 ± 0.329	2.45 ± 0.667

Table 4. RT-qPCR results of zebrafish testes treated with 10 and 20 µg/L BPA. Data are expressed as means ± SEM. All data were normalized against the expression levels of *rpl13* and *rplp0*. Asterisks (*) denote statistically significant differences with respect to the control (CTRL): * $p < 0.05$; (one-way ANOVA, $p < 0.05$, Dunnett's multiple comparison test). Relative levels of mRNA are reported in arbitrary units (a.u.).

Representation of the fold change of the genes analysed above (Figure 2).

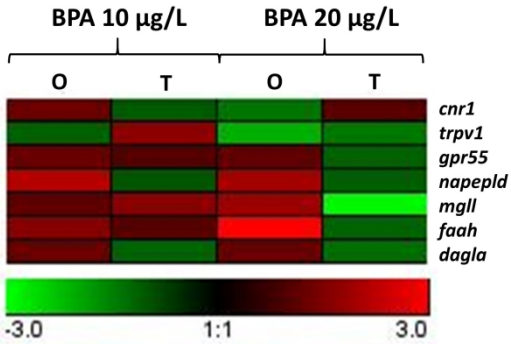


Figure 2. Heat maps for the ECS genes analysed in ovaries and testes. Red: up-regulation. Green: down-regulation. O: ovaries, T: testes.

3.4 Vitellogenin transcript levels in the liver.

The highest concentration of BPA increased the mRNA levels of vitellogenin (*vtg*) in the liver of both male (Figure 3a) and female (Figure 3b) zebrafish.

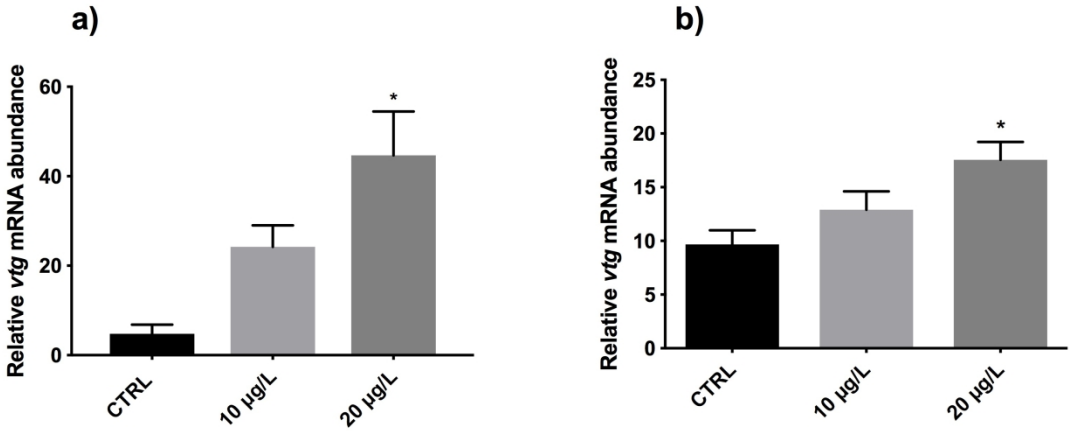


Figure 3. RT-qPCR results for *vtg* in zebrafish liver, in males (a) and females (b), following treatment with 10 and 20 µg/L BPA. Results are expressed as means dCt ± SEM. All data are normalized against the expression levels of *rpl13* and *rplp0* (male) and *rplp0* and *18s* (female). Asterisks (*) denote statistically significantly differences with respect to the control

(CTRL): * ($p < 0.05$); (one-way ANOVA, $p < 0.05$, Dunnett's multiple comparison test). Levels of mRNA abundance are reported in arbitrary units (a.u.).

3.5 Relative area covered by spermatozoa and spermatogonia.

The histological study of zebrafish testes revealed changes in zebrafish testicular development following exposure to BPA (Figure 4a-c). Using image analysis, the area occupied by spermatogonia and spermatozoa were quantified. The 20 µg BPA/L exposure induced a significant decrease of spermatogonia area (Figure 4d) while no effect was observed on the area occupied by spermatozoa (Figure 4e).

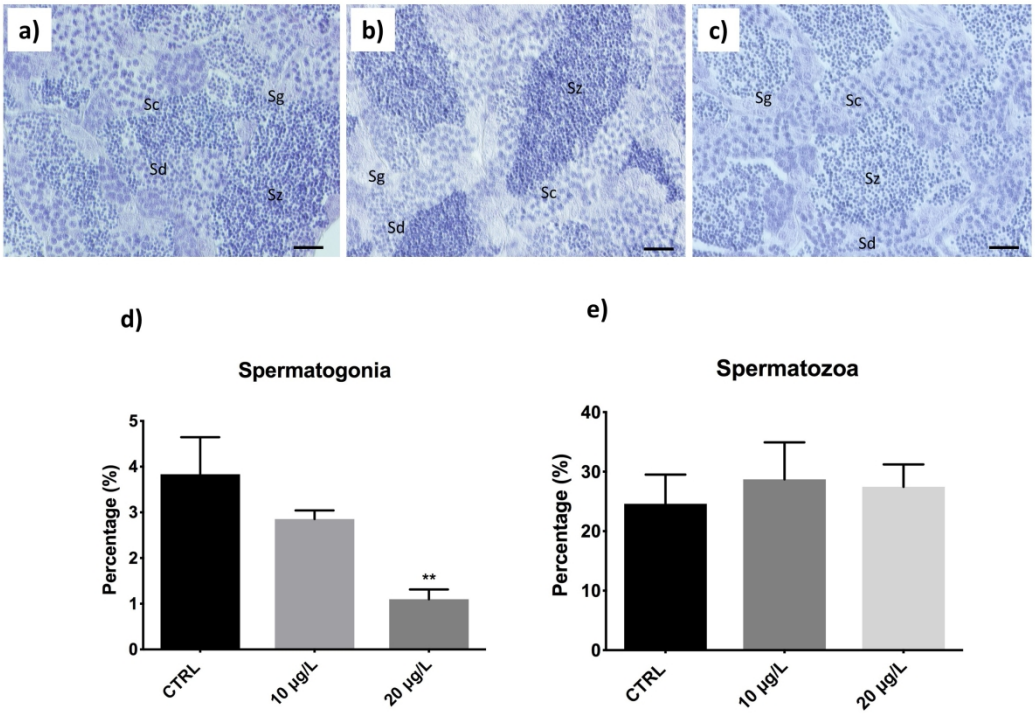


Figure 4. Histological sections of testis: CTRL group (a), 10 µg/L BPA (b) and 20 µg/L BPA (d). Eosin – Mayer's haematoxylin staining. Sg: spermatogonia; Sc: spermatocyte; Sd: spermatid; Sz: spermatozoa. Scale bar: 20 µm. Percentage of zebrafish testicular area occupied by spermatogonia (d) and spermatozoa (e). Data reported as means ± SEM. Asterisks (*) denote statistically significant differences with respect to the control (CTRL): ** $p < 0.01$, (one-way ANOVA, $p < 0.05$, Dunnett's multiple comparison test).

3.6 Changes in different classes of oocytes

Using histological analysis, we quantified the proportion of previtellogenic, vitellogenic and mature oocytes in the ovaries of zebrafish exposed to BPA (Figure 5a-c). The results demonstrate that exposure to 10 µg BPA/L significantly augmented the proportion of oocytes in vitellogenic stage, without affecting the percentage of previtellogenic and mature oocytes (Figure 5d-f).

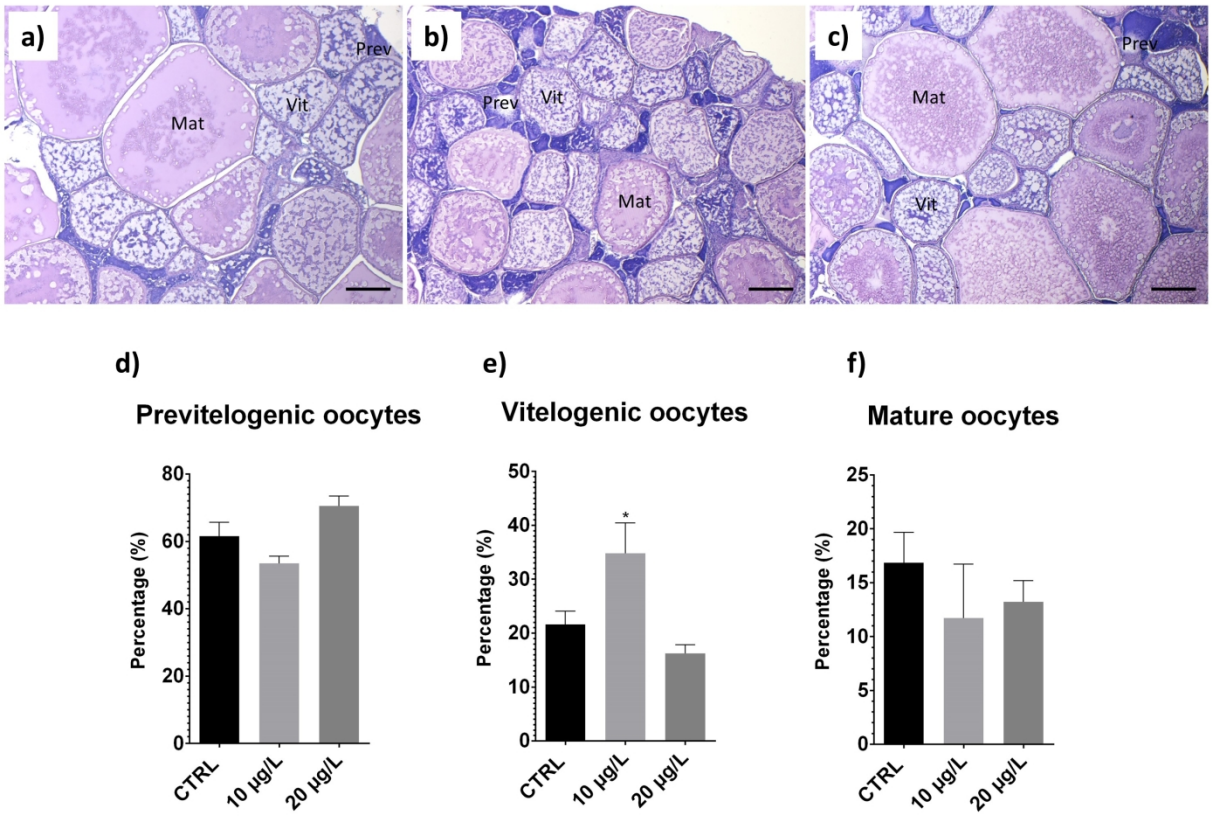


Figure 5. Histological sections of zebrafish ovaries, CTRL (a), 10 µg/L BPA (b) and 20 µg/L BPA (c). Eosin – Mayer's haematoxylin staining. Prev: previtelogenic oocytes; Vit: Vitellogenic oocytes; Mat: mature oocytes. Scale bar: 200 µm. Percentage of different classes of oocytes (d-f). Data are reported as mean ± SEM. Asterisks (*) denote statistically significant differences with respect to the control (CTRL): * $p < 0.05$, (one-way ANOVA, $p < 0.05$, Dunnett's multiple comparison test).

4. DISCUSSION

Due to the ubiquity of BPA in the environment, several studies have evaluated its adverse responses concerning male and female reproduction [49–51]. Here, we deciphered the effects of two environmental concentrations of BPA (10 and 20 µg/L) on zebrafish gonadal function with emphasis on the ECS. Firstly, our results demonstrate a dose - dependent increase of male GSI. In accordance with previous studies [26,52,53], this support the hypothesis that BPA can alter the testicular development in teleost species. In fact, our observations suggest that the presence of BPA can contribute to accelerate abnormal male testicular development and to unbalance gender patterns, as already reported in zebrafish for BPA and E_2 [54]. However, BPA action on the GSI cannot be generalized due to different results have been reported in different species [27,53,55,56]. It would

263 appear that duration, window of the exposure, species differences and BPA concentrations are
264 important factors influencing the effects of BPA on the GSI [53]. In this context, in murine models,
265 different sensitivities to BPA were observed depending on the mouse strain [57].
266 Hepatic VTG, a yolk precursor protein and a key signal for female reproduction, is another marker for
267 testing the effects of BPA estrogenicity, commonly used for teleosts [58]. Usually, males have low
268 basal or undetectable VTG levels, but exposure to external compounds with estrogen-like activity can
269 stimulate VTG production. Thus, VTG in male fish has become a useful biomarker to assess the
270 presence of environmental compounds with estrogen-like activity. Indeed, several studies have
271 correlated abnormal values of VTG or *vtg* with BPA in different teleost species [19,27,53,55,59–62].
272 Specifically, Mandich and coworkers (2007) reported an increase of VTG in plasma in common carps
273 after 14 days of BPA treatment. With zebrafish, 21 day exposure was enough to find an increased
274 gene expression of hepatic *vtg* in both sexes, similar to what was observed in *Sparus aurata* after a
275 3-week BPA exposure [26]. Curiously, the increase of *vtg* transcript (20 µg BPA/L) does not match
276 with the group showing the highest percentage of vitellogenic oocytes (10 µg BPA/L). This may be
277 explained as 1) oocyte maturation is a complex process where VTG is not the only player and other
278 hormones (*i.e.* FSH, LH) are involved, 2) VTG is a complex protein which undergoes a strong post-
279 transcriptional process, and additionally, as cited in [63], the RT-qPCR results do not inform whether
280 the mRNA will be or not be translated into protein.
281
282 The regulation of endocannabinoid tone during the different phases of reproduction is crucial in
283 numerous species [31,33,35,64–66]. However, little information is available regarding the role of AEA
284 tone during oocyte maturation [67]. It is tempting to hypothesize that the AEA produced from the
285 granulosa cells of growing follicles play a role in the control of oocyte maturation in mammals [31].
286 In the aquatic model, *Xenopus laevis*, AEA is associated with K^+ current supression in oocytes (stages
287 V or VI), and consequently, with the inhibition of oocyte responsiveness to gonadotropin and
288 progesterone required for final meiotic maturation [68]. Thus, we may assume that the augmented
289 AEA levels (10 µg BPA/L) may contribute to the signals inducing vitellogenesis but impairing the final
290 oocyte maturation. However, few information is available on the functional role of AEA in teleost
291 ovary, although our hypothesis would be consistent with a previous study [15] demonstrating that
292 10 µg BPA/L is associated with the down-regulation of oocyte maturation signals in female zebrafish.
293 Furthermore, in rodents and humans, the hormonal environment regulates the endocannabinoid
294 production in the gonads [38,69]. Additionally, we observed a decrease in ovarian OEA in the BPA

295 exposed groups. In this context, OEA, which is a AEA-related compound, may be involved in the
296 mammalian follicular maturation [70]. In addition, OEA also plays a role as an anti-inflammatory
297 signal molecule [71,72], suggesting an inflammatory process in the BPA ovaries. In this context, BPA-
298 induced inflammatory responses have been already reported in other species [73].
299
300 Concerning male ECS, reduction in AEA and 2-AG levels was observed following exposure to 20 µg
301 BPA/L in the testis. In male mouse germ cell cultures, the highest levels of 2-AG were observed in
302 spermatogonia and then, such levels were progressively decreased in spermatocytes and spermatids
303 [74]. The latter study is consistent with our findings where the lowest levels of 2-AG were found in
304 the BPA group associated with the reduced area of spermatogonia. In fact, CB2 signaling through 2-
305 AG contributes to the normal progression of the spermatogenesis [75]. Focusing on the other
306 endocannabinoid, AEA, Grimaldi and coworkers demonstrated a constant AEA concentration during
307 spermatogenesis (spermatogonia, spermatocyte and spermatids), whereas here we found a
308 decrease in testicular AEA following BPA exposure. Two factors may explain this finding: 1) the
309 observed reduction in AEA was the result of reduced spermatogonia induced by BPA, and/or 2) an
310 increased activity of FAAH (AEA catabolic enzyme) induced by the estrogen-like activity of BPA,
311 reduced the AEA levels. Based on the present results, we cannot distinguish between these two
312 possibilities. Though, similar results were obtained following E_2 treatment in mouse Sertoli cell [76].
313 Very few studies have quantified testicular cell types, and to date, there are no published
314 observations on the effects of environmental doses of BPA on testicular cell numbers. In zebrafish,
315 higher doses of BPA exclusively reduced the proportion of spermatocytes [18]. Interestingly, our
316 results are in agreement with a study in rats demonstrating that lower concentrations of BPA trigger
317 a reduction in the number of spermatogonia and spermatocytes [21,77]. Furthermore, in male
318 gonads, BPA can induce alterations in miRNAs [78–81] and epigenetic markers, as hyperacetylation
319 of histones and DNA hypermethylation [18], these changes can be also inherited by the offspring and
320 compromise early embryo development depending on the dose and the window of exposure [82,17].
321 Overall, the present results and those obtained by others in different species suggested that exposure
322 to BPA might be associated with low fertility in male zebrafish and reduced numbers of
323 spermatogonia and spermatogenesis.
324
325 **5. CONCLUSIONS**

21-day exposure to environmentally relevant concentrations of BPA induced changes on the ECS. As far as we know, the correct functioning of the ECS is crucial for the normal progression of the reproductive processes in both males and females. Despite of the ECS presence in teleost gonads and its potential role in reproduction, further studies will be needed to fully understand its involvement in fish oogenesis and spermatogenesis. Finally, our results suggested that the ECS may be used as a biomarker for EDC activity related to reproduction.

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☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Author statement

Isabel Forner-Piquer: conceptualization, formal analysis, investigation, writing-original draft, visualization;

Silvia Beato: formal analysis, investigation, writing-original draft, visualization;

Fabiana Piscitelli: methodology, formal analysis;

Stefania Santangeli: formal analysis, investigation;

Vincenzo di Marzo: methodology, resources, writing – review, supervision;

Hamid R. Habibi: methodology, resources, writing - review funding acquisition;

Francesca Maradonna: conceptualization, investigation;

Oliana Carnevali: conceptualization, methodology, validation, resources, writing - review, supervision, project administration, funding acquisition.

Table S1. Additional information of primers.

GENE	Exon span	Amplicon size	Ta (°C)	TISSUE	ZFIN ID	Reference
Cannabinoid receptor 1	No	181	55	ovary, testis	ZDB-GENE-040312-3	[1]
Transient receptor potential cation channel, subfamily V, member 1	Yes	129	59	ovary, testis	ZDB-GENE-030912-8	[2]
G protein-coupled receptor 55	No	91	53	ovary, testis	ZDB-GENE-051113-260	[3]
N-acyl phosphatidylethanolamine phospholipase D	No	121	57	ovary, testis	ZDB-GENE-030131-3856	[1]
Monoglyceride lipase	No	118	53	ovary, testis	ZDB-GENE-031006-9	[1]
Fatty acid amide hydrolase	No	202	60	ovary, testis	ZDB-GENE-070619-3	[4]
Diacylglycerol lipase alpha	No	58	59	ovary, testis	ZDB-GENE-070619-1	[1]
Abhydrolase domain containing 4	Yes	132	60	ovary, testis	ZDB-GENE-050417-83	[4]
Vitellogenin 1	Yes	209	60	liver	ZDB-GENE-001201-1	[5]
Ribosomal subunit 18s	No	90	55	ovary, liver	ZDB-GENE-070410-9	[6]
Ribosomal protein large P0	No	160	60	ovary, testis, liver	ZDB-GENE-000629-1	[5]
Ribosomal protein L13a	Yes	147	59	testis, liver	ZDB-GENE-030131-168	[7]

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