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Plastic and environmental safety: the effects of EDCs on metabolism, reproduction and epigenetic processes

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Dedicated to the memory of Dr. Marcello Spanò

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1. INTRODUCTION

1.1. Plastic additives

Plastic are synthetic organic polymers, derived from the polymerization of monomers extracted from oil or gas (Derraik 2002; Rios et al. 2007). From the development of modern plastic, in 1907, a number of inexpensive manufacturing techniques have been optimized, resulting in the mass production, in a surplus of lightweight, durable, inert and corrosion-resistant plastics (Cole et al. 2011). These attributes have led to the extensive use of plastic in almost inexhaustible applications leading to the growth of plastics production from approximately 1.9 tons in 1950, to approximately 330 million tons in 2013 (PlasticsEurope 2015).

Whilst the social benefits of plastic are far-reaching (Andrady and Neal 2009), this valuable commodity has been the subject of increasing environmental concern. In first place the durability of plastic, that makes it such an attractive material to use, but also, makes it highly resistant to degradation (Barnes et al. 2009b; Sivan 2011). We must also consider the copious use of "throw-away" plastic users, the proportion of plastic that contributes to municipal waste constitutes 10% of waste generated worldwide (Barnes et al. 2009a). An estimate up to 10% of plastic produced end up in the oceans, where they may persist and accumulate (Cole et al. 2011). The impact that large plastic debris, known as "macroplastics" can have on the marine environment include: the injury and death of marine birds, mammals, fish and reptiles resulting from plastic entanglement and ingestion (Derraik 2002), the transport of non-native marine species to new habitats on floating plastic debris, and the smothering of the seabed preventing gas-exchange (Gregory 2009).

Macroplastics will gradually break down into ever-smaller-pieces due to sunlight exposure, oxidation, and the physical action of waves, currents, and grazing by fish and birds (Zettler et al. 2013) forming tiny plastic fragments called secondary microplastics with a diameter smaller than 1 or 5 mm. Degradation of larger pieces of plastic is not the only way microplastics end up in the ocean. For example microbeads used as scrubbing in personal care products can escape water treatment facilities and pass into environment (Fendall and Sewell 2009).

Although plastics are typically considered as biochemically inert (Roy et al. 2011), plastic additives, also called plasticizers may be incorporated into plastics during manufacture to change their properties or extend the life

of the plastic by providing resistance to heat, oxidative damage and microbial degradation (Browne et al. 2007). These additives are of environmental concern since they both extend the degradation times of plastic and may, in addition, leach out, introducing potentially hazardous chemicals to biota (Barnes et al. 2009b; Lithner et al. 2009) through the interference with biologically important processes, potentially resulting in endocrine disruption (Barnes et al. 2009a; Lithner et al. 2009, 2011).

1.2. Endocrine disruptor compounds

An endocrine disrupting compound (EDCs) is defined by the United States Environmental Protection Agency (EPA) as *"an exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny, or (sub)populations"*. The term endocrine-disrupting chemicals is used to define a structurally diverse class of synthetic and natural compounds that possess the ability to alter various components of the endocrine system and potentially induce adverse health effects in exposed individuals and populations. EDC, have been reported to interfere with the endocrine system and ultimately disturb the normal function of tissues and organs, producing adverse developmental, reproductive, neurological, cardiovascular, metabolic and immune effects (Schug et al. 2011). Given their physicochemical differences and distinct biological effects, it is not surprising that a variety of mechanisms are used by EDCs to influence the endocrine system (Henley and Korach 2006). The main evidence suggesting that exposure to environmental chemicals can lead to disruption of endocrine function comes from changes seen in a number of wildlife species. Effects related to endocrine disruption have been reported in molluscs, crustacea, fish, reptiles, birds and mammals in various parts of the world (http://ec.europa.eu/environment/chemicals/endocrine/index_en.htm).

The hormonal activity of some chemical compounds was well known some decades ago. For instance, in 1938 an unidentified hormonal activity for bisphenol A (BPA) was published (Dodds and Lawson 1938). After World War II, scientists located high levels of xenoestrogens (or EDCs related with human activity) in the environment because of their increased use in industrial and domestic areas. In 1990 only some pesticides, catalogued as dangerous for other negative effects, formed the EDCs list. The endocrine disruptor concept, the rules for specific studies of endocrine activity, and the awareness of human health and environmental exposure were established at the European Workshop on the Impact of Endocrine Disruptors on Human Health and

Wildlife (Weybridge, U.K.) in 1996. Alkylphenols, bisphenols, phthalates, polychlorinated biphenyls, polycyclic aromatic hydrocarbons, dioxins–furans (PCDD/F), some metals (Pb, Cd, and Hg), and organotin compounds have been finally included in the EDCs list.

Chemicals with hormonal activity, and thus potential endocrine disruptors, include:

- **Natural hormones** from any animal, released into the environment, and chemicals produced by one species that exert hormonal actions on other animals. For example human hormones unintentionally reactivated during the processing of human waste in sewage effluent, may result in changes to fish.
- **Natural chemicals** including toxins produced by components of plants (the so-called phytoestrogens, such as genistein or coumestrol) and certain fungi.
- **Synthetically produced pharmaceuticals** intended to be highly hormonally active. For example the contraceptive pill and treatments for hormone-responsive cancers may also be detected in waste water.
- **Man-made chemicals** and by-products released into the environment. Laboratory experiments have suggested that some man-made chemicals might be able to cause endocrine changes. These include some pesticides (e.g. DDT and other chlorinated compounds), chemicals in some consumer and medical products (e.g. plastic additives), and a number of industrial chemicals (e.g. polychlorinated biphenols (PCBs), dioxins). The hormonal activity of these chemicals, is many times weaker than the body's own naturally present hormones, e.g. nonyl-phenol (a breakdown product of alkylphenol ethoxylate surfactants), found as a low level contaminant in some rivers in Europe, has an estrogenic activity only about one-ten thousandth that of the natural hormone, estrogen.

Numerous toxic compounds have environmental concentrations below the thresholds risk, according to the regulations, but it must take into account that the human and environmental health problem regarding EDCs is related to their bioaccumulation, low level chronic exposures, and synergistic effect of them, according to scientists. Some EDCs frequently identified in the migration tests were BPA, phthalates and 4-NP (4-nonylphenol) or t-butylphenol. The main non-EDCs identified, were solvents, antioxidants, and rubber-like compounds (Romero et al. 2002). Some chemicals can act on the endocrine system to disturb the homeostatic mechanisms of the body or to initiate processes at abnormal times in the life cycle. They are usually either natural products or synthetic chemicals that mimic, enhance (an agonist), or inhibit (antagonist) the action of hormones (Soto et al. 1995).

The mechanisms through which these chemicals can exert their effects are different:

- Due to structural similarity to the natural hormone, EDCs can mimic their actions in the body by binding its receptor or group of receptors on the cell surface, cytoplasm, or nucleus, acting as agonists and changing gene expression in a way characteristic for a given hormone (Roy et al. 2009);
- Mimicry can also make EDCs act as partial agonists, or antagonists (they can bind the receptor but not activate it) of particular receptor (Diamanti-Kandarakis et al. 2009);
- EDCs may alter receptor production;
- Activation of receptors can be modulated by EDCs through receptor phosphorylation or other ways (Lohse et al. 1990);
- EDCs may interfere with recruitment of transcriptional co-activators, in which receptor activity relies (especially nuclear receptors and aryl hydrocarbon receptor). (Swedenborg et al. 2009);
- Some EDCs can impose the disruption without direct receptor interaction. They can affect the level of enzymes, influencing directly or indirectly the production of hormone (e.g. aromatase) (Swedenborg et al. 2009);
- Concentration of the active hormone, reaching the receptor site with bloodstream, can also be altered by modulating hormone transport (they may bind to transport proteins in the blood, altering the amounts of natural hormones that are present in the circulation) or metabolism (Baker et al. 1998);
- EDCs can alter signaling pathways in different and complex ways, including pathway crosstalk or may act by mechanisms yet unknown (Safe and Wormke 2003).

Endocrine disruption has been demonstrated to occur in wildlife, particularly in aquatic species or in species that are connected to aquatic food chains, like “fish-eating” birds (Tyler et al. 1998; Vos et al. 2000). The questions being addressed today, are not whether endocrine disruption occurs in wildlife but rather in which concentration it occurs, what mechanism are involved, and whether disruption of the endocrine system will lead to ecologically relevant effects (Guillette 2006). To answer these questions, zebrafish, together with fathed minnow, *Pimephales promelas*, and medaka, *Oryzias latipes*, are under evaluation as model species for regulatory test programs in the environmental risk assessment of EDCs (Ankley and Johnson 2004). Since EDCs can lead to chronic alterations of development and reproduction, the short life cycle of small laboratory

fish, such as zebrafish, represents an important advantage of these species as experimental animals (Bresch et al. 1990).

1.3. Non-monotonic dose-response curves

“The dose makes the poison” is the dogma of modern toxicology, derived from a quote from Paracelsus, a XV-century physician and alchemist considered to be a founder of toxicology as a discipline. This basic principle is not only popularly recognized, but also intuitive. It can be easily assumed, that the relation between chemical exposure dose and the observed biological effect is consequentially logical: the higher the dose, the higher the effect. This assumption underlies the modern system of toxicity testing for new compounds (and other effect-searching screening methods), so the belief in the principle is not only theoretical, but it has also very practical implications. The screening, usually employs a range of high doses of a particular compound, and obtained effects that are extrapolated to lower doses. Performed tests include assessing the LD50s (the dose lethal for 50% of the experimental animals), MTD (maximum tolerated dose), LOAEL (lowest levels observed adverse effect) and NOAEL (the no observed adverse effect level). The safe dose for humans and wildlife is subsequently calculated by an algorithm providing some safety coefficient and based on the NOAEL value. Assuming that the dose-response relationship had been linear, the “dose-safe” could have been deduced by simple dose response test. The relationship of dose and effect corresponding to this dogma can be visualized as a **monotonic dose-response curve** (Fig 1.3.1.), which may take slightly different forms, however, the effect curve always maintains a direction of change.

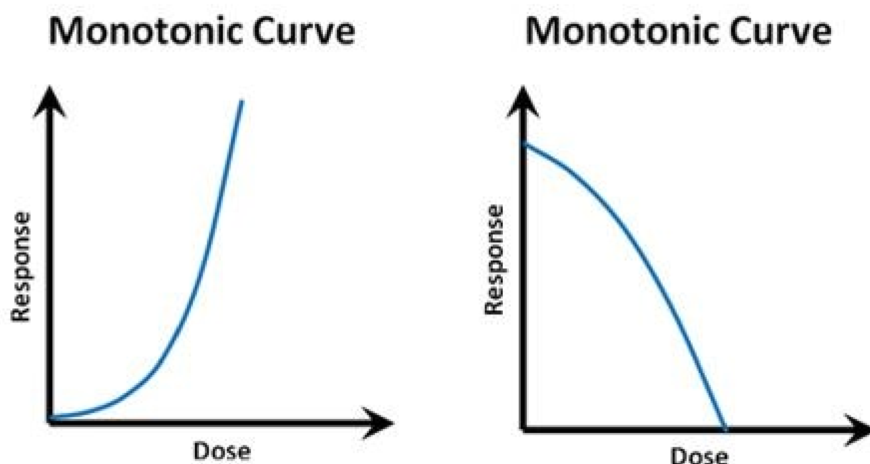

Figure 1.3.1. Examples of monotonic dose-response curves.

Even though accurate in many cases, the dogma does not hold true for some investigated compounds, and the number of them is rapidly increasing. It is possible for a chemical to elicit a more complex response pattern, than expected. While the number of possible response patterns shaped by complex molecular interactions is theoretically unlimited, it is common to observe a U-shaped or inverted-U-shaped dose-response curve characterized by the presence of a peak and subsequent effect decrease with a rising dose. That type of pattern is called a **non-monotonic dose-response curve** (NMDRC) (Fig. 1.3.2.), because the mathematical function defining this curve does not preserve the given order: the slope of the dose-response curve changes sign from positive to negative or conversely at some point of the range of examined doses, so the direction of biological effect change is not constant.

The consequence of the existence of non-monotonic dose-response curve phenomenon is that no toxicity testing results can be extrapolated to another range of doses, than those empirically tested. The lack of significant results observed during standard high dose chemical safety assessment does not entitle the researcher to assume safety of lower doses. This, however, undermines the paradigms of safety testing procedures, therefore questioning validity of conclusions reached from the majority of safety tests performed to this day and challenging the *status quo*. Even employing gold standard testing methods, it is necessary to expand extremely, the range of investigated compound concentrations in order to learn about its real potential to cause biologically relevant effects. The lack of such safety measures leads to an unrestricted use of

compounds, such as endocrine disruptor compounds, that later prove to be not safe, and often more toxic in low doses (which is recognized as a low-dose effect) (Vandenberg et al. 2012). It is important to underline that low doses are the kind of exposure experienced chronically by wildlife and humans in their living environment.

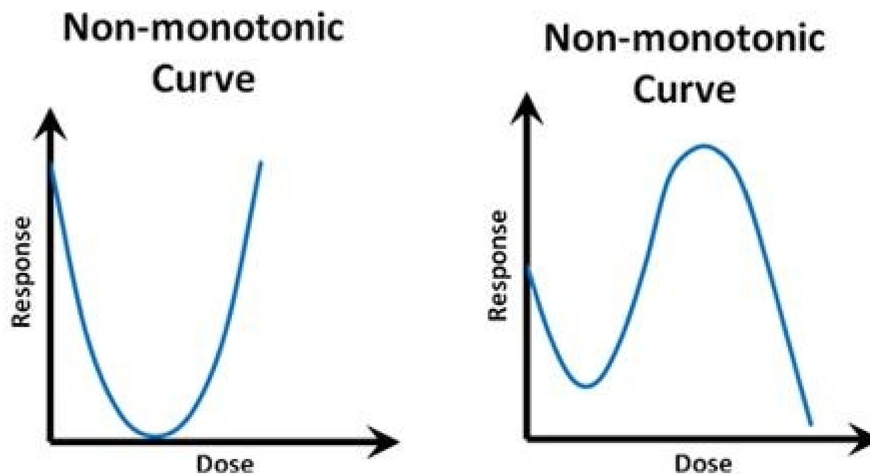

Figure 1.3.2. Examples of non-monotonic dose-response curves; there is a theoretically unlimited potential for different shapes of non-monotonic curves, influenced by complex molecular interactions.

Numerous molecular mechanisms are proposed to explain the existence of non-monotonic dose-response curves and low-dose effects. These include such as:

- Cofactors and receptors specific for a cell or tissue types: Some of the observed non-monotonic effects may be a result of overlapping monotonic effects, for example coming from a number of different cell subpopulations or tissues present in the sample or culture, that react to the hormone in a different way (Soto and Sonnenschein 2001) due to a different type and amount of receptors expressed (Morani et al. 2008) as well as the presence of specific cofactors capable of altering receptor selectivity and downstream signal processing (Jeyakumar et al. 2008), thus the response as well.
- Receptor selectivity: Many of the discussed compounds can act via multiple receptors and pathways depending on the concentration. They are activated, almost exclusively, by one type of receptor at low dose, and become less specific at high dose, weakly binding other kinds of receptor too (Moriyama et al. 2002) and altering the biological endpoints.

- Desensitization of receptors: For a wide range of hormones (or agents mimicking them) the decreased response to ligands by receptor had been observed as a result of biochemical inactivation after prolonged exposure.
- Down-regulation of receptor expression: Rising ligand levels often lead to an increase of inactivation and degradation of its receptors, at some point exceeding the capacity to produce new ones in the pace equal to the speed of degradation pathway (Ismail and Nawaz 2005). Also, receptor production can be regulated based on the level of circulating ligand, in a non-linear pattern. Additionally signaling from one receptor type can influence production process of another type (Kinyamu and Archer 2003).
- Competition for receptor sites: If the artificial compound is competing with a natural hormone for the same receptor, they can both find an unoccupied site, bind and activate the responses; however at a high dose of the compound it outcompetes the natural hormone, altering the response. Mathematical models were used to determine high probability of NMDRC in all such cases (Kohn and Melnick 2002).
- Negative endocrine feedback loops: Several animal hormones are regulated by positive or negative feedback loops: the circulating level of a given hormone or product of its action affects the production of this hormone based on activation of receptors related to it. Overstimulation of hormone receptor by other compounds decreases hormone production (Vandenberg et al. 2012).

Several studies demonstrates that EDCs do not follow the principle "the dose make the poison", but rather exhibit a U-shaped and inverted U-shaped, non-monotonic, dose response curve, where the strongest responses may be elicited by the lowest and highest doses, or by intermediate concentrations, respectively, probably due to the receptor down regulation induced by higher hormone levels (Tibbetts et al. 1998; Vandenberg et al. 2009, 2012).

1.4. Epigenetic effects of EDCs

The term *epigenetics* was described for the first time in 1942 by Conrad Waddington as "…the interaction of genes with their environment which brings the phenotype into being" (Waddington 1942). Epigenetic modifications are now described as heritable and reversible chemical modifications of chromatin, resulting in adjustment of its activity without change in the underlying DNA sequence. Epigenetic plays an important role in cellular differentiation, growth, metabolism, and regulation of gene expression by silencing and activating

specific genes (Bernstein et al. 2007; Esteller 2007; Jirtle and Skinner 2007). In addition, it was shown that epigenetic mechanisms facilitate interaction between gene transcription and environment (Esteller 2007). The main types of epigenetic modifications include DNA methylation and histone modification (Tammen et al. 2013).

Recent studies have demonstrates the ability of EDCs to have epigenetic effects (Anway and Skinner 2006; Ho et al. 2006). 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), polychlorinated biphenyls (PCBs), and phthalate esters, have been found to affect the reproductive system or induce tumor development by altering DNA methylation, and steroid hormone metabolism and signaling (McLachlan et al. 2006; Wu et al. 2004). Plastics induce a delayed female pubertal onset in F_1 rats but in F_2 generation. Plastics, dioxin and jet fuel promote early onset of puberty in female rats (Manikkam et al. 2012). BPA shows trans generational effects on sperm fertility and other reproductive parameters in male rats (Wolstenholme et al. 2012) and was additionally found able to interfere with both DNA methylation and histone modification processes (Doherty et al. 2010; Dolinoy et al. 2007; Kundakovic et al. 2013). Plasticizer such as BPA, DEHP and DBP can produce inheritance effects in F_3, such as testis, prostate, kidney and ovary effects, tumors and may induce obesity in subsequent generation (Manikkam et al. 2013).

1.5. The different groups of EDCs studied in this project

In this project, the different studies conducted, have together investigated the endocrine disrupting potential, of different compounds: Bisphenol A, nonylphenol, 4-tert-octylphenol, di-isononyl-phthalate and diethylene glycol dibenzoate. The current section will give a short general description of these four groups of endocrine disrupting compounds.

1.5.1. Bisphenol A

Bisphenol A (BPA) is a phenol formed by a hydroxyl residue directly bound to an aromatic ring (Flint et al. 2012) which was synthesized firstly in 1891 by the Russian chemist Alexander P. Dianin. Later in 1930, its estrogenic effects were discovered (Rubin 2011), while the use of this chemical in the plastics industry, started in 1940, since its ability to increase heat resistance and elasticity. Nowadays BPA is a key monomer in

production of epoxy resins and in the most common form of polycarbonate plastic and is one of the most produced chemicals in the world. According to recent estimates, a hundred ton of BPA per year can be found in the environment (Rubin 2011) through landfill or plastic degradation (Kang et al. 2006), realistically resulting in environmental waters concentrations ranging from 5 µg/L to 21 µg/L (Crain et al. 2007).

BPA is present in 95% of products containing epoxy resins and polycarbonates such as food-containers, bottles, toys, dental products, CD and DVD and water pipes. It is also used in the vinyl chloride and thermal paper production which are used for the manufacture of slips, books, booklets, ticket, kitchen rolls, toilet paper and food paperboard (Nam et al. 2010). For this reason, it is an important contaminant due to its ubiquitous presence and the increased exposure of humans and wild organisms via environment and food chain.

BPA is generally characterized as an oestrogen-like compound. Oestrogen classical action mode, is trough activation of the nuclear oestrogen receptors (ERs), ERα and ERβ. In addition to these nuclear receptors, estrogen-related receptor gamma (ERRγ) and the G-protein coupled oestrogen receptor (GPER) have been proposed as potential mediators of BPA-associated oestrogen disruption (Riu et al. 2011; Wang et al. 2012). Similar to other xenoestrogens, it is considered to be a selective oestrogen receptor modulators because its effects are pro-oestrogenic in some tissues and anti-oestrogenic in others (Kundakovic and Champagne 2011; Wetherill et al. 2007) while its capacity to bind also to membrane receptors make it harmful even at pico and nanomolar concentrations (Wetherill et al. 2007).

As an environmental pollutant, BPA is probably able, to alter epigenetic mechanisms through methylation of the CpG sites (Dolinoy et al. 2007). Even though we don't have a good knowledge on the matter, BPA is supposed, to be able to alter histone modification and, hence, the structure of chromatin correlated with either activation or repression of transcription (Kundakovic and Champagne 2011).

Many studies revealed ontogenetic and endocrine disruptions by BPA on aquatic organisms at environmental relevant concentrations and there is an emerging concern that this pollutant may adversely impacts reproduction, lipid metabolism as well as brain development not only in animal but also in human (Lam et al. 2011; Saal et al. 2012; Sheng et al. 2013).

1.5.2. Nonylphenol

Nonylphenol (NP) is a xenobiotic compound consisting of a phenol ring and nine-carbon chain on the *para*-position. Nonylphenol ethoxylates are highly "cost effective" surfactants with exceptional performance and consequently used widely in industrial, institutional, commercial and household applications such as detergents, emulsifiers, wetting and dispersing agents, antistatic agents, demulsifiers and solubilisers. Nonylphenol ethoxylates reach sewage treatment works in substantial quantities where they biodegrade into several by-product including nonylphenol. Due to its physical-chemical characteristics, such as low solubility and high hydrophobicity, nonylphenol accumulates in environmental compartments that are characterized by high organic content, typically sewage sludge and river sediments, where it persists. The occurrence of nonylphenol in the environment is clearly correlated with anthropogenic activities such as wastewater treatment, landfilling and sewage sludge recycling (Soares et al. 2008). The first evidence that alkylpehnols could be oestrogenic was published in 1938 (Dodds and Lawson 1938). Nonylphenol was found to mimic the natural hormone 17β-oestradiol by competing for the binding site of the oestrogen receptor (White et al. 1994). Recently it has also been established that nonylphenol has antiandrogenic activity (Lee et al. 2003) and it has been proposed to induce lipid accumulation in hepatocytes (Hao et al. 2012).

1.5.3. 4-tert-octylphenol

Para-Alkylphenols are present in the aquatic environment due to their use in pesticide formulations, from leaching of plastics and from degradation of alkylphenol ethoxylates. 4-tert-octylphenol (t-OP) is the most estrogenic of the 4-alkylphenols and in vitro studies have demonstrated that it binds to the estrogen receptor, activates estrogen responsive genes, and stimulates mitogenesis of MCF breast cancer cells (Routledge and Sumpter 1997; White et al. 1994). In "vivo" studies, have demonstrated the ability of this pollutant to cause estrogenic-like effects, including, disruption of spermatogenesis, increased incidence of sperm deformities and lowered serum concentration of testosterone and gonadotropins (Boockfor and Blake 1997) while there are limited information on their effects on the metabolic pathway (Pedersen and Hill 2000).

1.5.4. Di-isononyl-phthalate

Di-isononyl-phthalate (DINP) is a compound belonging to the phthalate family.

Phthalates are chemicals with a broad worldwide distribution which are used as solvent in products for personal care but also as additives in plastics, specifically, in plasticized polyvinyl chloride (PVC) (David et al. 2001; Koch and Calafat 2009; Schettler 2006). The annual world production of phthalates is more than 4 Mt, of which about 1 Mt is produced in Europe (Peijnenburg and Struijs 2006). The most important compound of this category was bis(2-ethylhexyl)phthalate (DEHP), that, given its proven toxicity for reproduction (Carnevali et al. 2010), has recently been replaced by other compounds, such as di-isononyl phthalate (DINP) (Clara et al. 2010). DINP became then one of the main plasticizer used for PVC production with a consequent increase in its presence in the environment. Environmental concentration of DINP was found at concentration from 0.52 μg/L (1.2×10^{-8} M) and upwards (Quinn-Hosey et al. 2012).

The formation of DINP occurs through an esterification reaction between phthalic anhydride and two molecules of isononyl alcohol, with the production of a water molecule. This compound contains two alkyl chains of nine carbon atoms which confers a high molecular weight. DINP is consequently more permanent, it has a lower solubility rate and a low rate of migration compared to other phthalates (Vieira et al. 2011).

Because of its composition and chemical structure, DINP appears to be an endocrine disrupter, playing mostly an anti-androgen action in men by blocking the receptors of male sex hormones and inhibiting their biological effects. Very little is known about the effect of DINP in teleost since most of studies have focused primarily on mammals but several studies have shown its ability to act on the androgen responsive tissues, causing retention of nipples, testicular atrophy, epididymal agenesis and the decrease of testosterone levels, in both testes and plasma, of male rats (Borch et al. 2004; Gray et al. 2000).

1.5.5. Diethylene glycol dibenzoate

Diethylene glycol dibenzoate (DGB) was recently approved by European Chemical Agency as an alternative to phthalates in the processing of plastic (Kermanshahi et al. 2009). DGB is characterized as a high solvating plasticizer intended for the use in the manufacturing of PVC, vinyl flooring, adhesives, latex caulks, sealants and elastomers. The main mechanism of environmental removal for this chemical is microbial degradation.

Dibenzoate, has a higher biodegradation rates than phthalates, however studies have shown that interaction of Rhodotorula rubra with dibenzoate-based plasticizers, resulted to an incomplete microbial degradation leading to the accumulation of monobenzoates, which have higher toxicity than the original plasticizers. The biodegradatioin of this commercial plasticizer by the yeast R. rubra, results in the formation of substantial amounts of metabolites, such as diethylene glycol monobenzoate, which is toxic and resistant to further degradation (Kermanshahi et al. 2009).

Only a handful of research papers, concerning DGB are available, focused on understanding the pathways and results of DGB metabolism in a living organism. These publications suggest, that while DGB does not last long in the organism, the intermediates of its detoxification do, and the results do not support the use of DGB as an environmentally safer alternative to phthalates (Gartshore et al. 2003; Kermanshahi et al. 2009).

1.6. Physiological processes studied in the present thesis work

It is well known that EDCs can interfere with vertebrate reproductive system (Carnevali et al. 2010; Crain et al. 2008; Patisaul and Adewale 2009). In addition to reproductive effects, there is also a growing concern that metabolic disorders may be linked with EDCs (Casals-Casas and Desvergne 2011). Oogenesis and lipid metabolism process were thus investigated in this project.

1.6.1. Oogenesis

In teleost fish, oogenesis is under the control of hypothalamic-pituitary-gonadal (HPG) axis. The principal component of the axis includes the hypothalamus and hypophysis (pituitary gland) in the brain, the gonads and the liver. These tissues are structurally linked in one or both of the following mechanism: a relatively fast neuronal linkage and a slower vascular linkage. In teleost, pituitary is directly innervated by hypothalamic neurons that secrete directly into the intercellular space of the hypophysis. The main HPG axis neurohormone, secreted by hypothalamus is gonadotropin-releasing hormone (GnRH) which stimulates the production and the release of two types of glycoprotein gonadotropic hormones: the follicle-stimulating hormone (FSH), which primarily induces oogenesis, and luteinizing hormone (LH), whose primary activity causes final gametes maturation and induction of ovulation.

The ovaries of principal teleost are paired or bilobed organs and we will focus on those which are asynchronous spawners. The principal cell types of the ovary are the germinative cells, in various stage of development, starting with the primary oogonia, which develop into mature oocytes. At any particular time, the asynchronous ovary contains oocytes at all stage of development. Surrounding the developing oocyte, is a spherical layer of cells called the granulosa, which assists the translocation of the egg-yolk lipoprotein vitellogenin into developing oocyte. Finally, in the stromal layers of the ovary resides a cell type called thecal cells, which are involved in the synthesis of steroid hormone.

The primary effects in the ovary of FSH and LH are to stimulate the thecal and granulosa cells to produce the female's steroid hormone 17β-estradiol. However, the synthetic pathway of this hormone includes the male hormone testosterone as an intermediate. Apparently, the thecal cells, under the stimulatory influence of LH, synthesize testosterone, which diffuses to the granulosa cells. FSH induces the enzyme aromatase in the granulosa cells, which converts testosterone to estrogen. The estrogen that is produced has several critical roles, including oocyte development and stimulation of the liver to produce and secrete vitellogenin into the bloodstream. Once in the ovary, the vitellogenin is translocated via the granulosa cells into the developing oocyte. Finally maturation is under control of LH which induces the production of progesterone and its receptors, accountable for inducing the first meiotic division, indicated as germinal vesicle break down. (Ankley and Johnson 2004; Nagahama 1994).

It is now evident that exposure to EDCs has the potential to adversely affect reproductive physiology in vertebrates. One of the most well studied is BPA which was found able to interfere with steroidogenic and apoptotic processes in the ovary (Peretz et al. 2011; Ziv-Gal et al. 2013) but also to delay or inhibit ovulation (Lahnsteiner et al. 2005). The critical question now is if wildlife and human health is at risk from chronic exposure to low doses of this compound, either alone or in mixture.

Furthermore the ability of these compounds to permanently affect the epigenome could be potentially catastrophic to the welfare of future generations and requires further attention by both toxicologists and endocrinologists.

1.6.2. Lipid metabolism

Recent studies have revealed the involvement of environmental chemicals in obesity. This chemicals are defined as obesogens, substances that increase weight gain acting directly on fat cells, or indirectly, by altering the regulators system of appetite and satiety, the metabolic rate and the energy balance and thus favoring calories storage (Alonso-Magdalena et al. 2011; Grün and Blumberg 2009; Janesick and Blumberg 2011). Many known obesogens are endocrine disruptor compounds (EDCs) often used for the manufacturing of plastic materials and then presents in the majority of the object of daily use (North and Halden 2013; Schug et al. 2011). Liver is a key metabolic organ which governs body energy metabolism. The maintenance of energy expenditure involves the dynamic integration of two metabolically opposed states, fasting and feeding. Fasting involves the induction of catabolic, or ATP generating pathways, whereas feeding engages anabolic or ATP-consuming pathways, that build carbon skeletons and store energy. The integration of these states is governed by endocrine signals, such as insulin and glucagon, nutrient signals such as long chain fatty acids and branched chain amino acids, adipose and gut-derived polypeptide hormones, such as leptin, and nutrient-sensing kinases.

In the feeding phase, food is digested in the gastrointestinal tract, and glucose, fatty acids, and amino acids are absorbed into the bloodstream and transported to the liver through the portal vein circulation system. Insulin is released from pancreatic β cells and suppresses hepatic glucose production; glucose is condensed into glycogen and/or converted into fatty acids or amino acids in the liver. Other nonesterified fatty acids (NEFAs) originate from chylomicrons, which are produced by enterocytes with dietary fat, and arrive to the liver by circulation where they are released through lipolysis process. In hepatocytes, free fatty acids are esterified with glycerol 3-phosphate to generate triacylglycerol (TAG) or with cholesterol to produce cholesterol esters. TAG and cholesterol esters are either stored in lipid droplets within hepatocytes or secreted into the circulation as very low density lipoprotein (VLDL) particles which deliver lipids to extra hepatic tissues through the circulation.

In the fasted state or during exercise, adipose tissue produces and releases NEFAs and glycerol via lipolysis, whereas muscle breaks down glycogen and proteins and releases lactate and alanine. Alanine, lactate, and glycerol are delivered in the liver and used as precursors to synthesize glucose through gluconeogenesis.

NEFAs are oxidized in hepatic mitochondria through fatty acid β oxidation and generate ketone bodies (ketogenesis). The liver also begins to produce glucose through glycogenolysis. These fuel substrates (glucose, Ketone bodies) are released from the liver into the circulation system and are essential for the extrahepatic tissues, including red blood cells and the brain.

The liver's energy metabolism is tightly controlled. Transcriptional regulation of the genes involved in fatty acid metabolism is presently considered as the major long-term regulatory mechanism that controls the lipid homeostasis. It is executed by a variety of transcription factors among which the SREBPs, the C/EBPs, and members of the nuclear receptor family are particularly active agents. It is therefore important that different process such as fatty acid synthesis, storage, oxidation and mobilization are well balanced (Clarke 1993; Karanth et al. 2009; Lodhi and Semenkovich 2014; North and Halden 2013; Rogers et al. 2014).

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2. OBJECTIVES

The present thesis has the ambition of applying an interdisciplinary approach to gaining an understanding on the effect of EDCs on reproduction and lipid metabolism. Different groups of EDCs were investigated to address the following objectives:

1. Determine the epigenetic effects of BPA on female reproduction and embryo development.
2. Determine the EDCs effects in fish reproduction.
3. Determine the EDCs effects on fish metabolism.

To achieve these objectives this study has been divided into 5 chapters plus a review:

1. **BPA-induced deregulation of epigenetic patterns: effects on female zebrafish reproduction.**

   The aim of this work is to determine the effects of BPA on female reproductive physiology and investigate whether changes in the expression levels of genes related to reproduction are caused by histone modification.

2. **Epigenetic changes in F1 zebrafish embryo from mother exposed to BPA.**

   This study had the aim to investigate the effects of BPA treated females crossed with control males on the subsequent generation embryos. General and locus specific methylation plus genes involved in embryo and gonadal development were especially investigated to answer this question.

3. **Effects of diisononyl phthalate on *Danio rerio* reproduction.**

   The purpose of this study was to determine the effect of exposure to different concentration of DiNP, starting from low environmental concentration (420 ng/l) to higher levels on female zebrafish reproductive parameters, including number and size distribution of ovarian follicles, apoptosis and autophagy signals, spatially resolved biochemical information of vitellogenic oocytes using fourier transform infrared (FTIR) imaging, and transcript levels for genes involved in reproduction.

4. **Obesogenic effects of environmental pollutants: disruption of lipid metabolism in *Danio rerio.***

   The aim of the present study is to investigate the effects of two EDCs involved in plastic manufacturing as the BPA, which nowadays is one of the main plasticizer used worldwide, and the DGB, approved by European Chemical Agency as an alternative to phthalates in the processing of plastic.

5. **Xenobiotic-contaminated diets affect hepatic lipid metabolism: implications for liver in *Sparus aurata* juveniles.**

   The present study investigated the effects of feed contaminated with BPA, NP, or t-OP on the lipid metabolism of gilthead sea bream (*Sparus aurata*). Considering their broad diffusion and the high potential for human exposure, these EDCs may pose a concrete risk to human health.

6. **Effects of BPA on female reproductive function: the involvement of epigenetic mechanism.**

   The purpose of this review is to summarize the effects of BPA on adult female reproduction with focus on epigenetic changes that can be heritable.

3. BPA-INDUCED DEREGULATION OF EPIGENETIC PATTERNS: EFFECTS ON FEMALE ZEBRAFISH REPRODUCTION

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Abstract

Bisphenol A (BPA) is one of the commonest Endocrine Disruptor Compounds worldwide. It interferes with vertebrate reproduction, possibly by inducing deregulation of epigenetic mechanisms. To determine its effects on female reproductive physiology and investigate whether changes in the expression levels of genes related to reproduction are caused by histone modifications, BPA doses consistent with environmental exposure were administered to zebrafish for three weeks. Effects on oocyte growth and maturation, autophagy and apoptosis processes, histone modifications, and DNA methylation were assessed by Real-Time PCR (qPCR), histology, and chromatin immunoprecipitation combined with qPCR analysis (ChIP-qPCR). The results showed that 5 μg/L BPA down-regulated oocyte maturation-promoting signals, likely through changes in the chromatin structure mediated by histone modifications, and promoted apoptosis in mature follicles. These data indicate that the negative effects of BPA on the female reproductive system may be due to its upstream ability to deregulate epigenetic mechanism.

1. Introduction

Endocrine Disruptor Compounds (EDCs) are chemicals that can interfere with vertebrate reproduction (Patisaul and Adewale, 2009). The vertebrate reproductive system is under the control of the hypothalamic-pituitary-gonadal axis, which operates through a hormonal cascade where production of gonadotropin-releasing hormone (GnRH) in the hypothalamus stimulates pituitary gonadotropin synthesis, eventually leading to production of steroid hormones by the gonads.

Bisphenol A (BPA) is commonly used in the manufacturing of polycarbonate plastics and epoxy resins, making it one of the most widespread EDCs worldwide. According to recent estimates, 100 ton of BPA per year may end up in the environment (Rubin, 2011) due to landfill or plastic degradation (Kang et al., 2006), resulting in concentrations of 5 μg/L to 21 μg/L in environmental water (Crain et al., 2007).

BPA alters gene expression by binding to nuclear estrogen receptors (ERs) ERα and ERβ. It is defined as a selective ER modulator because its effects are pro-estrogenic in some tissues and anti-estrogenic in others (Kundakovic and Champagne, 2011; Wetherill et al., 2007). Its ability to bind to membrane receptors makes it harmful even at pico- and nanomolar concentrations (Wetherill et al., 2007).

As an environmental pollutant, BPA is probably able to affect epigenetic mechanisms through methylation of CpG sites (Dolinoy et al., 2007). It also seems to induce histone modification, altering the chromatin structure and through it transcription activation and repression (Kundakovic and Champagne, 2011).

The first goal of the present study was to determine the effects of BPA concentrations consistent with environmental exposure (5, 10, and 20 µg/L) on reproductive physiology in female vertebrates. To do this, the expression of several genes critical for reproduction was monitored in the ovary: ERs *esr1* and *esr2a*, steroidogenic acute regulatory protein (*star*), and a member of the cytochrome P450 family 11 (*cyp11a1*)(Arukwe, 2008; Cotter et al., 2013; King et al., 2008; Palter et al., 2001) for follicle development and steroidogenesis; progesterone receptors *pgrmc1* and *pgrmc2* for oocyte growth and maturation; and bone morphogenetic protein 15 (*bmp15*) and growth differentiation factor 9 (*gdf9*) as local signals(Nagahama, 1994). Apoptosis, autophagy, and oocyte viability were also assessed.

The second goal of the study was to explore whether the changes in the expression levels of these genes were caused by histone modifications, as measured by lysine 4 and 27 trimethylation in the amino terminal of histone 3 (respectively H3K4me3 and H3K27me3). H3K4me3 is involved in the activation of transcription (Kouzarides, 2007), since it marks the Transcription Start Site (TSS) region, whereas H3K27me3 is chiefly associated with gene silencing (Lindeman et al., 2010). Based on the recent hypothesis of a possible interplay between histone modifications and DNA methylation patterns, where DNA methyltransferases (DNMTs) seem to play an important role (Cedar and Bergman, 2009), DNMT gene expression levels were also investigated.

The experimental model selected for the study, *Danio rerio,* has a fully sequenced genome and shares with humans 70% gene orthology and body plan similarity (Howe et al., 2013); these features make it an ideal model to assess the effects of EDCs on human reproduction. The issue is both urgent and topical, since several studies have reported a decline in human fertility, especially in western countries (Brannian and Hansen, 2006; Nyboe Andersen and Erb, 2006). Even though the decrease may be related to a variety of factors, a role for environmental EDCs is strongly suspected (Patisaul and Adewale, 2009).

2. Methods

2.1. Animals and BPA administration

A total of 48 adult female zebrafish (*D. rerio*, AB wild-type strain) were placed in eight 10-L aquaria (6 fish/tank) with oxygenated water under controlled conditions (28.0 ± 0.5 ˚C) and maintained on a 14/10 h light/dark cycle. They were fed 4 times a day, twice commercial food (Vipagran; Sera, Loessnitz, Germany) and twice *Artemia salina*. There were a control and 3 exposed groups, which received 5, 10, or 20 µg/L BPA (98% analytical purity, Sigma-Aldrich, Milano, Italy) for 3 weeks. All tanks were maintained in duplicate. After 3 weeks, fish were lethally anesthetized with 500 mg/l MS-222 (3-aminobenzoic acid ethyl ester, Sigma Aldrich) buffered to pH 7.4. Five half ovaries from each experimental group, in duplicate, were removed and fixed in Bouin's solution; the remaining ovaries were used for Real Time semi-quantitative Polymerase Chain Reaction (qPCR) (5 half ovaries) and Chromatin ImmunoPrecipitation (ChIP) analysis (7 whole ovaries). All procedures complied with Italian animal experimentation laws and were approved by the Ethics Committee of Padua University (Prot. 112/2015-PR).

2.2. Egg collection and fertility

Starting on the 8th day of treatment, the 3 groups of BPA-exposed females and control females were crossed with untreated males, and fertility was determined during the following 15 days. Fertilized eggs were counted and the fertility rate was calculated as the mean ± standard deviation (SD) of fertilized egg number / female / day from the 8th to the 21th day of treatment.

2.3. RNA extraction and cDNA synthesis

Total RNA was isolated from the ovary with RNAzol solution (Sigma Aldrich) according to the manufacturer's instructions. Its final concentration was determined using Nanophotometer TM P-Class (Implen GmbH, Mϋnich, Germany), whereas integrity was established by Gel Red staining of 28S and 18S ribosomal RNA fragments on 1% agarose gel.

Total RNA was treated with DNAse to remove genomic DNA (10 IU at 37 ˚C for 10 min; Fermentas MBI, Amherst, NY, USA). Then 1 µg total RNA was used for cDNAs synthesis with iScript cDNA Synthesis Kit (Bio-Rad, Milano, Italy), and kept at -20 °C until use.

2.4. Real-Time qPCR

Relative quantification of gene expression was performed with the SYBR Green method in an iQ5 Multicolor Real-Time PCR Detection system (Bio Rad). All samples were analyzed in duplicate. Reactions contained 1 µl cDNA diluted 1/10, 5 µl 2X SYBR Green PCR Master Mix (Bio Rad) containing SYBR Green as a fluorescent intercalating agent, 0.1 µM of forward and reverse primers (ED Table 1), and 3.8 µl of milliQ water. The thermal profile was as follows: enzyme activation at 95 ˚C for 3 min; 45 cycles of denaturation (10 sec at 95 ˚C) followed by 20 sec annealing at 60 ˚C for *star*, *dnmt6*, *esr2a*, *gdf9*, *ambra1a*, *beclin1*, *tp53*, *caspase3*, and *cyp11a1*, 59 ˚C for *dnmt1*, *dnmt8*, *dnmt3*, *dnmt5*, *fshr*, *pgrmc1*, *pgrmc2*, *bmp15*, and 58˚C for *dnmt4*, *dnmt7*, *esr1*, *esr2b*, and *lhcgr*, and 20 sec elongation at 72 ˚C. Fluorescence was monitored at the end of each cycle. Dissociation curves for primer specificity and absence of primer-dimer formation check were performed and consistently showed a single peak.

Genes *18S*rRNA (Tang et al., 2007) and *arp (*acid ribosomal protein) (Aursnes et al., 2011) were used as internal controls to enable result standardization by eliminating variations in mRNA and cDNA quantity and quality (Bustin et al., 2009). These genes were chosen because their mRNA levels did not vary either between experimental treatments or between follicular stages. No amplification product was observed in the negative control (absence of template). Data were analyzed using iQ5 Optical System version 2.1 (Bio-Rad). The quantification method was based on a ΔΔCt calculation implemented with the Pfaffl equation, to improve accuracy by accounting for varied reaction efficiencies depending on primers (Pfaffl, 2001; Vandesompele et al., 2002). All results are expressed with respect to control ovaries. Data were generated in duplicate from 5 biological replicates.

2.5. Histology

Five ovaries per fish group were fixed in Bouin's solution and prepared for histological examination using standard biological procedures. Gonads were embedded in paraffin and sectioned (7 µm) with a microtome.

Each ovary was fully sectioned, processed for hematoxylin-eosin staining, and observed at 200x final magnification under a light microscope (Leica Microsystems Inc., Milano, Italy). Atretic follicles were identified based on specific morphological markers of follicular atresia (i.e. granulosa cell hyperplasia, invagination and breakdowns of zona radiata, basal membrane disintegration, absorption of vitellus) as described by Üçüncü and Çakıcı (2009) (Üçüncü and Çakıcı, 2009).

Images (50x final magnification) were captured using a high-resolution digital camera (DC300F; Leica Microsystems) and atretic follicle number was counted and expressed as a percentage of the number of atretic/total follicles.

Student's t-test and ANOVA followed by Duncan's test for multi-group comparisons were performed, as appropriate, to assess the significance of differences. Data were expressed as percentages and reported as mean values ± SD.

2.6. Chromatin immunoprecipitation and antibodies

Chromatin was prepared from frozen ovaries following the instructions of the ChIP-IT High Sensitive Kit (Active Motif catalog no. 53040). Chromatin was fragmented by sonication with Active Motif's EpiShearSonicator (80% amplitude, pulse for 30 sec on and 30 sec off for a total sonication "on" time of 12 min –or 22 min of elapsed time) to produce fragments ranging from 200 to 600 bp. Approximately 200 µl of chromatin was used for each immunoprecipitation reaction. Then, 50 µl was removed from each sample and used as input control. ChIP was performed using antibodies specific for H3K4me3 (Abcam) and H27K4me3 (diagenode).

2.7. ChIP-qPCR

ChIP-qPCR was performed with real time PCR using the SYBR Green method. The reaction consisted in 5 µl SYBR Green Reaction Mix, 1 µl 0.1 µM primer pairs, 3 µl sterile water, and 1 µl DNA sample (ChIP or Input), for a total volume of 10 µl. Gene mapping and information on regions of interest were obtained using the UCSC Genome Browser. Primers were designed to target the TSS, the two flanking regions, and the gene body region using Pick Primers from the NCBI website, and were tested by *in silico* PCRs on the UCSC Genome Bioinformatics website. Primer sequences are reported in ED Table 2. For each pair, primer efficiency was

tested on zebrafish genomic DNA by means of a standard qPCR curve using serial dilutions of gDNA (from 4 ng/µl to 0.004 ng/µl). Each dilution was assayed in triplicate.

qPCR analyses were performed in a 7900 HT Fast Real-Time PCR System (Applied Biosystems) using technical triplicates. The thermal protocol was as follows: initial 10-min denaturation at 95 ˚C, followed by 40 cycles of 95 ˚C for 15 sec; 60 ˚C for 30 sec; and 72 ˚C for 30 sec. ChIP-qPCR signals were calculated as percentage of input and SD was measured and represented as error bars. Data were obtained from 7 biological replicates.

2.8. Statistical analysis

Statistical analysis of gene expression was performed with one-way ANOVA followed by Tukey's multiple comparison test. Significance was set at $p < 0.05$. All procedures were performed with GraphPad Prism 6. Numerator and denominator degrees of freedom are respectively 3 and 17. F and P values were also calculated for each gene analyzed and are reported in the legends.

3. Results

3.1. Fertility

Fertility was measured as mean number ± standard deviation (SD) of fertilized eggs per female per day from the 8th to the 21st day of BPA treatment. The results showed blocked ovulation (0 ± 0 eggs) 8 days into treatment in fish receiving the lowest BPA dose (5 µg/L), whereas the intermediate (10 µg/L) and the highest (20 µg/L) dose did not induce significant changes (respectively 314 ± 8 and 330 ± 9 eggs) compared with control fish (333 ± 12 eggs).

3.2. Molecular analysis of the genes related to oogenesis

The lowest of the 3 concentrations tested, 5 µg/L, induced the most severe effects. Molecular analysis demonstrated that BPA can inhibit reproduction, particularly by affecting oocyte maturation. The lowest concentration induced significant downregulation of *esr1* and *esr2a* (Fig. 1C,D), whereas it did not significantly affect the transcription pattern of the other genes involved in steroidogenesis and oocyte growth, *esr2b*, *star*, *cyp11a1*, and follicle stimulating hormone receptor (*fshr*) (Fig. 1A,B,E,F). It also significantly

reduced the transcripts of genes related to oocyte maturation and germinal vesicle breakdown processes (i.e. luteinizing hormone/choriogonadotropin receptor, *lhcgr,* and *pgrmc1*) (Fig. 2A,B), whereas there was no change in *pgrmc2* expression (Fig. 2C).

The 10 μg/L concentration significantly up-regulated the *cyp11a1* gene (Fig. 1B) and significantly down-regulated *esr2a* and *lhcgr* (Fig. 1D, 2A), whereas the remaining genes were not affected. The highest BPA concentration, 20 μg/L, positively and significantly interfered with the expression of *esr2b*, *star*, and *fshr*, the genes involved in steroidogenesis and oocyte growth (Fig. 1A,E,F), and of *lhcgr*, which has a role in oocyte maturation (Fig. 2A). Such changes were associated with a complete block of reproduction 8 days into treatment with the lowest BPA concentration, whereas no changes in fecundity were seen in the other groups.
Importantly, none of the BPA concentrations tested affected the expression of *bmp15* and *gdf9* (Table 3), which are considered as local signals.

The expression trends of autophagy/beclin-1 regulator 1a (*ambra1a1*) and *beclin1* (Table 3), two key signals involved in the autophagy process, were not affected. In contrast, apoptosis gene expression exhibited a differential pattern, where the effector *caspase3* (Table 3) was up-regulated by all 3 concentrations, while tumor protein 53 (*tp53*) (Table 3) was up-regulated only by the lowest.

3.3. Histological analysis

Oocyte viability was assessed by histology, to document the effects of 5 μg/L BPA on the ovary.
Findings showed that total follicle number did not change in any experimental group, and that pre-vitellogenic follicles grew normally and developed into vitellogenic and mature ones. However, nearly all exposed mature follicles exhibited morphological evidence of atresia, including membrane disintegration, follicle cell proliferation, zona radiata breakdown, and yolk resorption (Fig. 3).

3.4. ChIP-qPCR analysis

To establish whether the gene expression alterations caused by exposure to 5 μg/L BPA could be caused by epigenetic changes, H3K4me3 and H3K27me3 enrichment was assessed in the TSS region, in its two flanking

regions, and in other gene body regions by evaluating *star*, *fshr* and *lhcgr*, which are related to steroidogenesis, oocyte growth, and oocyte maturation.

H3K4me3 enrichment was reduced in the TSS and the *star*_4 region (Fig. 4A) of BPA-treated ovaries compared with control ovaries, whereas it was unchanged in the remaining regions (Fig. 4A). Similarly, H3K4me3 enrichment in the *fshr* TSS region was reduced in BPA-exposed compared with control ovaries and was unaffected in the other regions (Fig. 4B). Notably, the H3K27me3 epigenetic marks for *star* and *fshr* were weaker in treated than in control ovaries at all sites (Fig. 4A,B), reflecting similar trends in H3K4me3 and H3K27me3 histone modifications. As regards oocyte maturation, the H3K4me3 mark appeared to be fainter in the *lhcgr* TSS region of treated compared with control ovaries, whereas no changes were detected in the other regions (Fig. 4C). In contrast, increased H3K27me3 enrichment was documented in the TSS gene of treated compared with control fish (Fig. 4C). In addition, reduced and similar levels of H3K27me3 enrichment were found in the flanking regions and in the remaining regions of the TSS, respectively (Fig. 4C).

3.5. Molecular analysis of DNMTs

The expression of the DNMT isoforms involved in methylation maintenance, *dnmt1*, and de novo methylation, *dnmt3*, was significantly up-regulated in BPA-exposed compared with control ovaries (Table 4), whereas a reduction in the relative abundance of *dnmt4*, *dnmt6* and *dnmt7* transcripts was detected in treated ovaries (Table 4). The expression trend of *dnmt5* and *dnmt8* did not show significant differences between treated and control ovaries (Table 4).

4. Discussion

This study examined whether BPA concentrations similar to those found in the environment affect female reproduction by altering endocrine signaling and/or local factors at the level of the ovary, and whether the well-established harmful effects of BPA are exerted via deregulation of epigenetic mechanisms. These working hypotheses are based on a study (Wetherill et al., 2007) suggesting that even pico- and nanomolar BPA concentrations interact with the ERα or β isoforms, leading to gene expression alteration. Matsushima and colleagues found that the effect of such small concentrations relies on strong binding with estrogen-related receptor γ (ERR γ), and showed for the first time that this nuclear receptor forms a complex with BPA

(Matsushima et al., 2007). Moreover, BPA administration to pregnant Agouti viable yellow mouse (A^{vy}) induced DNA hypomethylation in the epigenome of the offspring, demonstrating a role for it in epigenetic changes (Dolinoy et al., 2007).

The relationship between histone modifications and BPA has been explored only by Doherty and co-workers (Doherty et al., 2010), who documented the ability of BPA to promote histone methyltransferase EZH2 (Enhancer of Zeste Homolog 2) protein translation in mouse mammary gland exposed to 5 mg/kg BPA. Changes in EZH2 protein synthesis are usually associated with an increase in H3K27me3, which typically leads to gene repression (Viré et al., 2006; Zhu et al., 2009). Kundakovic and Champagne (Kundakovic and Champagne, 2011) have suggested that BPA-induced gene expression, which depends on the chromatin structure, could be up- or down-regulated both by DNA methylation and histone modifications occurring in specific gene regulatory regions.

To shed some light on the issue, this study adopted a multidisciplinary approach including gene expression analysis and monitoring of histone modifications. The expression of some key genes involved in different phases of oogenesis was analyzed to elucidate the effect of BPA on the process. In particular, *star* and *cyp11a1* were selected as biomarkers of steroidogenesis. The former is involved in cholesterol transfer through the mitochondrial membrane (Arukwe, 2008) and the latter, a member of the cytochrome P450 family, in the conversion of cholesterol to pregnenolone (Quek and Chan, 2009). Indeed, given its role in early oogenesis, *cyp11a1* has proved to be a valuable biomarker of the effects of EDCs on fish (Arukwe, 2008).

The three ER isoforms found in zebrafish ovary (*esr1*, *esr2a*, and *esr2b*) were investigated for their important role in follicle development, given the ability of EDCs to interact with genes presenting the estrogen response element (ERE) region (Wetherill et al., 2007), while *fshr* was included because it is part of the hormonal cascade, which comprises the synthesis of 17β-estradiol in the gonads and of vitellogenin in the liver, that ultimately leads to oocyte growth (Kohli et al., 2005).

The signals responsible for oocyte maturation were also examined for BPA-induced effects. The most important are *lhcgr*, which is stimulated by luteinizing hormone and induces production of progesterone and its receptors (*pgrmc1* and *pgrmc2*), and *pgrmc1* and *pgrmc2*, which induce the first meiotic division, or germinal vesicle breakdown.

Even though oogenesis is mainly controlled by pituitary gonadotropins and their receptors, local factors such as *bmp15* and *gdf9* also play a large role, since they inhibit the early maturation of oocytes (Danforth, 1995; Ge, 2005; Hillier, 2001).

Finally, the demonstration by our group (Gioacchini et al., 2012) that autophagy - unlike apoptosis - increases energy recycling efficiency, exerting a favorable influence on zebrafish reproduction, prompted the investigation of two biomarkers for each of these processes.

The results showed higher mRNA levels of *caspase3* and *tp53*, two markers of apoptosis, in the group exposed to 5 µg/L BPA, and a lack of effect on the autophagy process, suggesting inhibition of reproduction. The other findings suggest that the lowest BPA dose was the most harmful, since it significantly down-regulated *esr1*, *esr2a*, *lhcgr*, and *pgrmc1*, but not *esr2b*, *star*, *cyp11a1*, and *fshr* expression.

Therefore, since the signals related to steroidogenesis and oocyte growth were not affected, it may be speculated that BPA damages oocyte maturation even at a concentration as low as 5 µg/L. These data agree both with the histology findings, which documented a greater incidence of atresia in mature oocytes from fish treated with 5 µg/L BPA compared with controls, and with the block of reproduction seen only in fish exposed to this concentration.

The results of ChIP-qPCR in zebrafish exposed to 5 µg/L BPA are also in line with the findings of molecular analyses, since enrichment of H3K4me3 and H3K27me3 histone modifications in the TSS region of the *star* and *fshr* genes decreased in the exposed groups. Their unaltered gene expression pattern may be explained by a balancing out of reduced levels of both promoter and repressor, resulting in unchanged gene transcription.

In contrast, *lhcgr,* which was down-regulated by the lowest BPA concentration, showed a different modulation of the epigenetic pattern, with a decrease and an increase, respectively, in H3K4me3 and H3K27me3 marks in its TSS region. Histone 3 lysine 4 trimethylation has been reported to promote gene transcription and specifically marks the TSS region, whereas the increment of histone 3 lysine 27 trimethylation in the promoter region often leads to repression of gene expression (Bannister and Kouzarides, 2011; Lindeman et al., 2011). Therefore, *lhcrg* downregulation in fish treated with the low BPA concentration is due to an increase of H3K27me3 in the TSS region, demonstrating the adverse effect of BPA on the epigenome.

DNMTs have recently been suggested to mediate the interplay between histone modifications and DNA methylation (Cedar and Bergman, 2009; Vaissière et al., 2008). However, both processes should thoroughly

be examined to gain insights into the toxic effects of pollutants on the epigenome (Kamstra et al., 2014). We therefore measured the gene expression levels of the zebrafish DNMT isoforms identified by Kamstra and colleagues (Kamstra et al., 2014) in fish exposed to the low BPA concentration. For reasons that are still unclear, zebrafish have a large number of DNMTs (*dnmt1* to *8*). It has been suggested that each has a specific function - *dnmt1* would maintain methylation while *dnmt3-8*, especially *dnmt3* and *dnmt8*, would be involved in de novo methylation and share similarities with mammalian DNMT3 (Kamstra et al., 2014).
Moreover, a relationship has been suggested between human disease and the deregulation of epigenetic mechanisms (Egger et al., 2004; Feinberg et al., 2006; Jones and Baylin, 2002).
The above considerations suggest that upregulation of *dnmt1* and *dnmt3* in fish exposed to 5 µg/L BPA could be related to the histone modifications, further supporting the notion of BPA-induced epigenetic deregulation.
In conclusion, the lowest BPA concentration consistent with environmental exposure proved to be the most harmful, affecting *D. rerio* oocyte growth and maturation, and down-regulating the signals involved in the last phase of oogenesis.

These findings agree with previous data highlighting that EDCs do not follow the principle “the dose makes the poison”, but rather exhibit a U-shaped and inverted U-shaped, non-monotonic, dose response curve, where the strongest responses may be elicited by the lowest and highest doses, or by intermediate concentrations, respectively (Vandenberg et al., 2009). BPA may thus act in a U-shaped fashion, with low doses inducing the strongest response. The hypothesis is supported by several studies, which have suggested that such curve shapes may depend on receptor downregulation induced by higher hormone levels (Medlock et al., 1991; Tibbetts et al., 1998; Vandenberg et al., 2012).

Even though the present data are preliminary, and further work is needed, particularly to gain additional information on the methylation pattern of the genes involved in reproduction, these findings strongly suggest that the adverse effects of BPA on reproduction are due to its upstream ability to deregulate epigenetic mechanisms. Given the high synteny between the zebrafish and human genome, this evidence may be a starting point to explore the role of EDCs in the decline of human fertility (Brannian and Hansen, 2006; Nyboe Andersen and Erb, 2006), and to shed light on the BPA-induced epigenetic changes affecting human ovary.

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Author contributions

S.S. and O.C. conceived and designed the experiments; S.S., F.M., G.C. and C.C.P. performed the experiments; S.S., F.M., G.G., G.C., C.C.P., L.D.V., and O.C. analyzed the data; G.C., L.D.V., and O.C. contributed reagents / materials / analysis tools; S.S. and O.C. wrote the paper.

Competing financial interests

The authors declare no competing financial interests.

Table 1. Lists of primers used in gene expression analyses by Real-Time qPCR.

Gene	Forward primer (5'- 3')	Reverse primer (5'- 3')	Acc number	Gene ID
18S rRNA	TCGGAAAACGGTGAACCTG	AAGGTCTTTGAACCCACGG	NM_001098396.1	100037361
arp	CTGAACATCTCGCCCTTCTC	TAGCCGATCTGCAGACACAC	NM131580.2	58101
star	CCAAGTGCAGATGACCCCAA	GGAAGGTGTGTGCCCTTGTT	NM_131663.1	63999
cyp11a1	GCAGGATTGCCGAGACTGA	TCTGCTGGCATTCAGTGGT	AF527755.1	80374
esr1	GGTCCAGTGTGGTGTCCTCT	AGAAAGCTTTGCATCCCTCA	NM_152959.1	259252
esr2a	TAGTGGGACTTGGACCGAAC	TTCACACGACCACACTCCAT	NM_180966.2	317734
esr2b	TTGTGTTCTCCAGCATGAGC	CCACATATGGGGAAGGAATG	NM_174862.3	317733
fshr	GATTCTTCACCGTCTTCTCC	TGTAGCTGCTCAACTCAAACA	NM_001001812.1	195820
lhcgr	GGCGAAGGCTAGATGGCACAT	TCGCAATCTGGTTCATCAATA	NM_205625.1	402920
pgrmc1	CGGTTGTGATGGAGCAGATT	AGTAGCGCCAGTTCTGGTCA	NM_001007392.1	492520
pgrmc2	ACAACGAGCTGCTGAATGTG	ATGGGCCAGTTCAGAGTGAG	NM_213104.1	406378
bmp15	AGGGTGACCGGATCACTATG	TGCTGCCAGACTTTTTAGACC	NM_001020484.1	334183
gdf9	CGACCACAACCACCTCTCTCC	GGGACTGAGTGCTGGTGGATGCC	NM_001012383.1	497643
caspase3	GTGCCAGTCAACAAACAAAG	CATCTCCAACCGCTTAACG	NM_131877.3	140621
ttp53	GGCTCTTGCTGGGACATCAT	TGGATGGCTGAGGCTGTTCT	AF365873.1	30590
beclin1	GGACCACTTGGAACAACT	CCGAAGTTCTTCAGTGTCCATC	AB266448.1	393846
ambra1a	TCTTTCGAGAAATGGCACCT	CTCTCTGCGTTAGGGACAGG	HE602022.1	100332642
dnmt1	GAGCCTGTGAAGCAGGAGAA	CATGAATGGCACTGCACAGA	NM_131189.2	30430
dnmt3	AAACAACGCGCTTCCACG	TTCCATAACCACCACCGTCC	AF135438.1	30659
dnmt4	GCGTCAGAAGTATGCGAGGA	GACCTTTCCTAGCAGGGTTGA	AB196915.1	317744
dnmt5	GCTCCATCACATCTCAGCCC	CAAATCCGACACCGGCAAAG	XM_009296722.1	323723
dnmt6	AGAAAACCCATTCGCGTCCT	GTGCCCTCGTAGAGACCTTTT	AB196917.1	553189
dnmt7	ATCCGACATCTCTTTGCACC	GTGAAGTGAATTTGCAGAAAGC	AB196918.1	321084
dnmt8	GGACGTATTGTGTCCTGCT	ATCACCAAACCACATGACCC	AB196919.1	553187

Table 1. *arp*: acidic ribosomal protein; *star*: steroidogenic acute regulatory protein; *cyp11a1*: cytochrome P450, family 11, subfamily A; *esr1*: estrogen receptor 1; *esr2a*: estrogen receptor 2a; *esr2b*: estrogen receptor 2b; *fshr*: follicle stimulating hormone receptor; *lhcgr*: luteinizing hormone/choriogonadotropin receptor; *pgrmc1*: progesterone membrane receptor component 1; *pgrmc2*: progesterone membrane receptor component 2; *bmp15*: bone morphogenetic protein 15; *gdf9*: growth differentiation factor 9; *tp53*: tumor protein 53; *ambra1a*: autophagy/beclin-1 regulator 1a; *dnmt1* to *8*: DNA (cytosine-5-)-methyltransferases.

Table 2. Sequences, location and product size of primers used in Chip-qPCR analysis.

Oligo name	Sequence 5' to 3'	PCR product location	Product size (bp)
fshr_1	For: AACACCGAAGACACACTTGC Rev: CTTGTTGCCCAGACAGATGA	chr12: 26245412+26245492	81
fshr_2	For: GACACAGGAGAGTGGGTTTT Rev: ACCTGCTTGAGAAACCAGTG	chr12: 26249492+26249616	125
fshr_3	For: AATGCAGAACCATACTGACCG Rev: CTTGGACAGGCTTCTGAACT	chr12: 26264368+26264451	84
fshr_4	For: AGCCTGATTTGACTGGGTTG Rev: TGTAATGACAGCAGCCGTTT	chr12: 26272745+26272876	132
fshr_5	For: CCCTACACATAAACCGCAAGA Rev: AACATAGCCAACTGAACCCA	chr12: 26242191+26242307	117
lhcgr_1	For: CTTTCTGCTGTGGAGTGTGT Rev: TACAGTCTGCTGAGGCTCTT	chr13: 48032912+48033020	109
lhcgr_2	For: CCTTCAATAACCTGCCGAAC Rev: TTTTGTTCCAGTCTGCGGTA	chr13: 48053608+48053757	150
lhcgr_3	For: TTCAGCCTGTCTGCAATCTC Rev: GTCGATGCTACCGAAAGTGT	chr13:48076229+48076318	90
lhcgr_4	For: TCGCATGCTTATCAATGGCA Rev: TCATCGCCTCTGAAGAAACC	chr13: 48030973+48031116	144
lhcgr_5	For: ACGTGTTTACGGCCATTAGA Rev: TCCAGAGATTTGGGTGTGTG	chr13: 48037498+48037628	131
star_1	For: TCTGTAAGAAATGCCAGCACA Rev: AGGGCTATAAAGGGGCTGAA	chr8: 46837041+46837190	150
star_2	For: GTGTTCACCAGGGTCACAAT Rev: ACCAGTGCTATGTGCAACAA	chr8: 46835252+46835396	145
star_3	For: GCGTCTGCCAAATGCATAAA Rev: CCTGTATTGTCATGCGACCA	chr8: 46833862+46833973	112
star_4	For: CTCCTTCTTCGGATGTGGTT Rev: ATGATTGCCATCCACCATGA	chr8: 46836840+46836919	80
star_5	For: ACAGTGTCAGGCCATACCATA Rev: CAGTGTTGGGCTGATTACGTT	chr8: 46837645+46837779	135

Table 2. *fshr*: follicle stimulating hormone receptor; *lhcgr*: luteinizing hormone/choriogonadotropin receptor; *star*: steroidogenic acute regulatory protein.

Table 3. Analysis of local factors and genes involved in apoptosis and autophagy.

Gene	Ctrl	BPA 5 µg/L	BPA 10 µg/L	BPA 20 µg/L
bmp15	2.56 ± 0.25^{a}	2.51 ± 0.94^{a}	2.29 ± 0.42^{a}	2.01 ± 0.65^{a}
gdf9	2.33 ± 0.33^{a}	2.50 ± 0.95^{a}	2.77 ± 0.44^{a}	3.31 ± 0.39^{a}
caspase3	1.65 ± 0.45^{a}	3.54 ± 0.25^{b}	3.03 ± 0.47^{b}	3.61 ± 0.09^{b}
tp53	2.40 ± 0.45^{a}	3.73 ± 0.61^{b}	2.43 ± 1^{a}	$2.93 \pm 0.27^{a,b}$
ambra1a	3.23 ± 0.54^{a}	2.41 ± 0.75^{a}	2.34 ± 0.81^{a}	2.22 ± 0.54^{a}
beclin1	1.43 ± 0.15^{a}	1.83 ± 0.56^{a}	1.9 ± 0.38^{a}	1.6 ± 0.37^{a}

Table 3. *bmp15*: bone morphogenetic protein 15 (F = 0.7821; P = 0.5222); *gdf9*: growth differentiation factor 9 (F = 2.513; P = 0.0931); *caspase3* (F = 35.65; P = 0.0001); *tp53*: tumor protein 53 (F = 5.594; P = 0.0081); *ambra1a*: autophagy/beclin-1 regulator 1a (F = 2.336; P = 0.1101); *beclin1* (F = 1.460; P = 0.2607). Data are expressed as mean ± standard deviation; letters indicate differences between treatments ($p < 0.05$ compared with untreated controls; ANOVA followed by Tukey's multiple comparison tests). Data were generated in duplicate from five biological replicates.

Table 4. Transcriptional profiles of DNA (cytosine-5-)-methyltransferase genes.

Gene	Ctrl	BPA 5µg/L
dnmt1	1.69±0.84	19.14±0.9*
dnmt3	2.12±1.04	5.41±0.64*
dnmt4	3.86±1.06	1.58±0.55*
dnmt5	2.47±1	2.82±0.66
dnmt6	2.61±0.33	1.64±0.32*
dnmt7	5.51±0.9	2.16±1.15*
dnmt8	2.16±0.63	2.18±0.51

Table 4. *dnmt1 to 8*: DNA (cytosine-5-)-methyltransferase genes 1 to 8. Data are expressed as mean±standard deviation*$p < 0.05$ compared with untreated controls. Data were generated in duplicate from five biological replicates.

Figure Legends

Fig. 1: Transcription profiles of genes involved in steroidogenesis and oocyte growth. Letters above each column indicate statistical differences among groups ($p < 0.05$ vs. untreated controls; ANOVA followed by Tukey's multiple comparison test). A: *star*: steroidogenic acute regulatory protein (F = 6.710; P = 0.0049); B: *cyp11a*: cytochrome P450, family 11, subfamily (F = 4.608; P = 0.0178); C: *esr1*: estrogen receptor 1 (F = 5.192; P = 0.0099); D: *esr2a*: estrogen receptor 2a (F = 5.350; P = 0.0115); E: *esr2b*: estrogen receptor 2b (F = 5.890; P = 0.0066); F: *fshr*: follicle stimulating hormone receptor (F = 4.586; P = 0.0257). Data were generated in duplicate from five biological replicates.

Fig.2: Transcription profiles of genes involved in oocyte maturation and germinal vesicle breakdown. Letters above each column indicate statistical differences among groups ($p < 0.05$ vs. untreated controls; ANOVA followed by Tukey's multiple comparison test). A: *lhcgr*: luteinizing hormone/choriogonadotropin receptor (F = 29.18; P = 0.0001); B: *pgrmc1*: progesterone membrane receptor component 1 (F = 3.382; P = 0.0462); C: *pgrmc2*: progesterone membrane receptor component 2 (F = 0.1755; P = 0.9114). Data were generated in duplicate from five biological replicates.

Fig. 3: Histological analysis of ovaries from control fish (A) and fish exposed (B) fish to 5 µg/L BPA. Ovarian sections show different follicular stages and atretic follicles. (C) A follicle with the morphological markers of atresia: ZR*: Zona radiata breakdown; YR*: Yolk resorption; FC*: Follicular cell proliferation. (D) Percentage of atretic follicles in ovary from control fish (CTRL) and fish exposed (BPA) to 5 µg/L BPA.

Fig. 4: ChIP-qPCR analysis of H3K4me3 and H3K27me3 trimethylation of lysine 4 (K4) and 27 (K27) in the amino terminal of histone 3 (H3) enrichment. A**:** ChIP-qPCR results for *star* (steroidogenic acute regulatory protein) gene associated with H3K4me3 and H3K27me. B**:** ChIP-qPCR results for *fshr* (follicle stimulating hormone receptor) gene associated with H3K4me3 and H3K27me. C**:** ChIP-qPCR results for *lhcgr* (luteinizing hormone/choriogonadotropin receptor) gene associated with H3K4me3 and H3K27me.

Fig.1

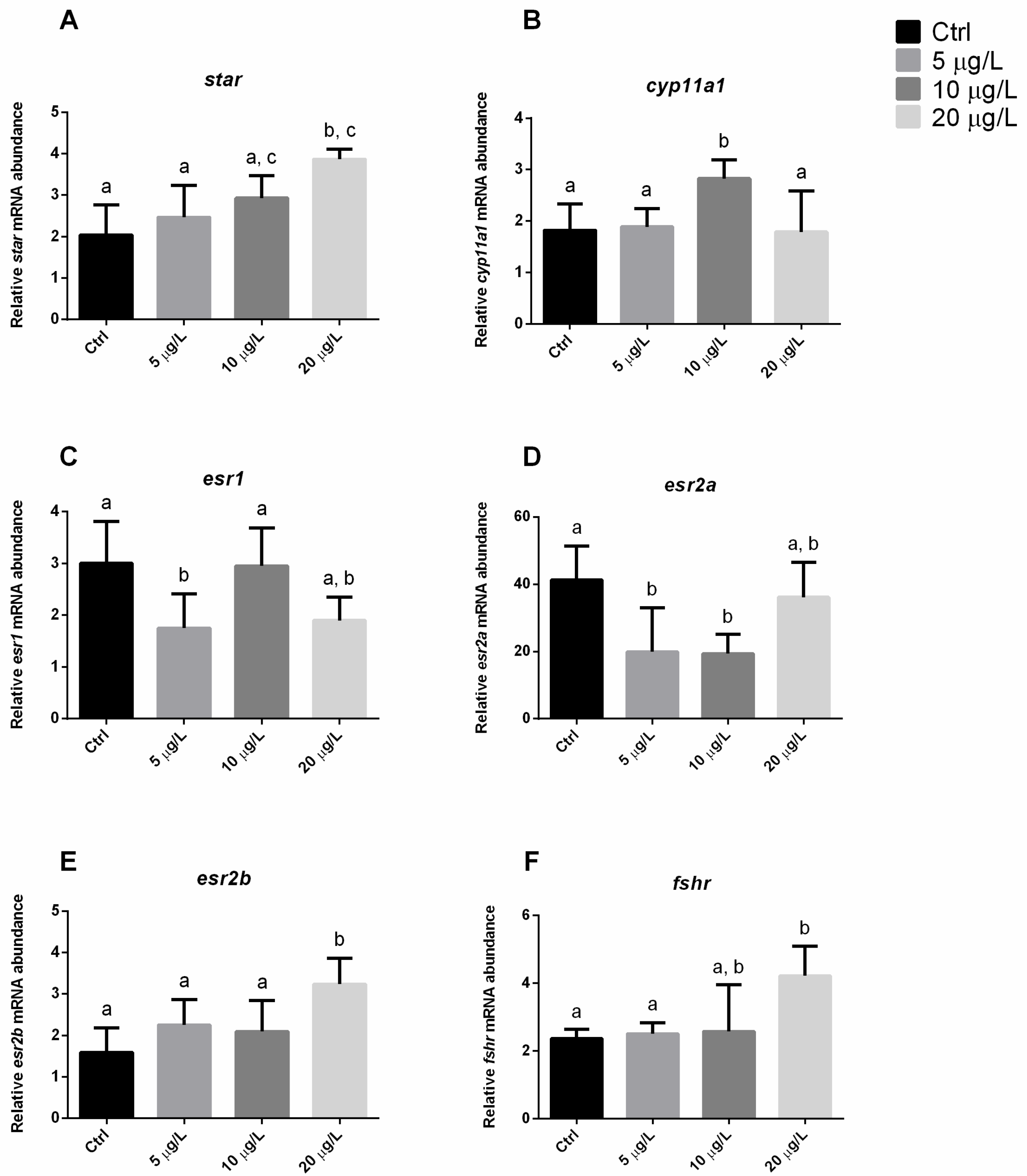


Fig.2

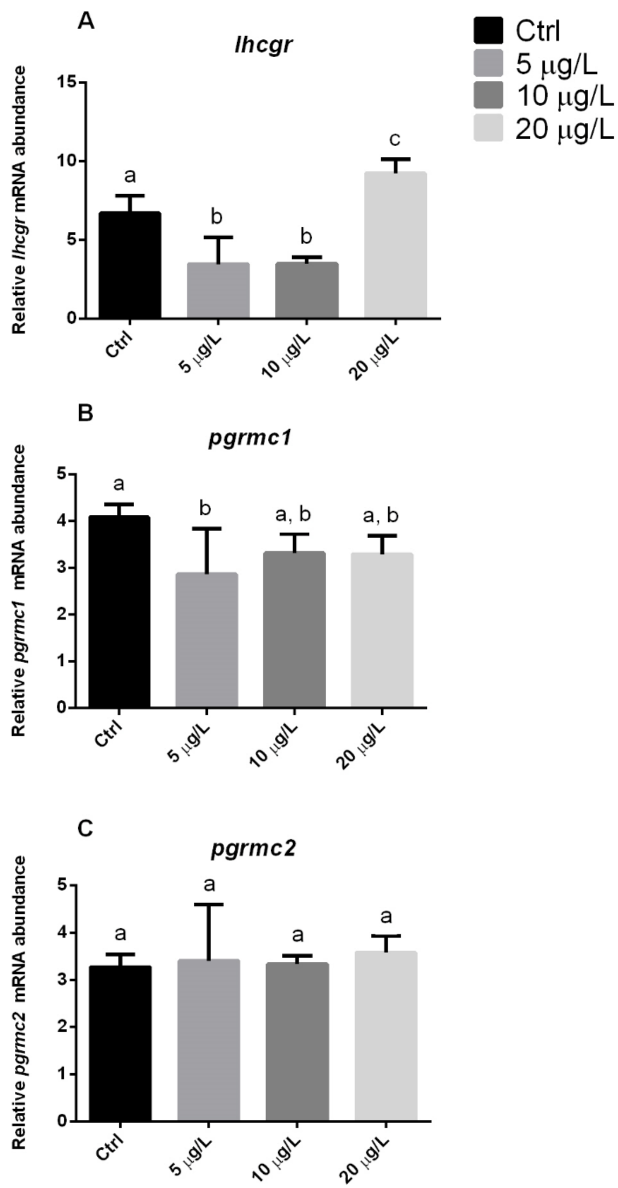


Fig.3

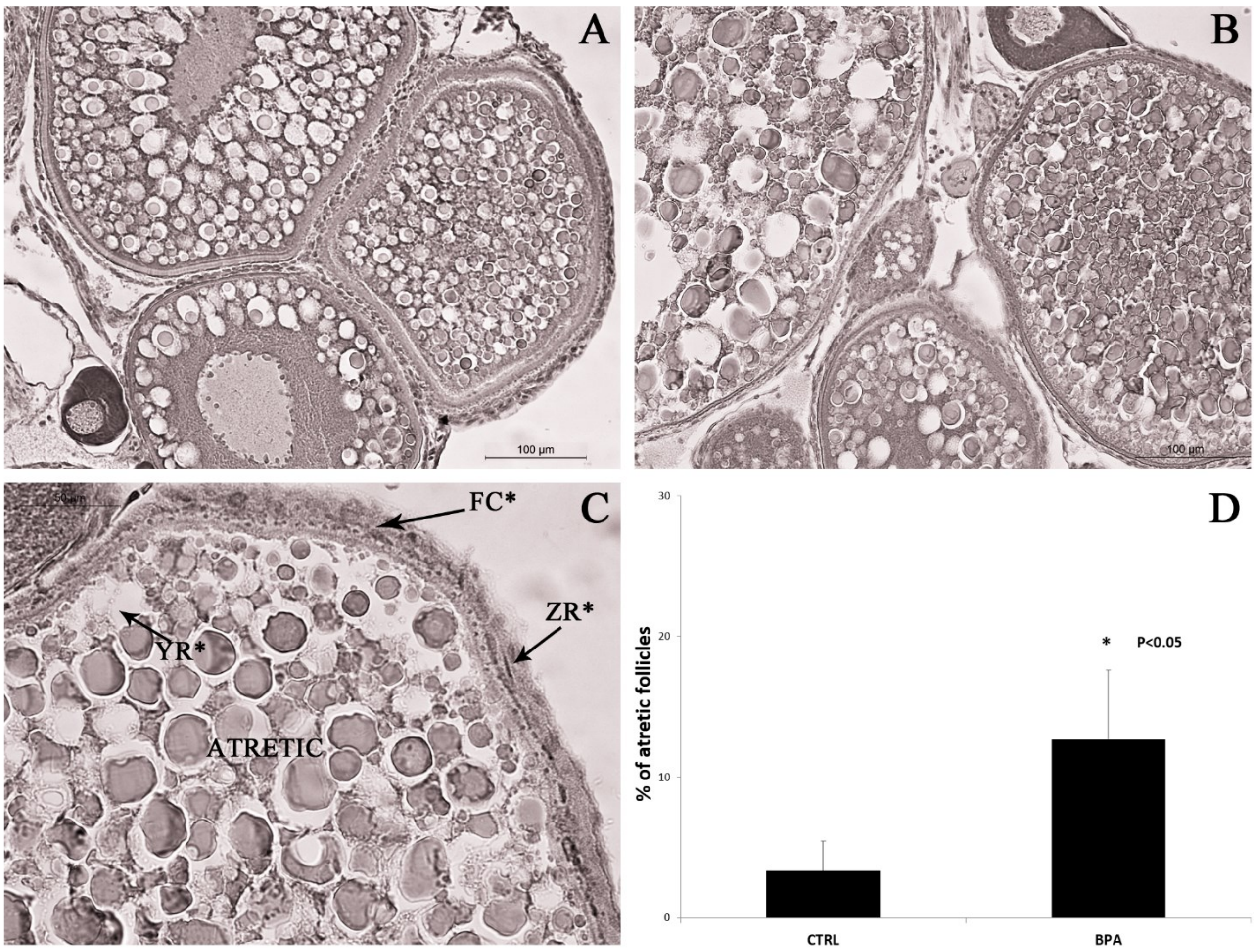
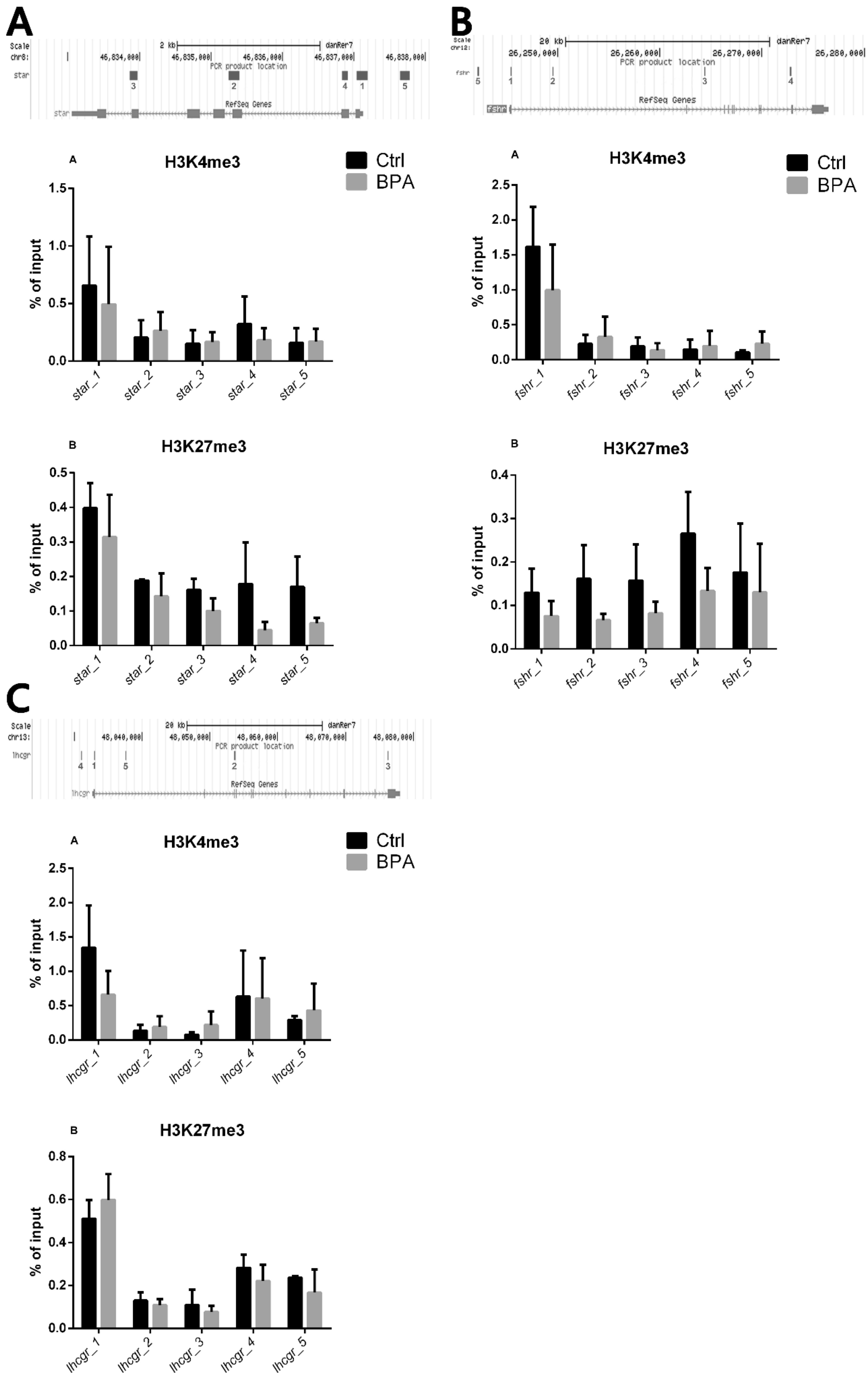


Fig.4



4. EPIGENETIC CHANGES IN F1 ZEBRAFISH EMBRYO FROM MOTHER EXPOSED TO BPA

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Abstract

Bisphenol A is a phenol with a worldwide distribution due to its use in the manufacturing of plastic materials. It is able to interfere with epigenetic mechanism and his transgenerational effects have also been demonstrated. To determine its effects on the subsequent generation, females of zebrafish were treated with two doses of BPA (10^{-7} M and 10^{-5} M) for four weeks and then crossed with untreated males. 24 hours post fertilization (hpf) embryos were collected and the effects of maternal exposure to BPA on embryo development and gonadal formation, in addition to epigenetic effects were assessed by Real-Time PCR (q-PCR) and bisulfite pyrosequencing. The results showed that both BPA doses interfere with gonadal formation in zebrafish embryos also by interfering with the CpG island methylation level of *amh* promoter regions, while only the lowest BPA dose interfere with DNA methyltransferases gene expression. These data clearly suggested that the changes due to BPA exposure of adult females can be inherited by F1 generation and interfere with gonadal formation and with the general methylation status of the developing embryos.

1. Introduction

Bisphenol A (BPA) is a phenol formed by a hydroxyl residue directly bound to an aromatic ring (Flint et al., 2012). It is present in 95% of product containing epoxy resins and polycarbonates, but is also used in the vinyl chloride and thermal paper production (Nam et al., 2010). BPA is released mainly in landfill or in plastic degradation process (Kang et al., 2006), it has been detected in the urine of 95% of adult in USA and Asia (Laing et al., 2016) and is present in the aquatic environment with a concentration up to 21 μg/L (Crain et al., 2007).

BPA has been demonstrated to interact with estrogen signaling pathways by binding to the estrogen receptors ERα and ERβ (Naciff et al., 2002; Wetherill et al., 2007). Low dose effects and non-monotonic dose response curves have been reported (Honma et al., 2002; Zhang et al., 2014) and, in addition to the well-established estrogenic mode-of-action, further mechanism have been proposed, including potential anti-androgenic activity (Bonefeld-Jørgensen et al., 2007).

Recently, increasing evidence suggests that BPA may alter the epigenetic regulation of gene expression having the ability to cause DNA hypermethylation/hypomethylation at CpG (cytosine-guanine dinucleotide) islands

near gene promoter regions, histone modifications, and expression of non-coding RNAs, including microRNAs (Singh and Li, 2012). Nowadays several studies suggest that exposure to endocrine-disrupting chemicals (EDCs) may have cumulative adverse effects on future generations, and that these effects could be mediated by epigenetic mechanism (Kundakovic and Champagne, 2011). Considering previous studies highlighting the capacity of BPA to interfere with female reproduction, mediated by its capacity to interfere with the epigenetic processes (Laing et al., 2016; Santangeli et al., 2016a), we aimed at investigate whether the administration of BPA to adult females could affect embryo development, and in particular interfere with those genes involved in the gonad differentiation. To achieve this aim, was first investigated the expression level of several isoforms of DNA methyltransferases (DNMTs) (Cedar and Bergman, 2009; Kamstra et al., 2014) and genes involved in gonadal formation as DEAD (Asp-Glu-Ala-Asp) box polypeptide 4 (*ddx4)*, anti-Mullerian hormone (*amh*) and cytochrome P450, family 19, subfamily A, polypeptide 1a (*cyp19a1)* (Bouwmeester et al., 2016; Potok et al., 2013; Rodríguez-Marí et al., 2005). Furthermore, we examined other genes involved in embryo development such as goosecoid (*gsc*), piwi-like RNA-mediated gene silencing 1 (*piwil*) and homeobox B1b (*hoxb1b*) were investigated (Kamstra et al., 2014; Prince et al., 1998; Wu et al., 2011). Lastly the percentage of methylation marking CpG island in the promoter region of *dnmt1*, *ddx4* and *amh* was investigated.

The experimental model chosen for this study was *Danio rerio.* This specie, has well-known advantages, among these, the complete-genome sequence, the 70% of orthology of the gene between the zebrafish and humans and the similarity between body plan (Howe et al., 2013) that make it an ideal candidate to predict the effects of the EDCs also on human.

2. Methods

2.1. Animals and BPA administration

A total of 15 adult female zebrafish (*D. rerio* wild-type strain) were placed in two 100-L aquaria (5 fish/tank) with oxygenated water under controlled conditions (28.0 ± 0.5 ˚C) and maintained on a 14/10 h light/dark cycle. They were fed 4 times a day, twice with commercial food (Vipagran; Sera, Loessnitz, Germany) and twice with *Artemia salina*. There were a control group and two exposed ones, which received 10^{-7} and 10^{-5} M

BPA (98% analytical purity, Sigma-Aldrich, Milano, Italy) for 4 weeks. BPA was administered to fish via water in a static system and his concentration and half-life was determined by high-performance liquid chromatography (HPLC) analysis. According with these data BPA was renewed every four days. All tanks were maintained in duplicate.

After 4 weeks, BPA treated females, designed as F0 generation, plus control females, were crossed with control males and 24hours post fertilization (hpf) embryos, designed as F1 generation, were collected. Ten eppendorf containing pool of twenty 24 hpf embryos each were collected from each group, in duplicate, for analysis of Real Time semi-quantitative Polymerase Chain Reaction (q-PCR) (five biological replicates for group) and for bisulfite pyrosequencing (five biological replicates for group).

2.2. RNA extraction and cDNA synthesis

Total RNA was isolated from pool of 24 hpf embryos with RNAzol solution (Sigma Aldrich) according to the manufacterer's instructions. Its final concentration was determined using the Nanophotometer TM P-Class (Implen GmbH, Mϋnich, Germany), while its integrity was verified by GelRed staining of 28S and 18S ribosomal RNA fragments on a 1% agarose gel. Total RNA, has been treated with DNAse to remove genomic DNA (10 IU at 37 ˚C for 10 min; Fermentas MBI, Amherst, NY, USA). DNAs treated total RNA was used for cDNA synthesis using SuperScript-II kit (Invitrogen,Life Technologies), and the samples were kept at -20°C until use.

2.3. Real-Time qPCR

The relative quantification of gene expressions was performed with the SYBR green method in an Eco Real-Time PCR System (Illumina). All samples were analyzed in triplicates. The reactions consisted in 1 µl of diluted (1/10) cDNA, 5 µl of 2X SYBR Green PCR Master Mix (Thermo Scientific), supplemented with 0,02 µl of ROX, containing SYBR Green as a fluorescent intercaling agent, 0.3 µM of both forward and reverse primers and 3.4 µl of milliQ water (Table 1). The thermal profile for all reactions consisted in i) enzyme activation at 95 ˚C for 3 min, ii) 45 cycles of denaturation (10 s at 95 ˚C) followed by a 20-s annealing at 60˚C for 18s, *arp*, *ddx4, piwil1, gsc, hoxb1b, cyp19a*, *dnmt6*, 59˚C for *amh*, *dnmt1, dnmt8, dnmt3, dnmt5* and 58˚C

for *dnmt7* and iii) 20 s elongation at 72˚C. Fluorescence was monitored at the end of each cycle. Dissociation curve for primer specificity and absence of primer-dimer formation check was performed and, in all cases, it showed a single peak. We used, *18S* rRNA (Tang et al., 2007) and *arp,* acid ribosomal protein (Aursnes et al., 2011) as internal controls with the aim of enabling results standardization by eliminating variations in mRNA and cDNA quantity and quality (Bustin et al., 2009). These were chosen because their mRNA levels did not vary either between experimental treatments or between follicular stages. No amplification product was observed in the negative control (absence of template). Data were analyzed applying quantification method based on a ΔΔCt calculation implemented with the Pfaffl equation to improve accuracy by accounting for a varied reaction efficiencies depending on primers (Pfaffl, 2001; Vandesompele et al., 2002). All results are expressed with respect to control group.

2.4. DNA extraction

DNA was extracted from pool of 24 hpf embryos using the Quick-gDNA MiniPrep (Zymo Research, Irvine, CA, USA) according to the manufacturer's instructions except for the first part in which we homogenized embryos, immersed in 500 µl of Genomic Lysis Buffer, with 15 stroke of a dounce homogeneizer, before moving to the next step. The DNA concentration was determined at 260/280 nm (DNA-50) using a spectrometer (Nanodrop, Thermo Fisher Scientific, Waltham, MA, USA).

2.5. Locus-specific DNA methylation

Locus-specific DNA methylation analysis was performed through pyrosequencing of a PCR product of bisulfite converted DNA. The DNA Methylation-Gold Kit (Zymo Research, Irvine, CA, USA) was used according to the manufacturer's instructions (conversion efficiency > 99%), using 500 ng DNA as input. Converted DNA was eluted with 20 µl of M-Elution Buffer. The concentration of bisulfite converted DNA was determined using Nanodrop set to RNA-40. Once converted, the target sequences in the gene of interest promoter region were amplified by PCR using Hot Start PCR 5X Master MIX (Euroclone). Primers, PCR conditions, position and amplicon for *dnmt1* and *amh* were obtained by the work of Laing and colleagues (Laing et al., 2016). Primers, PCR conditions, position and amplicon for *ddx4* were obtained by the work of Bouwmeester and colleagues (Bouwmeester et al., 2016). Based on signal strength in a pilot run, up to 22 µl

of the biotinylated PCR product was used. Cleaned biotinylated template was annealed to the sequencing primer (0,3 µM) at standard conditions. The assay setup and dispensation order of the nucleotides were generated using PyroMark Q24 Software (Quiagen, version 2.0). The dispensation order was based on the expected sequence in the amplicon and in the bisulfite controls, to evaluate the efficiency of the bisulfite treatment. Pyrosequence was conducted with a PyroMark Q24 System (Qiagen), and the methylation level of each CpG site was calculated as the ratio of peak heights in the pyrogram.

2.6. Statistical analysis

Statistical analysis was performed with one-way ANOVA followed by Tukey's multiple comparison test. Significance was set at $p < 0.05$. All procedures were performed with GraphPad Prism 6.

3. Results

In order to study if BPA could affect embryo development, zebrafish females were fed with two different concentration of this compound: 10^{-7} and 10^{-5} M.

Analyzing the expression of embryo's *dnmt* genes, we observed that the lowest dose of BPA decreased the transcription level of *dnmt1* (Fig.1A), while increased *dnmt3* and *dnmt7* (Fig.1 B,E) gene expression. The other isoform of DNMTs were not affected by any of these doses (Fig.1 C,D,F). On the contrary, the highest dose of BPA did not affect any of the DNMTs isoform analyzed in this study (Fig.1).

Regarding the genes involved in embryo development, both BPA doses, were exerting the same effects, decreasing the gene expression level of those genes involved in gonadal formation, *ddx4* (Fig. 2A), *amh* (Fig.2 B) and *cyp19a1* (Fig. 2 C) while *gsc*, *hoxb1b* and *piwil* were not affected by the treatment (Fig. 2 D,E,F).

In order to investigate if transcription level reduction of *dnmt1, ddx4* and *amh* genes was due to a modification (hypermethylation) of DNA methylation of the promoters of these genes, the percentage of 5mC of their sequences was investigated. In the case of *dnmt,* embryos from BPA females and from control group did not show any difference in CpG DNA methylation, and (Fig. 3) the same result was obtained for *ddx4* gene(Fig 4). Contrarily the promoter region of *amh* revealed that exposure to both BPA doses, caused a significant increase in embryo DNAmethylation (Fig 5).

4. Discussion

The current study was conceived to examine the action of BPA, administered chronically to zebrafish female's brood stock, designed as F0 generation, on embryo development of the subsequent F1 generation. Our working hypothesis was based on the evidence that exposure to EDCs may activate or deactivate selectively some part of the genome, causing epigenetic effects. The altered epigenome can be transferred to the subsequent generations by germline highlighting the importance to study the epi-genotype (Shahidehnia, 2016). Laing and colleagues found that BPA is able to interfere with zebrafish reproduction trough the alteration in the transcription of a number of genes involved in epigenetic regulation, but also by the decreasing in global DNA methylation (Laing et al., 2016). We previously demonstrated the ability of this pollutant to interfere with the reproductive process in zebrafish females through the deregulation of histone modification and DNMTs gene expression (Santangeli et al., 2016a). Dolinoy and collaborators also demonstrated that maternal BPA exposure was able to shift the coat color distribution of viable yellow agouty mouse offspring, towards yellow, by decreasing the CpG methylation of the IAP's sequence upstream of the *Agouty* gene, highlighting the effects of this pollutant on the subsequent generation (Dolinoy et al., 2007). To shed some light on the issue, the present study adopted molecular techniques in order to analyze gene expression and monitoring the percentage of DNA methylation marking the promoter region of specific gene involved in embryo development and gonadal development to elucidate the effects of BPA on F1 embryos. In particular, *ddx4, amh and cyp19a1* were chosen to check the effects of BPA on gonadal development. The first is essential for germline development and is used as a marker gene for the germ cell population (Castrillon et al., 2000; Fang et al., 2013), the second causes the regression of the Müllerian ducts in males (Rodríguez-Marí et al., 2005), eventually the *cyp19a1* is essential for the regulation of gonadal sex differentiation and sex change in most teleost fishes (Guiguen et al., 2010). Furthermore *piwil*, which is important for germ-line functions (Potok et al., 2013), *hoxb1b*, which is involved in the central nervous system (CNS) development like hindbrain development and the development of otic vesicle (Lindeman et al., 2011; Wu et al., 2011) and finally the *gsc,* involved in the dorsalization process (William et al., 2013) were investigated. The results obtained by q-PCR analysis clearly shows that both BPA doses are able to downregulate gene expression levels of those gene involved in gonadal development as *ddx4*, *amh* and *cyp19a1*, leading us to hypothesize some impairment in

gonadal formation that could, in the future, affects the reproductive fitness. Furthermore, for *amh*, we were able to demonstrate that the deregulation in gene expression is due to the capacity of BPA to hypermethylate the CpG island present in the promoter region.

DNA methylation consists in the addition of a methyl group to the 5th ring carbon of the cytosine. It occurs mainly at locations rich in cytosine and guanine bases known as CpG islands, which are usually present in the promoter region of genes. This process is catalyzed by the DNMTs. Zebrafish possesses eight different isoforms of DNMTs: *dnmt1* which is involved in de maintenance methylation; *dnmt3*, *dnmt4* and *dnmt7* (orthologs of DNMT3b of mammals) and *dnmt6* and *dnmt8* (orthologs of DNMT3a of mammals) which are involved in de novo methylation (Kamstra et al., 2014).

We found that the lowest BPA dose decreases the expression of *dnmt1,* involved in the maintenance of methylation process and increases the expression of *dnmt3* and *dnmt7,* both involved in the novo methylation supporting the hypothesis that this BPA dose impair the normal methylation patterns occurring in embryo development, both interfering with de novo and the maintenance of methylation.

In conclusion, our study demonstrated that the chronical BPA's exposure of adult females, can cause impairment in embryo development evidencing the maternal role in the epigenetic control of embryo development. Even if other studies are required to better elucidate the transgenerational effects of EDCs, this study can be considered an important starting point to clarify the effects of a widespread pollutant as BPA on subsequent generation and its mechanism of action.

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Table 1. Lists of primers used in gene expression analyses by Real-Time qPCR.

Gene	Forward primer (5'- 3')	Reverse primer (5'- 3')	Acc number
18S rRNA	TCGGAAAACGGTGAACCTG	AAGGTCTTTGAACCCACGG	NM_001098396.1
arp	CTGAACATCTCGCCCTTCTC	TAGCCGATCTGCAGACACAC	NM131580.2
dnmt1	GAGCCTGTGAAGCAGGAGAA	CATGAATGGCACTGCACAGA	NM_131189.1
dnmt3	AAACAACGCGCTTCCACG	TTCCATAACCACCACCGTCC	AF135438.1
dnmt5	GCTCCATCACATCTCAGCCC	CAAATCCGACACCGGCAAAG	XM_009296722.1
dnmt6	AGAAAACCCATTCGCGTCCT	GTGCCCTCGTAGAGACCTTTT	AB196917.1
dnmt7	ATCCGACATCTCTTTGCACC	GTGAAGTGAATTTGCAGAAAGC	AB196918.1
dnmt8	GGACGTATTGTGTCCTGCT	ATCACCAAACCACATGACCC	AB196919.1
ddx4	AAGTCCTTTCAGGGGCCCAA	CGTGGGTTGAAGCCTGTTGT	NM_131057.1
amh	AGCCTCATTTGAAACGTCACAG	GCTGTCTCTGAGGGAACACAA	AY677080.2
cyp19a1	TAAGCAAGTCCTCCGCTGTG	ACTTCTGGAGACCTGGACCT	NM_131642.1
gsc	CAAGAGACGACACCGAACCA	TCGCTTTTGCCCTCCTCAAT	NM_131017.1
hoxb1b	CAGAAGAAGCGCGAGAAGGA	GCTAGTTGAGTGGTCTGCGT	NM_131142.2
piwil	TGGCAACCAGGTTCTTCTCG	CAGAACTCGGGGACAAGCAT	NM_183338.1

Table 1. *arp*: acidic ribosomal protein; *dnmt1* to *8*: DNA (cytosine-5-)-methyltransferases; *ddx4:* DEAD (Asp-Glu-Ala-Asp) box polypeptide 4; *amh:* anti-Mullerian hormone; *cyp19a1:* cytochrome P450, family 19, subfamily A, polypeptide 1a; *gsc:* goosecoid; *hoxb1b:* homeobox B1b; *piwil:* piwi-like RNA-mediated gene silencing 1.

Figure Legends

Fig. 1: Transcription profiles of DNA (cytosine-5-)-methyltransferase genes 1 to 8 (*dnmt1-8*). Letters above each column indicate statistical differences among groups ($p < 0.05$ vs. untreated controls; ANOVA followed by Tukey's multiple comparison test). A: *dnmt1*; B: *dnmt3*; C: *dnmt5*; D: *dnmt6*; E: *dnmt7*; F: *dnmt8*. Data were generated in duplicate from five biological replicates.

Fig. 2: Transcription profiles of genes involved in embryo development.. Letters above each column indicate statistical differences among groups ($p < 0.05$ vs. untreated controls; ANOVA followed by Tukey's multiple comparison test). A: *ddx4:* DEAD (Asp-Glu-Ala-Asp) box polypeptide 4; B: *amh:* anti-Mullerian hormone; C: *cyp19a1:* cytochrome P450, family 19, subfamily A, polypeptide 1a; D: *gsc:* goosecoid; E: *hoxb1b:* homeobox B1b; F: *piwil:* piwi-like RNA-mediated gene silencing 1. Data were generated in duplicate from five biological replicates.

Fig.3: A: Gene specific DNA methylation profiles for 11 CpG sites in the promoter region of DNA (cytosine-5-)-methyltransferase 1 (*dnmt1*) in 24 hours post fertilization embryos, obtained crossing females treated with two BPA doses (10^{-5}M and 10^{-7}M) with control males. Data are presented as the mean of the percentage in methylation for the CpG sites analyzed. Asterisk indicates significant differences between embryos obtained crossing BPA treated females and embryos obtained crossing control females with control males ($p < 0.05$ vs. untreated controls; ANOVA followed by Tukey's multiple comparison test). B: example pyrogram of 11 CpG sites in the 5' flanking regions of the *dnmt1* gene.

Fig.4: A: Gene specific DNA methylation profiles for 5 CpG sites in the promoter region of DEAD (Asp-Glu-Ala-Asp) box polypeptide 4 (*ddx4*) in 24 hours post fertilization embryos obtained crossing females treated with two BPA doses (10^{-5}M and 10^{-7}M) with control males. Data are presented as the mean of the percentage in methylation for the CpG sites analyzed. Asterisk indicates significant differences between embryos obtained crossing BPA treated females and embryos obtained crossing control females with control males ($p < 0.05$ vs. untreated controls; ANOVA followed by Tukey's multiple comparison test). B: example pyrogram of 11 CpG sites in the 5' flanking regions of the *ddx4* gene.

Fig.5: A: Gene specific DNA methylation profiles for 5 CpG sites in the promoter region of anti-Mullerian hormone (*amh*) in 24 hours post fertilization embryos obtained crossing females treated with two BPA doses (10^{-5}M and 10^{-7}M) with control males. Data are presented as the mean of the percentage in methylation for the CpG sites analyzed. Asterisk indicates significant differences between embryos obtained crossing BPA treated females and embryos obtained crossing control females with control males ($p < 0.05$ vs. untreated controls; ANOVA followed by Tukey's multiple comparison test). B: example pyrogram of 11 CpG sites in the 5' flanking regions of the *amh* gene.

Fig.1

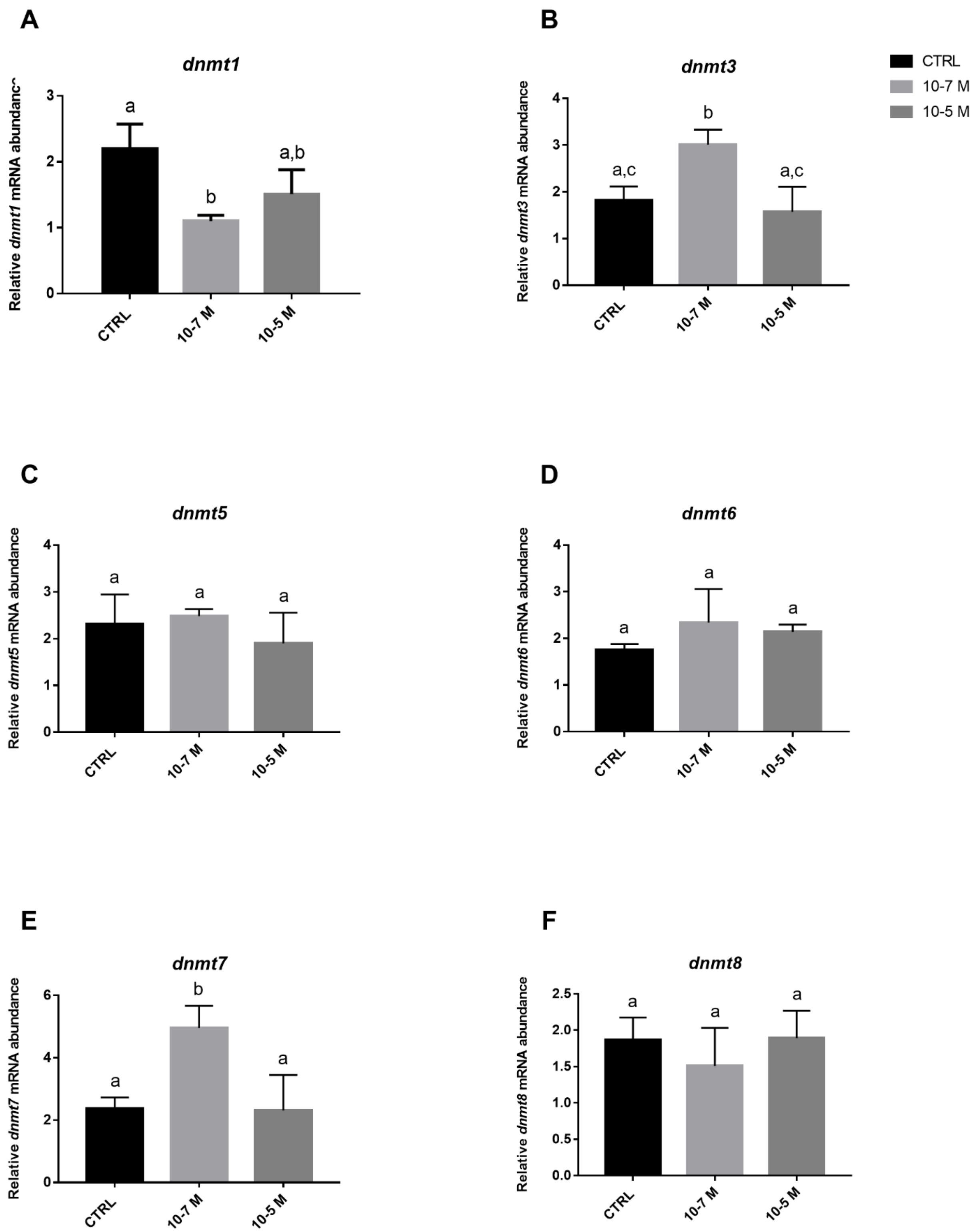


Fig.2

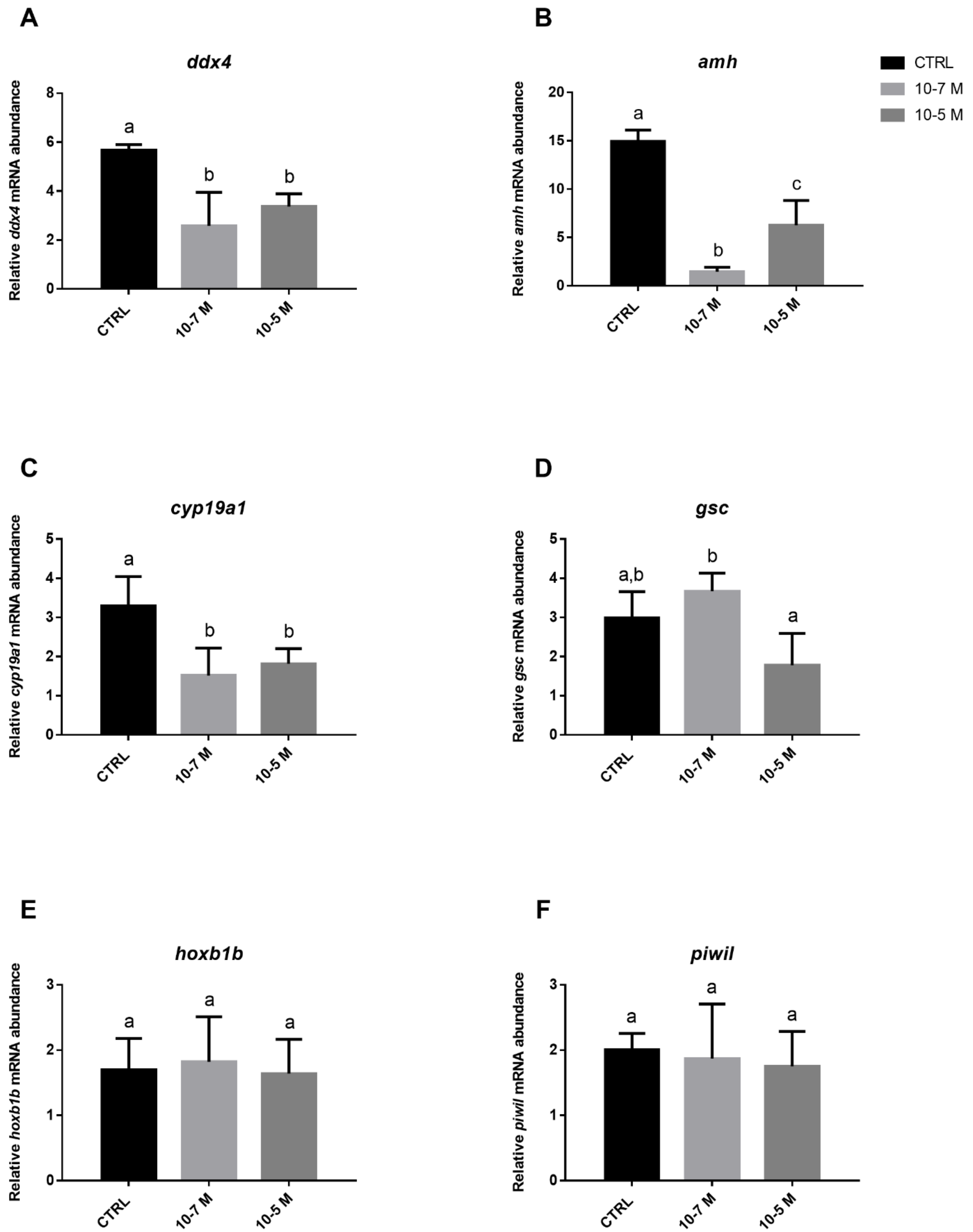


Fig.3

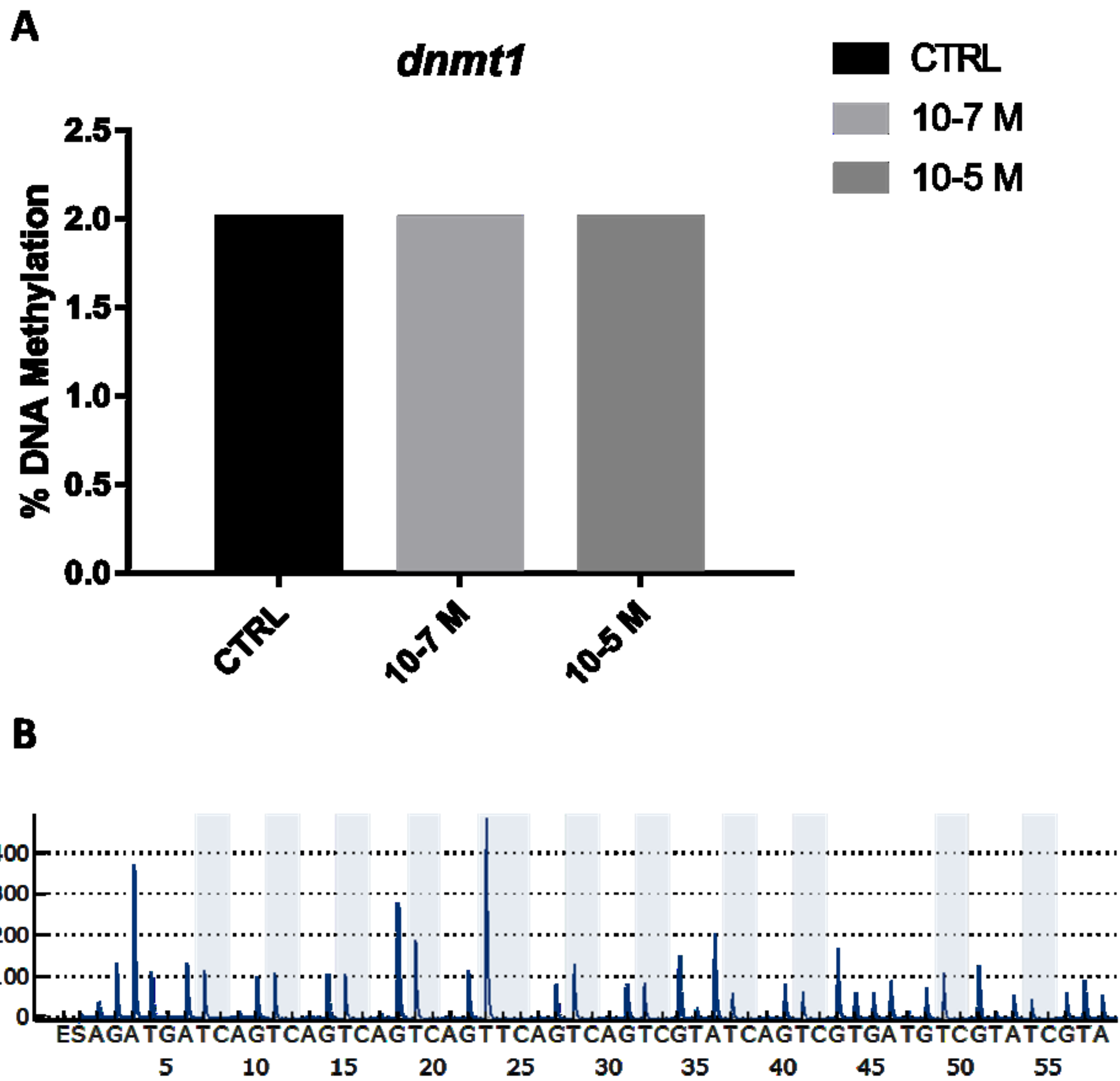
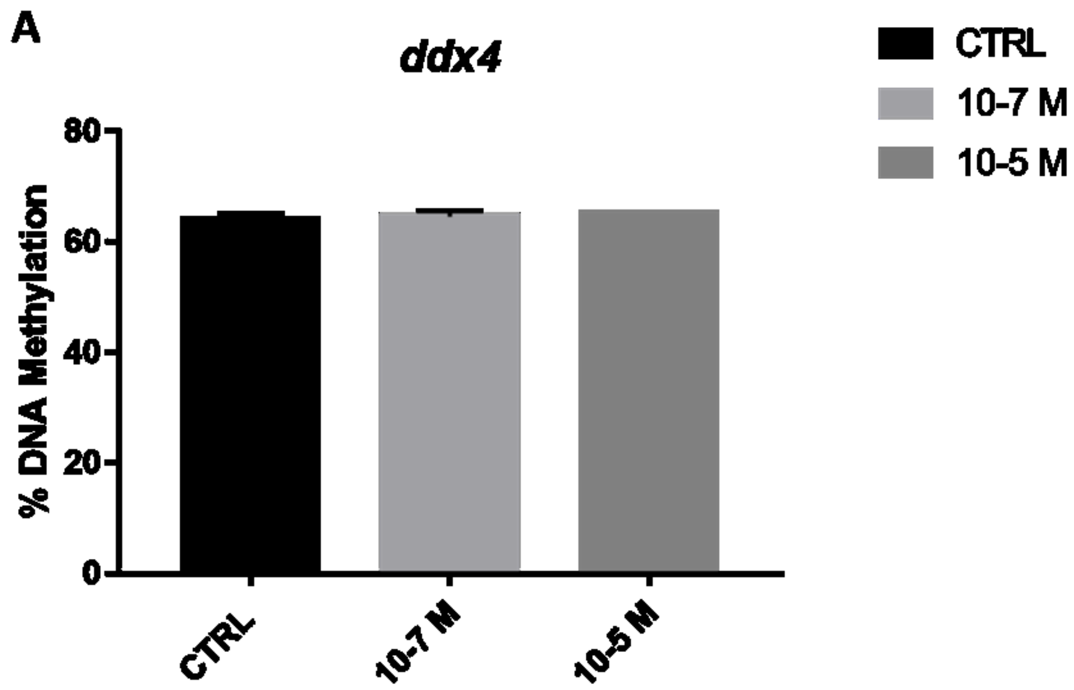


Fig.4



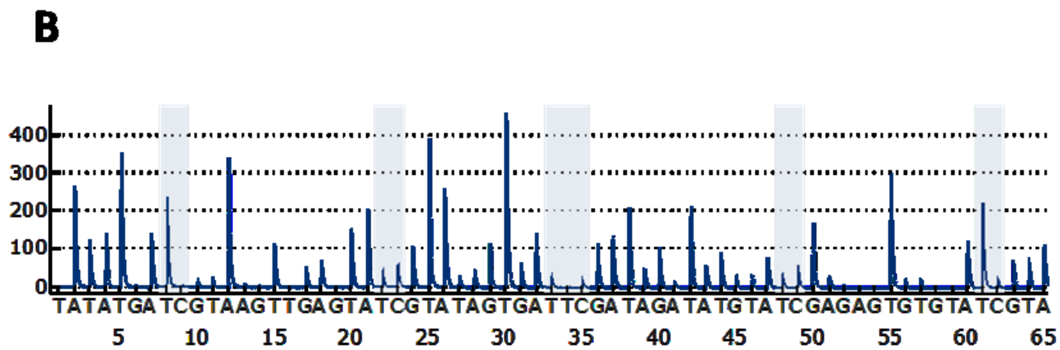
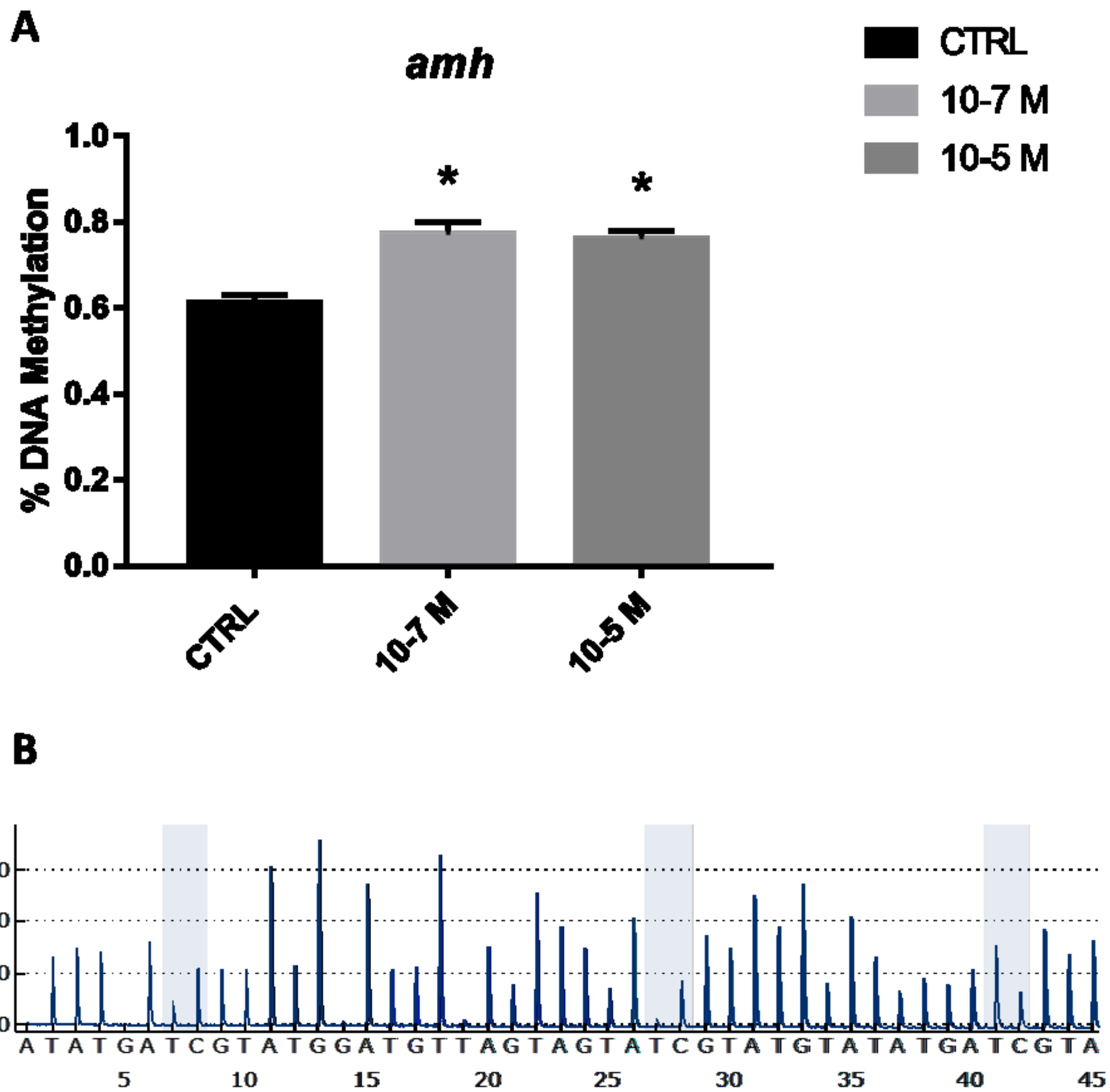


Fig.5



5. EFFECTS OF DIISONONYL PHTHALATE ON *DANIO RERIO* REPRODUCTION

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Abstract

Di-isononyl phthalate (DiNP) is a high molecular weight phthalate commonly used as a plasticizer. It was introduced as a replacement for bis (2-ethylhexyl)phthalate (DEHP) which is used in the production of plasticized polyvinyl chloride (PVC). The purpose of this study was to investigate for the first time the effect of DiNP on female reproductive physiology in zebrafish. Fish were exposed to five different doses of DiNP (4200 µg/L; 420 µg/L; 42 µg/L; 4,2 µg/L; 0,42 µg/L) for a period of three weeks. We evaluated transcript levels for genes involved in steroidogenesis, oocyte growth, oocyte maturation, autophagic and apoptotic processes, as well as, changes in morphology and chemical composition of vitellogenic oocytes by means of qPCR, histology and fourier transform infrared imaging. The results demonstrate a non-monotonic dose response relationship, and greater differences at the lower and higher doses. The findings provide evidence that exposure to DiNP significantly alter steroidogenesis, oocytes growth and maturation leading to disruption of reproduction in zebrafish.

1. Introduction

Phthalates are used as solvent in personal care products, and additives in plasticized polyvinyl chloride (PVC) (David et al., 2001; Koch and Calafat, 2009; Schettler, 2006). The annual global production of phthalates is more than 4 Mt, of which about 1 Mt is produced in Europe (Peijnenburg and Struijs, 2006). The most important compound of this category is bis (2-ethylhexyl) phthalate (DEHP), that was shown to disrupt reproduction (Oliana Carnevali et al., 2010), and has recently been replaced by other compounds, such as di-isononyl phthalate (DiNP) (Clara et al., 2010). DiNP has a high molecular weight and has longer half life. It has a lower solubility rate and slower rate of migration compared to other phthalates (Vieira et al., 2011). In the last few years, DiNP has become one of the main plasticizers in Europe with environmental levels at concentrations from 0,52 µg/L (1,2 X 10^{-8} M) and upwards (Quinn-Hosey et al., 2012) and it is therefore important to have information on its safety.

Several studies in male rats have shown that exposure to DiNP alters activity of androgen responsive tissues, cause retention of nipples, testicular atrophy, epididymal agenesis, and lower level of testosterone in both testes and plasma (Borch et al., 2004; Gray et al., 2000). This is the first study of the effect of DiNP on female

reproductive physiology, using zebrafish as a suitable model organism. The main objective of the present study was to determine the effect of exposure to different concentration of DiNP, relying on the assumption that this pollutant act as endocrine disruptor compounds (EDCs), starting from low environmental concentration (420 ng/lit) to higher levels on female zebrafish reproductive parameters, including number and size distribution of ovarian follicles, apoptosis and autophagy signals, spatially resolved biochemical information of vitellogenic oocytes using fourier transform infrared (FTIR) imaging, and transcript levels for genes involved in reproduction.

2. Methods

2.1. Animals and DiNP administration

The experiments were carried out on adult female zebrafish (*Danio rerio*, wild-type strain). Fish were maintained in 100-L aquaria with oxygenated water under controlled conditions (28.0 ± 0.5˚C under a 14/10 hours of light/dark period) and were fed four times per day, twice with commercial food (Vipagran; Sera, Loessnitz, Germany) and twice with *Artemia salina*. A total of 60 females were equally distributed into six aquaria (one control and five DINP experimental groups) with a total of 10 female fish each and treated for a three-week time with five different concentrations of DiNP (0,42 µg/L; 4,2 µg/L; 42 µg/L; 420 µg/L; 4200 µg/L (Sigma-Aldrich). DINP was administered to fish via water in a static system and it was renewed every four days. After twenty-one days of treatment, fish were lethally anesthetized with MS-222 (3-aminobenzoic acid ethyl ester(Sigma Aldrich) buffered to pH 7.4. according to University of Calgary animal care protocol for care and use of experimental animals). Ovaries from each experimental group were removed and used for FT-IR imaging analysis, transcript measurement by qPCR, and part of it was fixed in Bouin solution for further histological preparation.

2.2. Egg collection and fertility

Starting on the 8th day of treatment, the 5 groups of DiNP-exposed females and the control females were crossed with untreated males, and fertility was determined during the following 15 days. Fertilized eggs were

counted and the fertility rate was calculated as the mean ± standard deviation (SD) of fertilized egg number / female / day from the 8th to the 21th day of treatment.

2.3. RNA extraction and cDNA synthesis

Total RNA was isolated from ovary with RNAzol solution (Sigma Aldrich) according to the manufacterer's instructions. Its final concentration was determined using the Nanophotometer TM P-Class (Implen GmbH, Mϋnich, Germany), while its integrity was verified by GelRed staining of 28S and 18S ribosomal RNA fragments on a 1% agarose gel. Total RNA was treated with DNAse to remove genomic DNA (10 IU at 37 ˚C for 10 min; Fermentas MBI, Amherst, NY, USA). DNAs treated total RNA was used for cDNA synthesis using SuperScript-II kit (Invitrogen,Life Technologies), and the samples were kept at -20°C until use.

2.4. Real-Time qPCR

The relative quantification of gene expressions was performed with the SYBR green method in an iQ5 multicolor real-time PCR (BioRad). All samples were analyzed in duplicates. The reactions consisted in 1 µl of diluted (1/10) cDNA, 5 µl of 2X SYBR Green PCR Master Mix (Bio Rad) containing SYBR Green as a fluorescent intercaling agent, 0.1 µM of both forward and reverse primers and 3.8 µl of milliQ water (Table 1). The thermal profile for all reactions consisted in i) enzyme activation at 95 ˚C for 3 min, ii) 45 cycles of denaturation (10 s at 95 ˚C) followed by a 20-s annealing at 60˚C for *star*, *esr2a*, *gdf9*, *ambra1a*, *beclin1*, *tp53*, *caspase3* and *cyp11a1*, 59˚C for *fshr*, *pgrmc1*, *pgrmc2*, *bmp15*, and 58˚C for *esr1*, *esr2b*, and *lhcgr*, and iii) 20 s elongation at 72˚C. Fluorescence was monitored at the end of each cycle. Dissociation curve for primer specificity and absence of primer-dimer formation check was performed and, in all cases, it showed a single peak. We used, *18S* rRNA (Tang et al., 2007) and *arp,* acid ribosomal protein (Aursnes et al., 2011) as internal controls with the aim of enabling results standardization by eliminating variations in mRNA and cDNA quantity and quality (Bustin et al., 2009). These were chosen because their mRNA levels did not vary either between experimental treatments or between follicular stages. No amplification product was observed in the negative control (absence of template). Data were analyzed using iQ5 Optical System version 2.1 (Bio-Rad). Applied quantification method was based on a ΔΔCt calculation implemented with the Pfaffl equation to

improve accuracy by accounting for a varied reaction efficiencies depending on primers (Pfaffl, 2001; Vandesompele et al., 2002). All results are expressed with respect to control ovaries.

2.5. Histology

The ovaries (n=5/experimental group) were fixed in Bouin and prepared for histological examination using standard biological procedures. Gonads were embedded in paraffin and sectioned (5 μm) with a microtome (Leitz 1512). Each ovary was fully sectioned and processed for hematoxylin-eosin staining and observed (at x50 final magnification) under a light microscope (Zeiss Axioskop). Images (x50 final magnification) were captured using a high resolution digital camera (Canon EOS 6D). From the analysis of ovary's images, oocytes were counted, using imege J software (https://imagej.nih.gov/ij/), and divided into three stages: previtellogenic, vitellogenic and mature oocytes (Fig. 4).

2.6. Fourier transform infrared FT-IR

Fourier transform infrared (FTIR) imaging is a powerful and well-evaluated vibrational technique that can provide spatially resolved biochemical information of biological samples, exploiting IR microspectrometers, coupled with visible light microscopes and bidimensional detectors (Burattini et al., 2007). This technique was optimized in our group and characterized the spectral markers for each maturation stage of zebrafish follicles (Carnevali et al. 2009, 2010a, 2011; Gioacchini et al. 2012b; Giorgini et al. 2010). This approach here enabled investigation of the effects of DINP (0,42 μg/L; 4,2 μg/L; 42 μg/L; 420 μg/L) on chemical composition of vitellogenic oocytes In the present study, FT-IR measurements were carried out by using a Bruker VERTEX 70 interferometer coupled with the Hyperion 3000 Vis-IR micro-scope and equipped with a liquid nitrogen cooled bidimensional focal plane array (FPA) detector (area size 64 × 64 pixels). For each sample, images were acquired in transmission mode using a 15× condenser/objective, therefore achieving a pixel resolution of about 2.65 μm. On each section, a specific region was selected, containing oocytes III class, on which IR images were taken acquiring simultaneously groups of 4096 spectra, averaging 256 scans for each detector pixel with a spectral resolution of 4 cm^{-1}. Background single channel images were acquired on clean regions of the CaF_2 windows. Bigger images were done by defining a grid of images, until a maximum of 36,864 microspectra. By using OPUS 7.2 software (Bruker), chemical maps were generated for each sample,

integrating under the lipids stretching region (3100–2800 cm^{-1}), the amide I and II modes (1720–1480 cm^{-1}), and phosphate groups stretching region (1100-1065 cm^{-1}). 30 spectra were extracted from IR images of each sample (OPUS 6.5, Bruker), selecting the oocytes inner zone, in order to avoid the influence of the plasmatic membrane. All the extracted spectra were two-point baseline linear fitted in the spectral range of 4000–900 cm^{-1}, compensated from the atmospheric contributions of aqueous vapour and CO_2, and vector normalized. They were analysed in the following spectral regions: 3030-2800 cm^{-1} for lipids and =CH groups, 1800–1480 cm^{-1} for proteins and COO groups, and 1137-1060 cm^{-1} for phosphate groups. The lipids region added to the 1775-1065 cm^{-1} portion of the spectrum represented the total amount of chemical components of the sample, called "cell". On all the spectra collected from oocytes of each experimental group, was performed an integration of the bands. In order to normalize all the peculiar characteristics of each spectrum in terms of intensity and absorbance, we divided the numerical value of the integrated band by the *cell* value.

2.7. Statistical analysis

All infrared FT-IR results were analysed using one-way ANOVA, followed by Dunnett's post-hoc test, in order to compare the differences between the control and each of the DiNP doses used in the study. The transcript abundance results was performed with the one-way ANOVA analyses of variance followed by Turkey multiple comparison tests. Significance was set at $p < 0.05$. Results are presented as mean ± standard deviation. Histological results were analyzed with ANOVA followed by Duncan's test for multi-group comparison. Results are expressed in percentage and reported as mean ± standard deviation. All statistical procedures were run using GraphPad Prism 6.

3. Results

3.1. Fertility.

The fertility rate of each female were estimated as the mean of fertilized eggs number per female per day from the 8th to the 21st day of treatment. The results were expressed in terms of fecundity (Fig.1) and shows that all the tested doses were able to provoke a statistically significant decrease of the fecundity. The lowest and the highest concentrations of DiNP elicited the greatest differences compared to control (Fig. 1).

3.2. Molecular analysis of genes related to oogenesis.

Exposure to DiNP resulted in modulation of genes involved in steroidogenesis, but the differences did not follow a simple dose-response relationship. The *star* and *cyp11a1* transcript level response to DiNP was non-monotonic, with greater difference observed at the lowest and highest concentrations (Fig. 2A and B). For *fshr* transcript only exposure to the lowest concentration of DiNP resulted in significant difference from control (Fig. 2C). For *esr1* and *esr2b* only higher dose of the contaminant significantly reduced the transcript levels, whereas the *esr2b* response was non-monotonic with both lowest and highest concentration of DiNP reducing the transcript level (Fig. 2 D, E, F).

Among the genes involved in oocyte maturation transcript level for, *pgrmc2* did not change for any of the doses tested, whereas *lhcgr* and *pgrmc1* transcript levels only reduced at the higher doses of DiNP (FIG. 3A,B, C). Conversely, *bmp15* transcript level was significantly increased following the lowest dose of DiNP tested, but was not affected at higher doses (Fig. 3D). The *gdf9* transcript level was not affected following exposure to DiNP (Fig. 3D, E).

Transcript levels for genes involved in apoptosis, *caspase 3*, *tp53* remained unchanged following exposure to DiNP (Table 2). However, exposure to DiNP significantly increased autophagy gene *ambra1a* at the lowest concentration tested, but was without effect on *beclin1* (Table 2).

3.3. Histological analysis.

For the histological analysis, oocytes were counted and divided into three classes: previtellogenic, vitellogenic and in maturation. The results reveled no significant changes in the number of previtellogenic oocytes between treated groups and control one except for the dose of 42 µg/L, in which we observed a higher number of previtellogenic oocytes (Fig. 4A). The number of vitellogenic oocytes, however, was significantly decreased in ovary treated with the 3 lower dose of DiNP (42 µg/L, 4,2 µg/L, 42 µg/L) compared to control while exposure to the higher doses (420 µg/L and 4200 µg/L) were without effect (Fig. 4B). The number of mature oocytes was lower in the ovary of fish exposed to 0,42 µg/L, 420 µg/L and 4200 µg/L of DiNP. No differences were observed to 4,2 µg/L and 42 µg/L of DiNP (Fig. 4C).

3.4. Fourier transform infrared FT-IR.

For all ovary sections, specific areas containing III class oocytes were selected, and photomicrographs and corresponding chemical maps were acquired. The spectra extracted from the chemical maps were integrated under 2990-2836 cm^{-1} (lipids), 1723-1481cm^{-1} (Amide I and Amide II), 1137-1060 cm^{-1} (phosphate groups), 1765-1723 cm^{-1} (COO groups), 3027-2995 cm^{-1} (=CH groups). All these values were divided by the *cell* one. These bands were chosen in order to quantitatively locate the presence and the characteristics of vitellogenin's derived proteins inside the oocytes. Chemical maps integrated under lipids, proteins and phosphate groups (Fig. 5B, D, F) visually show with assigned colour scale. The intensity of the signal associated with a specific band, provide information on both the presence and the localization of molecular/chemical group. A quantitative analysis was also performed as showed in figure 5 for lipids, proteins and phosphate groups which were significantly decreased in all the experimental groups following exposure to DINP. In particular, lipids composition were significantly decreased following exposure to DINP at all doses tested (Fig. 5A. The total amount of proteins also decreased with the exception of the group exposed to 42 μg/L DiNP (Fig. 5C). Finally, phosphate groups amount was found to decrease in the groups exposed in t to DiNP treated groups similar to proteins (Fig. 5E). All these results can be attributed to macromolecules in vitellogenic stage oocytes, presumably associated with vitellogenin derivates because of its nature of phospholipoglycoprotein. In the present study, we also investigated the amount of =CH for the lipids and COO groups for the proteins. As it can be deduced by the numerical data there is an increase in the amount of these two chemical groups, especially at the lowest and the highest doses of DiNP (Fig 6A, B).

4. Discussion

This study provide novel information on potential harmful effects of exposure to DiNP in female zebrafish. This may have wider implications since there is 70% gene orthology between zebrafish and humans as well as other similarities (Howe et al., 2013). The present results are consistent with recent findings showing decreasing trend in human fertility, even in young women (Brannian and Hansen, 2006; Nyboe Andersen and Erb, 2006). The present results provide evidence that chronic exposure to DiNP at low environmentally

relevant concentrations can disrupt normal reproduction in zebrafish by affecting gene expression pattern, chemical composition of follicles as well changes in ovarian morphology.

Considering the widespread use of DiNP in Europe, mainly due to its introduction as a substitute for DEHP, the present results are alarming. The present results are also consistent with earlier studies demonstrating that in testis, DiNP acts similarly to DEHP by reducing genes involved in steroid synthesis such as *StAR*, *P450scc* and *CYP17*, leading to reduced testosterone levels (Boberg et al., 2011; Borch et al., 2006). Furthermore, Hannon et al. (2015) demonstrated the ability of DEHP to induce atresia in cultured mouse antral follicles and to upregulate apoptotic biomarkers such as *Capase3* and *Caspase8*. The latter authors also observed a decrease in estradiol level, which is consistent with the observed ability of DEHP to modify mRNA levels of the estradiol biosynthesis enzymes, such as *Cyp19a1* and *Star* (Hannon et al., 2015). In addition, DEHP was found able to decrease the expression of two genes important in the control of oogenesis including LHR and mPRβ (Oliana Carnevali et al., 2010).

In the present study we investigated transcript levels for genes involved in reproduction, including *star* and *cyp11a1* which are involved in the transfer of cholesterol through the mitochondrial membrane (Arukwe, 2008) and of cholesterol into pregnenolone (Quek and Chan, 2009), repectively. *Fshr* is important to oocyte growth, while the three estrogen receptor subtypes in zebrafish ovary (*esr1*, *esr2a*, *esr2b*) are important mediator of ovarian development (Kohli et al., 2005). To check whether DiNP could interfere with oocytes maturation, we investigated *lhcgr* transcript level which is involved in the production of LH-mediated progesterone production as well as two subtypes of membrane progesterone receptors (*pgrmc1* and *pgrmc2*) which are involved in the first meiotic division. Local factors such as *bmp15* and *gdf9*, involved in the block of precocious oocytes maturation, have also been investigated (Danforth, 1995; Ge, 2005; Hillier, 2001). We also investigated transcript levels for genes involved in the control of apoptosis and authophagy including *caspase3*, *tp53* and *ambra1a*, *beclin1* because of the evidence that autophagy – unlike apoptosis- increases energy recycling efficiency, exerting a positive effects on zebrafish reproduction (Giorgia Gioacchini et al., 2012).

Changes observed in transcript abundance for genes involved in the control of reproduction following exposure to DiNP provide evidence for interference of this contaminant and disruption of reproductive endocrine system in zebrafish. Interesting results were also obtained on non-monotonic effects of exposure to DiNP. For a

number of parameters measured more significant effects were observed following exposure to lowest and higher doses of DiNP. The observed effect at the lowest doses tested is particularly alarming since it implies that the presence of and use of DiNP could in time cause severe environmental impact on fish population and cause adverse health effects in fish and possibly higher vertebrates. Non-monotonic mode of action, also indicates that DiNP, like many environmental contaminants, including DEHP work through multiple receptor pathways (Jordan et al., 2012; Kinch et al., 2015). Non-monotonic response is likely resulting from overlap of specificity of DiNP at higher concentrations by possibly acting on multiple receptor systems and/or biological pathways (Jordan et al., 2012; Kinch et al., 2015). In this case the observed non-monotonic is consistent with previous studies (Vandenberg et al., 2012). It is alarming that the results demonstrate that in a number of studies, the strongest effects were observed following exposure to the lowest (0,42 µg/L) dose of DiNP which is environmentally relevant concentration. The results suggests that prevalence of environmental DiNP could potentially cause health impairment in aquatic species, and possibly other vertebrates exposed to this contaminants. The present results demonstrate that DiNP does not affect the regulation of oocyte maturation directly, but significantly altered steroidogenesis and oocyte growth which is similar to the effects reported for DEHP (Boberg et al., 2011; Borch et al., 2006). The present results are in agreement with studies reported by Hannon et al. 2015 who demonstrated the ability of DEHP to downregulate steroidogenic processes, leading to a reduction in the estradiol production in mouse. While there are similarities between results obtained in zebrafish and mouse, observation in the present study regarding apoptotic and autophagic indicators are different from mouse (Hannon et al., 2015).

The capacity of the higher doses of DiNP to reduce gene's expression levels of *lhcgr* and *pgrmc2* is in agreement with the results obtained by Carnevali and co-workers demonstrating reduction of *lhcgr* and *pgrmc2* transcript levels following exposure to DEHP (Oliana Carnevali et al., 2010) which provide further evidence that both DEHP and DiNP cause adverse effects on female reproductive physiology in similar ways.

The observed transcript results are supported by histological analysis, which shows a significant reduction in the number of vitellogenic oocytes in the ovary of the fish treated with the lowest DiNP dose, as well as reduction in the number of mature oocytes following exposure to highest dose of DiNP. Reduction in number of mature oocytes is reflected in the observed decrease in the number of ovulated eggs.

The observed decrease in lipids, phosphates and proteins by FT-IR measurements, suggests that exposure to DiNP reduce uptake of vitellogenin which is also consistent with lower number of eggs observed. Moreover, it is interesting to highlight that the trends of =CH and COO, typical of lipids that have undergone a lipid peroxidation, are similar, with an increase in the amount of these two chemicals groups, especially at the lowest and at highest doses of DiNP. These observations suggest that adverse effects of DiNP include lower uptake of vitellogenin and changes in composition of lipids inside the follicles.

5. Conclusions

In summary, the present study demonstrates that DiNP adversely affected female reproductive physiology acting in a non-monotonic fashion. The lowest dose mainly interfere with steroidogenesis and oocyte growth, while the highest doses impair the oocyte maturation. Taking into account previous work that demonstrate similar effects of DEHP on zebrafish reproduction (Oliana Carnevali et al., 2010; Hannon et al., 2015), we can conclude that DiNP may not provide a safe substitute of DEHP and more studies on DiNP safety will be needed on safety of DiNP in fish and other organisms.

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Table 1. Lists of primers used in gene expression analyses by Real-Time qPCR.

Gene	Forward primer (5'- 3')	Reverse primer (5'- 3')	Acc number	Gene ID
18S rRNA	TCGGAAAACGGTGAACCTG	AAGGTCTTTGAACCCACGG	NM_001098396.1	100037361
arp	CTGAACATCTCGCCCTTCTC	TAGCCGATCTGCAGACACAC	NM131580.2	58101
star	CCAAGTGCAGATGACCCCAA	GGAAGGTGTGTGCCCTTGTT	NM_131663.1	63999
cyp11a1	GCAGGATTGCCGAGACTGA	TCTGCTGGCATTCAGTGGT	AF527755.1	80374
esr1	GGTCCAGTGTGGTGTCCTCT	AGAAAGCTTTGCATCCCTCA	NM_152959.1	259252
esr2a	TAGTGGGACTTGGACCGAAC	TTCACACGACCACACTCCAT	NM_180966.2	317734
esr2b	TTGTGTTCTCCAGCATGAGC	CCACATATGGGGAAGGAATG	NM_174862.3	317733
fshr	GATTCTTCACCGTCTTCTCC	TGTAGCTGCTCAACTCAAACA	NM_001001812.1	195820
lhcgr	GGCGAAGGCTAGATGGCACAT	TCGCAATCTGGTTCATCAATA	NM_205625.1	402920
pgrmc1	CGGTTGTGATGGAGCAGATT	AGTAGCGCCAGTTCTGGTCA	NM_001007392.1	492520
pgrmc2	ACAACGAGCTGCTGAATGTG	ATGGGCCAGTTCAGAGTGAG	NM_213104.1	406378
bmp15	AGGGTGACCGGATCACTATG	TGCTGCCAGACTTTTTAGACC	NM_001020484.1	334183
gdf9	CGACCACAACCACCTCTCTCC	GGGACTGAGTGCTGGTGGATGCC	NM_001012383.1	497643
caspase3	GTGCCAGTCAACAAACAAAG	CATCTCCAACCGCTTAACG	NM_131877.3	140621
ttp53	GGCTCTTGCTGGGACATCAT	TGGATGGCTGAGGCTGTTCT	AF365873.1	30590
beclin1	GGACCACTTGGAACAACT	CCGAAGTTCTTCAGTGTCCATC	AB266448.1	393846
ambra1a	TCTTTCGAGAAATGGCACCT	CTCTCTGCGTTAGGGACAGG	HE602022.1	100332642

[a]**Table 1.** *arp*: acidic ribosomal protein; *star*: steroidogenic acute regulatory protein; *cyp11a1*: cytochrome P450, family 11, subfamily A; *esr1*: estrogen receptor 1; *esr2a*: estrogen receptor 2a; *esr2b*: estrogen receptor 2b; *fshr*: follicle stimulating hormone receptor; *lhcgr*: luteinizing hormone/choriogonadotropin receptor; *pgrmc1*: progesterone membrane receptor component 1; *pgrmc2*: progesterone membrane receptor component 2; *bmp15*: bone morphogenetic protein 15; *gdf9*: growth differentiation factor 9; *tp53*: tumor protein 53; *ambra1a*: autophagy/beclin-1 regulator 1a.

Table 2. Transcriptional profiles of genes involved in apoptosis and autophagy.

Gene	Ctrl	DINP 0,42 µg/L	DINP 4,2 µg/L	DINP 42 µg/L	DINP 420 µg/L	DINP 4200 µg/L
caspase3	2,94±0,45[a]	2,05±0,58[a]	2,72±1,09[a]	3,23±1,02[a]	2,91±0,95[a]	2,97±0,85[a]
tp53	2,58±0,83[a]	2,18±0,99[a]	2,55±1,18[a]	3,37±1,60[a]	3,24±1,41[a]	4,52±1,78[a]
ambra1a	1,75±0,99[a]	6,78±2,35[b]	4,22±1,92[a,b]	3,86±1,75[a,b]	2,06±0,67[a]	2,05±0,78[a]
beclin1	2,24±0,63[a]	2,25±0,61[a]	1,66±0,64[a]	2,71±0,32[a]	2,14±0,47[a]	2,30±0,65[a]

[b]**Table 2.** *caspase3* (F = 0.9603; P = 0.4651); *tp53*: tumor protein 53 (F = 1.733; P = 0.1710); *ambra1a*: autophagy/beclin-1 regulator 1a (F = 7.365; P = 0.0003); *beclin1* (F = 1.596; P = 0.2047). Data are expressed as mean ± standard deviation; letters indicate differences between treatments ($p < 0.05$ compared with untreated controls; ANOVA followed by Tukey's multiple comparison tests). Data were generated in duplicate from five biological replicates.

Figure Legends

Fig. 1: Mean of fertilized eggs number per female per day during the last two weeks of treatment.

Results are expressed as the mean ± standard deviation of fertilized egg number / female / day from the 8th to the 21th day of treatment. Letters above each column indicate statistical differences among groups.

Fig. 2: Transcription profiles of genes involved in steroidogenesis and oocyte growth.

Letters above each column indicate statistical differences among groups ($p < 0.05$ vs. untreated controls; ANOVA followed by Tukey's multiple comparison test). A: *star*: steroidogenic acute regulatory protein (F = 18.11; P = 0.0001); B: *cyp11a*: cytochrome P450, family 11, subfamily (F = 8.513; P = 0.0003); C: *fshr*: follicle stimulating hormone receptor (F = 5.490; P = 0.0018); D: *esr1*: estrogen receptor 1 (F = 9.220; P = 0.0005); E: *esr2a*: estrogen receptor 2a (F = 10.27; P = 0.0001); F: *esr2b*: estrogen receptor 2b (F = 5.601; P = 0.0028); Data were generated in duplicate from five biological replicates.

Fig. 3: Transcription profiles of genes involved in oocyte maturation and germinal vesicle breakdown.

Letters above each column indicate statistical differences among groups ($p < 0.05$ vs. untreated controls; ANOVA followed by Tukey's multiple comparison test). A: *lhcgr*: luteinizing hormone/choriogonadotropin receptor (F = 6.04; P = 0.0012); B: *pgrmc1*: progesterone membrane receptor component 1 (F = 3.182; P = 0.0330); C: *pgrmc2*: progesterone membrane receptor component 2 (F = 6.540; P = 0.0017); **.** *bmp15*: bone morphogenetic protein 15 (F = 6.068; P = 0.0013); *gdf9*: growth differentiation factor 9 (F = 5.255; P = 0.0028). Data were generated in duplicate from five biological replicates.

Fig. 4: Histological analysis of ovaries from control fish and fish exposed to five doses of DiNP (0,42 µg/L; 4,2 µg/L; 42 µg/L; 420 µg/L; 4200 µg/L).

A: Percentage of previtellogenic oocyte on the total oocytes counted in the ovary of treated fish with different DINP doses respect to control; **B**: Percentage of vitellogenic oocyte on the total oocytes counted in the ovary of treated fish with different DINP doses respect to control; **C**: Percentage of oocyte in maturation on the total oocytes counted in the ovary of treated fish with different DiNP doses respect to control; **D**: Picture of control ovary: oocytes in previtellogenic stage are pointed as pvtg; oocyte in vitellogenic stage are pointed as vtg; oocyte in maturation are pointed as mat.

Fig. 5: quantitative analysis of lipids (A), proteins (C) and phosphate (E) groups.

B: chemicals map integrated under the lipids stretching region (3100–2800 cm^{-1}); **D**: chemicals map integrated under proteins group (1800–1480 cm^{-1}); **F**: chemicals map integrated under phosphate groups stretching region (1100-1065 cm^{-1}).

Fig. 6: quantitative analysis of CH and COO groups.

Fig.1

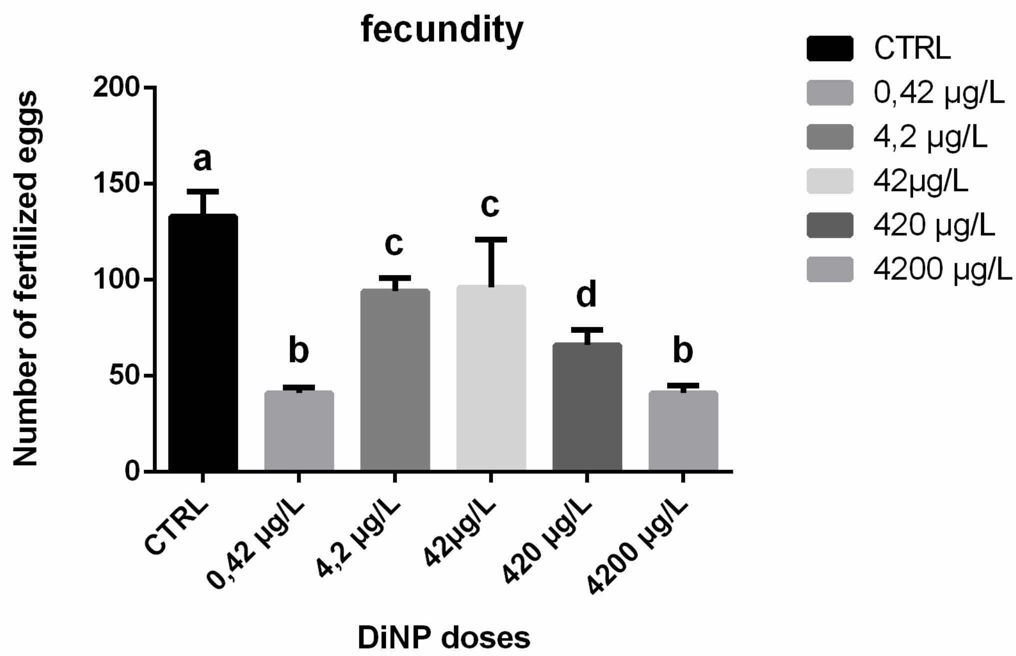
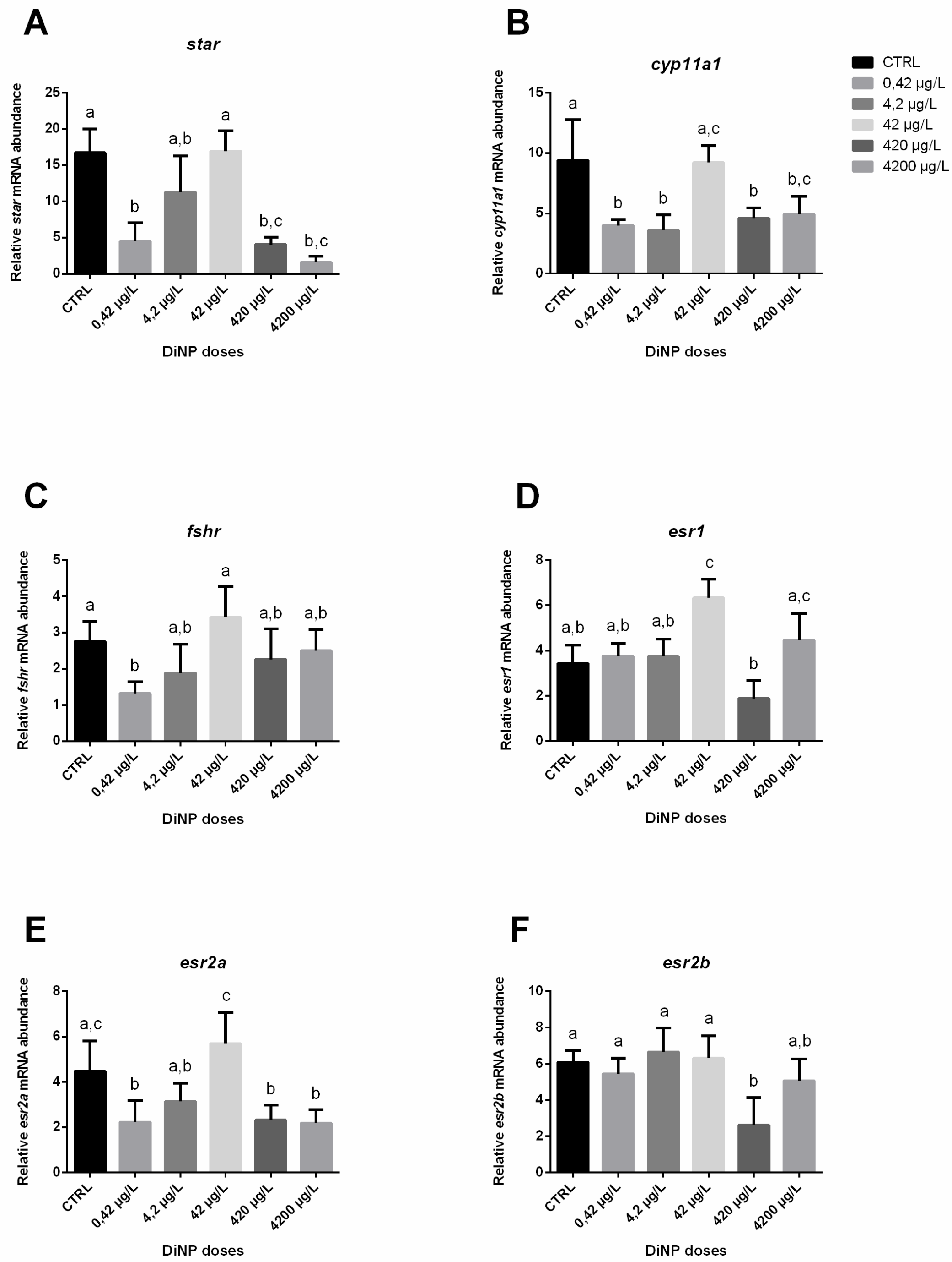


Fig.2



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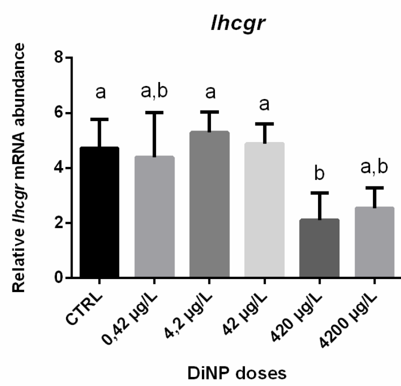
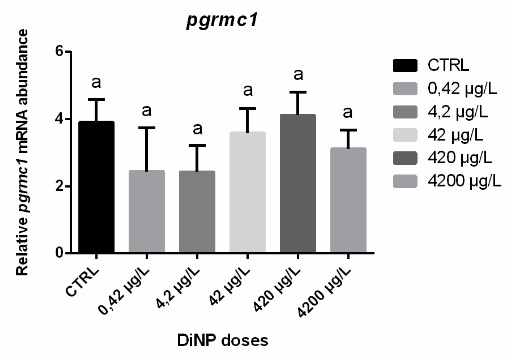
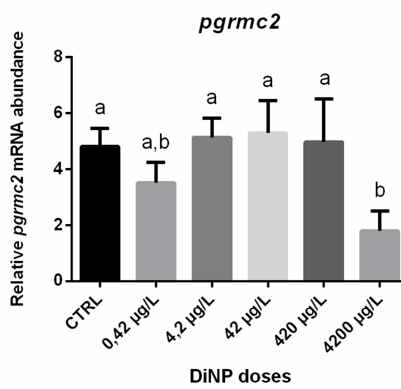
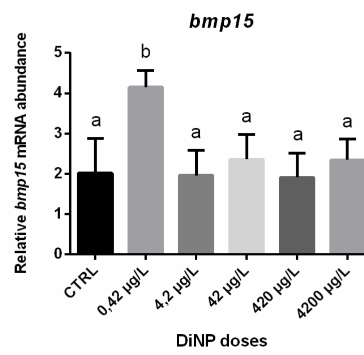








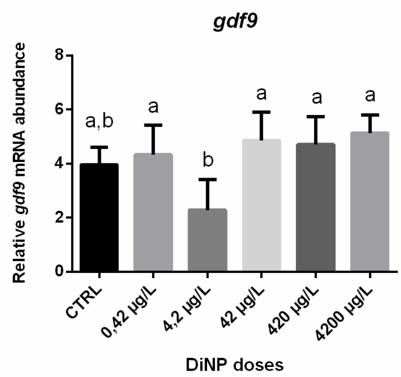
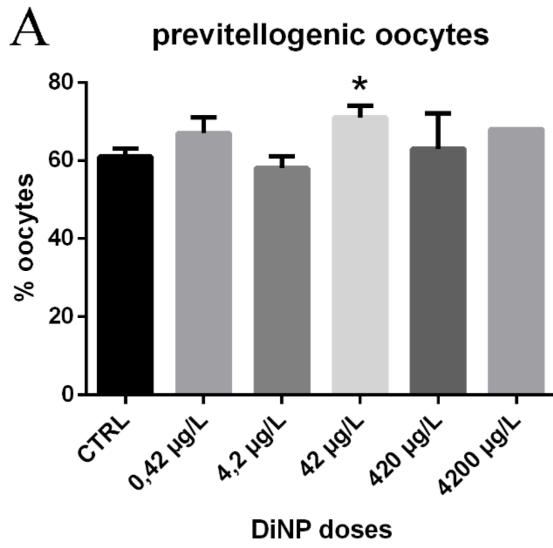
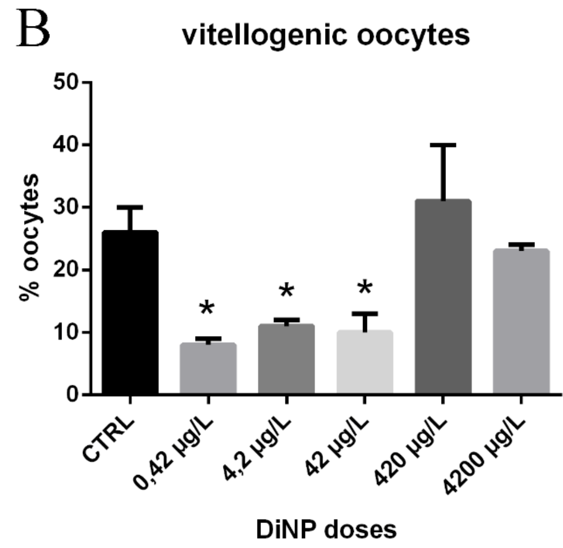
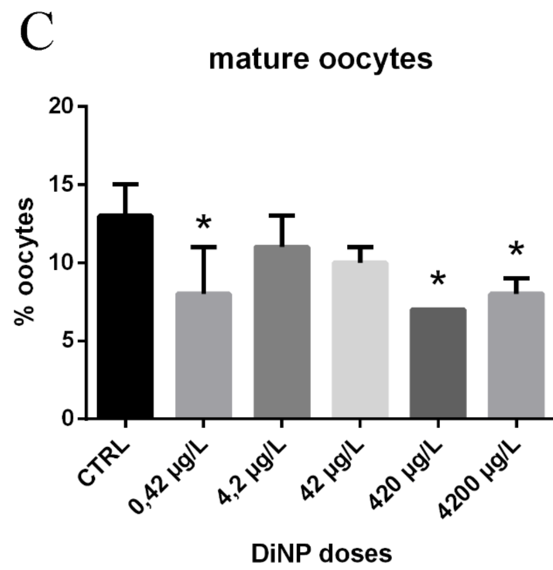


Fig.4







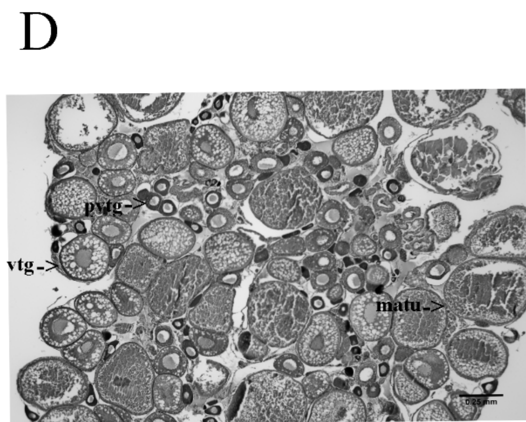


Fig.5

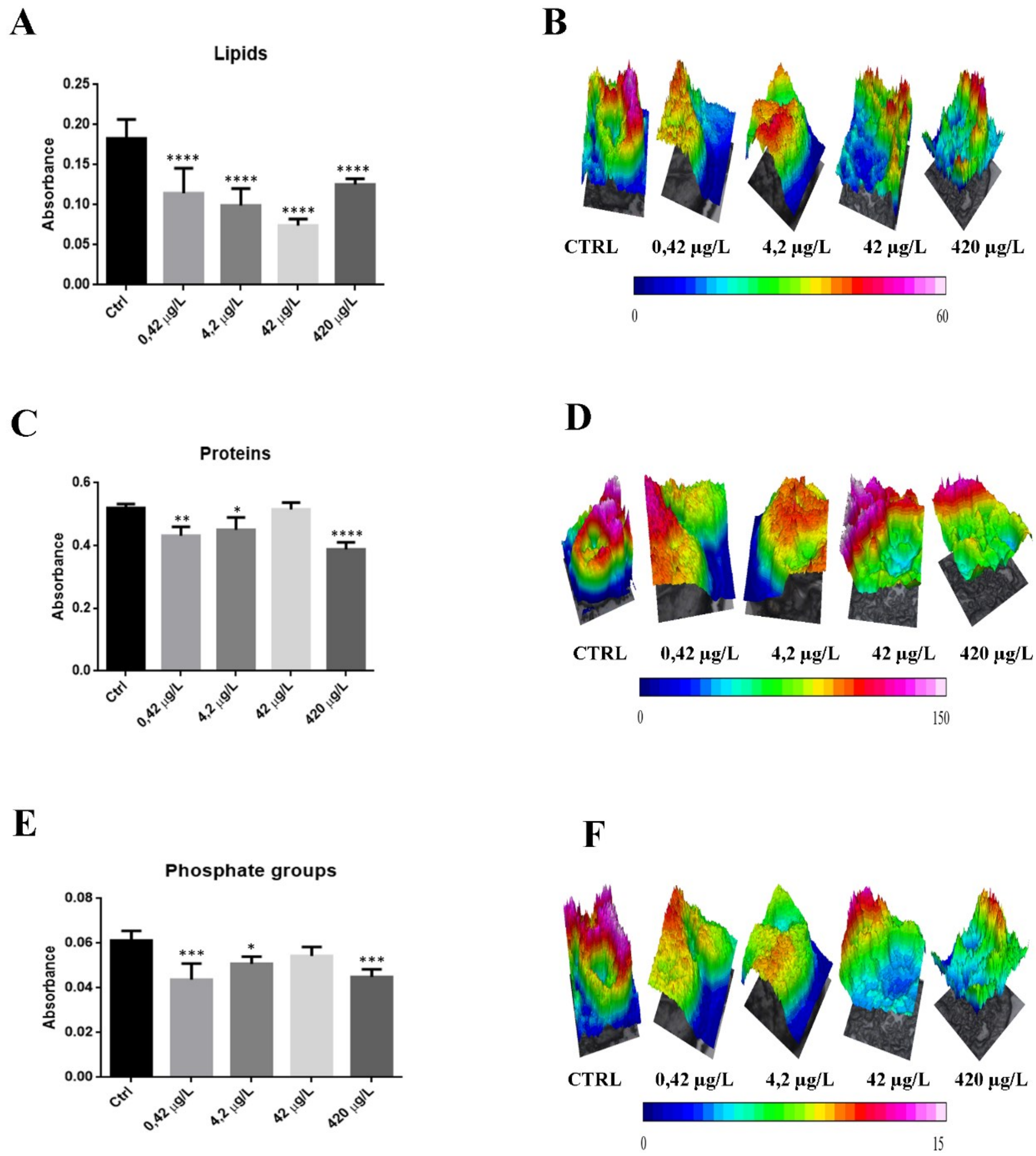
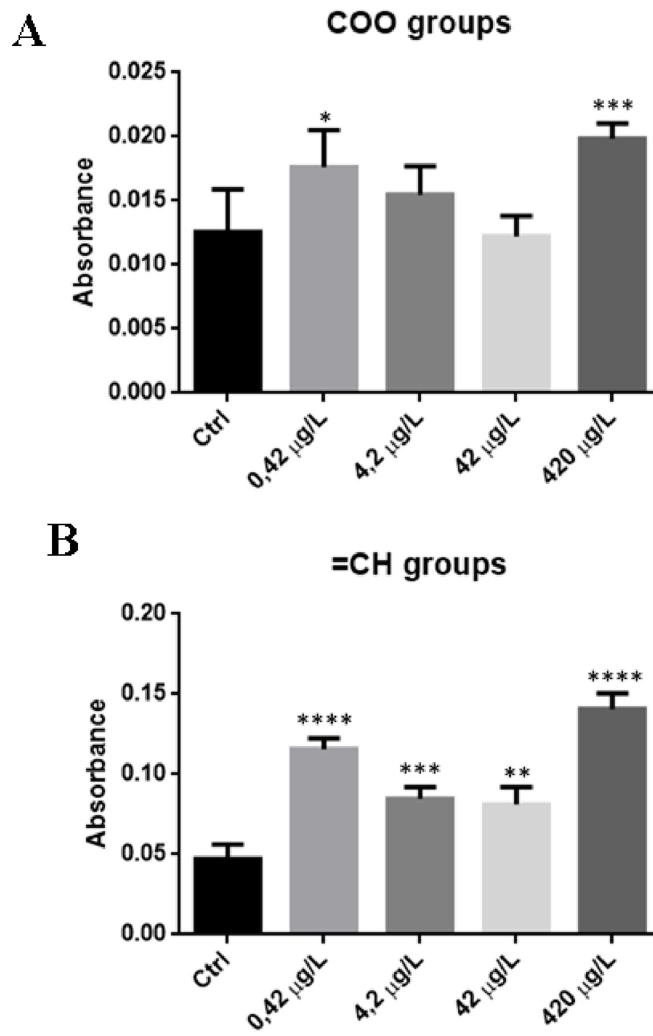


Fig.6



6. OBESOGENIC EFFECTS OF ENVIRONMENTAL POLLUTANTS: DISRUPTION OF LIPID METABOLISM IN *DANIO RERIO*

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Abstract

Traces of compounds and additives used in the manufacturing of plastic and latext materials can be found in our environment and everyday products, including plastic bottles, metal food cans, detergents, food additives, toys, cosmetics, and pesticides. Here we investigate the adverse effects of Bisphenol A (BPA) which is used to make polycarbonate plastic and epoxy resins, and Dhietylene glycol dibenzoate (DGB) which is used in latex products, industrial coatings and certain printing inks. BPA and related chemicals are used to give plastic products a balance of toughness, heat and electrical resistance, and optical clarity. Therefore BPA is among the most aboundant contaminants in the world. DGB is less aboundant but can be found in aquatic media because reasonable water solubility. The focus of the present study is to investigate the adverse effects of BPA and DGB on lipid metabolism in liver which can be a contributing cause of obesity. In the present study, we exposed fish to three concentrations of BPA (5; 10; 20 µg/L) and five concentrations of DGB (0,01; 0,1; 1; 10; 100 µg/L) for a period of three weeks.

The results provide evidence for detrimental effects of BPA and DGB on lipid metabolism. Lower concentration of BPA was found to increase the storage of triglycerides and promote fatty acid synthesis. Exposure to DGB was also found to affect lipid metabolism leading to increased lipid production and their mobilization. The findings link BPA and DGB to adverse lipid metabolism in fish, and provide an insight into potential cause of environmentaly-induced obesity which is a serious problem in developed countries.

1. Introduction

Obesity is a major health burden, troubling more than 20% of Western populations both in the United States and worldwide (Wang et al. 2014). In agreement with the World health Organization, approximately 310 million people worldwide are obese (Bessesen 2008). This finding is particularly worrying due to the strong association between obesity and comorbid metabolic and chronic disease (Wang et al. 2014).

Obesity is defined as the expansion of adipose mass and tissue through adipocyte hypertrophy and hyperplasia (Siriwardhana et al. 2013). It is a multifactorial complex disease, affected by lifestyle, behavior, environmental and genetic factors, which originates from an imbalance of energy due to the excessive caloric intake compared to the actual energy expenditure. This conditions can be caused by a sedentary lifestyle combined with the lack

of physical activity, although there are other contributing factors including genetic susceptibility and family as well as environment interactions (Bouchard 2010; Heber 2010).

Recent studies have linked a number of environmental chemicals to obesity. This chemicals, defined as obesogens, are substances that increase weight gain acting directly or indirectly on fat cells by altering the regulators of appetite and satiety, or changing the metabolic rate and the energy balance and thus favoring the calories storage (Alonso-Magdalena et al. 2011; Grün and Blumberg 2009; Janesick and Blumberg 2011). A number of known obesogens have hormone-like activity and function as endocrine disrupting chemicals (EDCs). These include chemicals used for the manufacturing of plastic materials which are widly aboundant (North and Halden 2013; Schug et al. 2011). The aim of the present study is to investigate the effects of two EDCs involved in plastic and latext manufacturing, including BPA which is one of the main copunds used in plastic manufacturing worldwide (Crain et al. 2007; Kang et al. 2006; Rubin 2011), and DGB which is an approved alternative to phthalates in the processing of plastic and latex (Kermanshahi et al. 2009).
To achieve this aim, we investigated the expression of a number of genes involved in fatty acid synthesis, storage, oxidation and transport (Chang et al. 2009; Clarke 1993; Dai et al. 2014; Desvergne and Wahli 1999; Flynn et al. 2009; Jensen-Urstad and Semenkovich 2012; Karanth et al. 2009; Lee et al. 2003; Lodhi and Semenkovich 2014; Zeng et al. 2014) in the liver of zebrafish exposed to different concentrations of BPA (5 μg/L; 10 μg/L; 20 μg/L) and DGB (0,01 μg/L; 0,1 μg/L; 1 μg/L; 10 μg/L; 100 μg/L). We measured transcript aboundance by qPCR and assessed lipid composition in the liver by Fourier transform infrared (FTIR) imaging. The latter technic represents a robust and well-assessed vibrational technique, which makes use of IR microspectrometers coupled with visible light microscopes and bidimensional detectors to give spatially resolved biochemical information of biological samples (Burattini et al. 2007).
Adult zebrafish (*Danio rerio)* was used as a suitable vertebrate animal model for medically relevant studies (Schlegel and Stainier 2007). Zebrafish adipose tissues and skeletal muscle are organized in a similar way to humans (Oka et al. 2010). In addition, the neuronal and endocrine signals that regulat food intake as well as nature of response to small molecules, drugs and environmental toxicants in zebrafish are similar to mammals (Gorissen et al. 2009; Nishio et al. 2008). Thus, zebrafish would be a suitable model organism for assessing adverse impact of contaminants on metabolism and the results would be relevant to mammals (Lam et al. 2011; Oka et al. 2010).

2. Methods

2.1. Animals and BPA and DGB administration

The experiments were carried out in adult female zebrafish (*Danio rerio*, wild-type strain). Fish were maintained in 100-L aquaria with oxygenated water under controlled conditions (28.0 ± 0.5˚C under a 14/10 hours of light/dark period) and were fed four times per day, twice with commercial food (Vipagran; Sera, Loessnitz, Germany) and twice with *Artemia salina*. For the BPA experimentation, a total of 40 females were equally distributed into four aquaria (one control and three BPA experimental groups) with a total of 10 female fish in each one and treated for a three-week time with three different concentrations of BPA (BPA, 98% analytical purity, Sigma-Aldrich) (5 μg/L; 10 μg/L; 20 μg/L). For the DGB experimentation, a total of 60 females were equally distributed into six aquaria (one control and five DGB experimental groups) with a total of 10 female fish in each one and treated for a three-week time with five different concentrations of DGB (DGB, 90% analytical purity, Sigma-Aldrich) (0,01 μg/L; 0,1 μg/L; 1 μg/L; 10 μg/L; 100 μg/L). BPA and DGB were administered to fish via water in a static system and they were renewed every four days. After twenty-one days of treatment, fish were lethally anesthetized with 500 mg/l of MS-222 (3-aminobenzoic acid ethyl ester, Sigma Aldrich, Milano, Italy) buffered to pH 7.4 (according to University of Calgary animal care protocol for care and use of experimental animals) and liver samples were collected and weighted to calculate the hepatosomatic index (HSI; the ratio of liver weight to body weight). Five liver from each experimental group were fixed for Real Time semi-quantitative Polymerase Chain Reaction (qPCR) and five were fixed for FT-IR imaging analysis.

2.2. RNA extraction and cDNA synthesis

Total RNA was isolated from the liver with RNAzol solution (Sigma Aldrich) according to the manufacturer's instructions. Its final concentration was determined using the Nanophotometer TM P-Class (Implen GmbH, Mϋnich, Germany), while its integrity was verified by GelRed staining of 28S and 18S ribosomal RNA fragments on a 1% agarose gel. Total RNA was treated with DNAse to remove genomic DNA (10 IU at 37 ˚C for 10 min; Fermentas MBI, Amherst, NY, USA). Then an amount of 1 μg of total RNA was used for cDNAs

synthesis employing the SuperScript-II kit (Invitrogen,Life Technologies), which were then kept at -20°C until use.

2.3. Real-Time qPCR

The relative quantification of gene expressions was performed with the SYBR green method in an iQ5 Multicolor Real-Time PCR Detection system (Bio Rad). All samples were analyzed in duplicates. The reactions consisted in 1 µl of diluted (1/10) cDNA, 5 µl of 2X SYBR Green PCR Master Mix (Bio Rad) containing SYBR Green as a fluorescent intercaling agent, 0.1 µM of both forward and reverse primers and 3.8 µl of milliQ water (Table 1). The thermal profile for all reactions consisted in i) enzyme activation at 95 ˚C for 3 min, ii) 45 cycles of denaturation (10 s at 95 ˚C) followed by a 20-s annealing at 60˚C for *apoA4*, *apoBa*, *fasn*, *fit2*, 59˚C for *apoAla*, *pparaa*, *pparg*, 58˚C for *hnf4a*, *dgat2*, *srebf2*, 57˚C for *rxraa*, *cebpa*, *fabp11a* and 56˚C for *agpat4*, *acat2*, *srebf1*, and iii) 20 s elongation at 72˚C. Fluorescence was monitored at the end of each cycle. Dissociation curve for primer specificity and absence of primer-dimer formation check was performed and, in all cases, it showed a single peak.

Two different genes, namely *18S* rRNA genes (Tang et al. 2007) and *arp,* acid ribosomal protein (Aursnes et al. 2011), were used as internal controls with the purpose of allowing standardization of the results, by eliminating variations in quality and quantity of mRNA and cDNA (Bustin et al. 2009). These were chosen because their mRNA levels did not vary neither between experimental treatments nor between follicular stages. No amplification product was observed in the negative control (absence of template). Data were analysed using iQ5 Optical System version 2.1 (Bio-Rad). Applied quantification method was based on a ΔΔCt calculation implemented with the Pfaffl equation to improve accuracy by accounting for a varied reaction efficiencies depending on primers (Pfaffl 2001; Vandesompele et al. 2002). All results are expressed with respect to control livers.

2.4. Fourier transform infrared FT-IR

FT-IR measurements were carried out by using a Bruker VERTEX 70 interferometer coupled with the Hyperion 3000 Vis-IR microscope and equipped with a liquid nitrogen cooled bidimensional focal plane array (FPA) detector (area size 64 × 64 pixels) on livers treated with three concentrations of BPA (5; 10; 20 µg/L)

and four concentrations of DGB (0,01; 0,1; 10; 100 μg/L). For each sample, images were acquired in transmission mode using a 15× condenser/objective, therefore achieving a pixel resolution of about 2.65 μm. On each section, we picked three randomly-selected regions, on which IR images were taken acquiring simultaneously groups of 4096 spectra, averaging 256 scans for each detector pixel with a spectral resolution of 4 cm^{-1}. Background single channel images were acquired on clean regions of the CaF_2 windows. By using OPUS 7.2 software (Bruker), chemical maps were generated for each sample, integrating under the lipids stretching region (3100–2800 cm^{-1}), the Amide I and II modes (1720–1480 cm^{-1}), the phosphate groups stretching region (1286-1191 cm^{-1}) and the ester C=O stretching vibration (1769-1718 cm^{-1}). All the 4096 spectra were extracted from IR images of each chemical map (OPUS 6.5, Bruker): no selection of spectra was performed, thanks to the homogeneity of the hepatic tissue. All the extracted spectra were two-point baseline linear fitted in the spectral range of 4000–900 cm^{-1}, compensated from the atmospheric contributions of aqueous vapor and CO_2, and vector normalized. Spectra were analyzed in the following spectral regions: 3030-2800 cm^{-1} for lipids and =CH groups, 1800–1480 cm^{-1} for proteins and C=O groups, and 1286-1191 cm^{-1} for phosphate groups. The lipids region added to the 1775-1065 cm^{-1} portion of the spectrum represented the total amount of chemical components of the sample, called "cell". On all the spectra collected from livers of each experimental group, an integration of the bands of interest was performed. In order to normalize all the peculiar characteristics of each spectrum in terms of intensity and absorbance, we divided the numerical value of the integrated band by the cell value.

2.5. Statistical analysis

All infrared FT-IR results were analysed using one-way ANOVA, followed by Dunnett's post-hoc test, in order to compare the differences between the control and each of the BPA and DGB concentrations used in the study. The transcript abundance results was performed with the one-way ANOVA analyses of variance followed by Turkey multiple comparison tests. Significance was set at $p < 0.05$. Results are presented as mean ± standard deviation. Histological results were analyzed with ANOVA followed by Duncan's test for multi-group comparison. Results are expressed in percentage and reported as mean ± standard deviation. All statistical procedures were run using GraphPad Prism 6.

3. Results

3.1. Hepatosomatic index.

Exposure to BPA significantly increased the HSI at the lowest concentraion tested (5 µg/L). Higher concentraions were without effect, compared to control (Fig. 1A). Exposure to DGB also increased HIS at low concentration of 0.1 µg/L. Higher concentrations of DGB were without effect compared to control (Fig. 1B).

3.2. mRNA abundance of genes related to lipid metabolism.

The overall results obtained by qPCR demonstrate that both BPA and DGB have the ability to interfere with lipid metabolism. The lowest BPA concentration (5 µg/L) significantly increased the transcription level of fat storage-inducing transmembrane protein2 (*fitm2*), acyl-coenzime A cholesterol acyltransferase (*acat2),* fatty acid binding protein 1A (*fabp11a*), sterol regulatory element-binding protein 1 (*srebf1*) and CCAAT/enhancer binding protein α (*cebpa*). In contrast, the mRNA levels for genes involved in the production of triacylglycerol (TAG), including diglyceride acyltransferase (*Dgat2*) and in fatty acid oxidation, as peroxisome proliferator-activated receptors α (*pparaa*) were decreased compared to control. All other gene transcript levels analyzed in zebrafish exposed to 5 µg/L of BPA, including fatty acid synthase (*fasn,)*, sterol regulatory element-binding protein 2 (*srebf2*), 1-acylglycerol-3-phosphate o-acyltransferase (*agpat4)*, peroxisome proliferator-activated receptors γ (*pparg*), retinoid X receptor α (*rxraa*), hepatocyte nuclear factor 4α (*hnf4a*), apolipoprotein a-IV (*apoA4b.1*), apolipoprotein B (*apoBa*) and finally apolipoprotein Ala (*ApoAla*) are not showing any change in gene expression respect to controls (Table 2).

At 10 µg/L of BPA increased transcript level of *apoA4b*.1, *fabp11a* and *srebf2*, decreased gene mRNA level for *dgat2*, *acat2*, *apoAla*, *srebf1*, *pparaa* and *cebpa.* BPA treatment was without effect on the remaining genes tested (Table 2).

The highest concentration of BPA (20 µg/L), however, increased transcript levels for *fasn*, *fitm2*, *apoA4b.1*, *apoAla*, *fabp11a* and *srebf2*, decreases *dgat2*, *srebf1* and *ceba*, but was without effect on other genes tested (Table 2).

Exposure to DGB at 0.01 µg/L significantly decreased transcript levels for *apoAla* and *cebpa,* and increased mRNA level for all other genes tested except for *agpat4b.1*, *fitm2*, *srebf1*, *pparaa*, *rxraa* and *hnf4a*, which remained unchanged compared to control (Table 3). Exposure to DGB at 0.1 µg/L, also increased *agpat4*, *dgat2*, *acat2*, *apoA4b.1*, *apoBa*, *fabp11a srebf2*, *pparg*, and reduced *fasn* and *cebbpa* transcript levels. (Table 3). At 1 µg/L, DGB increased *fabp11a* and *rxraa*, reduced *cebpa* mRNA levels. At 10 µg/L, DGB exposure increased transcript levels for *fasn*, *agpat4*, *apoBa*, *srebf2*, *rxraa* and decreased *dgat2*, *fitm2*, *acat2* and *cebpa* (Table 3). At the highest concentration tested (100 µg/L) DGB increased transcript levels for *agpat4, dgat2, fit2, acat2, apoA4b.1, apoBa, apoAla, fabp11a, srebp1, srebp2, pparaa, pparg, rxraa, ceba, hnf4a,* compared to control, and decreased mRNA for *fasn* and *pparg*. Transcript levels for *agpat4*, *fitm2*, *acat2*, *srebf1* and *hnf4a*, remained unchanged (Table 3).

3.3. Fourier transform infrared FT-IR.

Chemical maps integrated under lipids (Fig.2B; Fig.3B) give a visible display with a color scale. The intensity of the signal associated with a specific integrated band, enlightening about the localization of that molecule/chemical group. A quantitative analysis was performed via integration of extracted spectra; results are reported in Figures 2 and 3. Exposure to the lowest concentration of BPA (5 µg/L) significantly increased total lipid concentration in the liver samples. However, exposure to higher concentrations of BPA did not affect the amount of lipid molecules (Fig.2A; Fig.2B). Use of infrared FT-IR enabled us to estimate the quantity and investigate the characteristics of lipids. We calculated the amount of $–CH_2$ groups of lipids, in order to evaluate the length of aliphatic chains composing fatty acids: the comparison of integrated areas of this band among the experimental groups do not show any modification (Fig.2F). Integration of 1769-1718 cm^{-1} range is used to determine the amount of ester C=O groups of lipids: the dedicated histogram displays how at the lowest concentration (5 µg/L) C=O groups increased their quantity, while the other two concentrations (10 µg/L and 20 µg/L) decreased this chemical group (Fig.2E). We also evaluated the amount of total proteins in the samples and observed no significant effects of BPA on protein composition of livers (Fig.2D). As shown in figure 2C, the analysis of phosphate groups indicate that only the lowest concentration of BPA (5 µg/L) was effective, and decreased this class of chemical group.

Exposure to all concentrations of DGB also increased total lipid content in the liver samples, with the exception of the 10 µg/L (Fig.3A; Fig.3B). Exposure to DGB increased the intensity of the band assigned to $–CH_2$ group, indicating an increase in the length of aliphatic chains of the fatty acids (Fig.3F). We also observed increased bands associated with the C=O groups which reflects the degree of esterification of the lipids (Fig.3E). The analysis of AI and AII bands showed a modification in protein amount and/or composition: specifically, with all the concentrations of DGB (except for the 10 µg/L). We found a significant decrease in the intensity of AI and AII bands (Fig.3D). Phosphate groups did not change among the experimental groups (Fig.3C).

4. Discussion

In the present study we investigated the effects of environmental pollutants, BPA and DGB, on female lipid metabolism in the liver of zebrafish. Liver is a key metabolic organ which governs body energy metabolism. Maintaining energy expenditure involves the dynamic integration of two metabolically opposed states, fasting and feeding. Fasting involves the induction of catabolic or ATP generating pathways, whereas feeding engages anabolic or ATP-consuming pathways that built carbon skeleton and store energy. (Desvergne and Wahli 1999; Lee et al. 2003; Rui 2014). The major long-term regulatory control mechanism of lipid homeostasis, is the transcriptional regulation of genes involved in fatty acid metabolism which is executed by a variety of transcription factors among which the *srebps*, the *cebps*, and other members of the nuclear receptor family are particularly active agents. It is therefore important that different processes such as fatty acid synthesis, storage, oxidation, and mobilization are balanced (Desvergne and Wahli 1999; Desvergne et al. 2006; Miranda et al. 2011; Sheng et al. 2013).

To elucidate the effects of endocrine disruptors, used in the manufacturing of plastics, on lipid metabolism and particularly, if they can have any obesogenic effects, we investigated target genes which are crucially important for this processes.

Fasn is an enzyme that catalyzes the biosynthesis of saturated fatty acids from simple precursors in de novo lipogenesis. Its expression is activated by a specific binding in its promoter with srebf, hnf4α and liver X receptor (LXR) (Clarke 1993; Jensen-Urstad and Semenkovich 2012). Zebrafish possess two isoforms of *srebf*: *srebf1*, which is mostly involved in fatty acid metabolism, and *srebf2*, that on the contrary, is involved

more in cholesterol metabolism (Jeon and Osborne 2012; Walker et al. 2010; Xu et al. 2013). Another important gene involved in the conversion of cholesterol in cholesterol esters is *acat2* whose major function is to protect cells against unnecessary built up of cholesterol in the membranes (Chang et al. 2009; Rogers et al. 2014). Similarly to *srebf1* also *hnf4α* affects the expression of genes involved in glucose, fatty acid, cholesterol metabolisms, bile acid biosynthesis and transport of low density lipoproteins from the liver (Hayhurst et al. 2001; Watt et al. 2003).

Except for *hnf4α,* the nuclear receptors of the metabolic sensor class are active as heterodimers with rxraa which is ligand dependent. Rxraa is a nuclear receptor that functions as a transcription factor by binding to specific six-base-pair sequences of DNA in the promoter regions of genes. It is an obligatory partner for many nuclear receptors such as *ppars* (Dawson and Xia 2012; Desvergne and Wahli 1999; Desvergne et al. 2006). *Ppars* are recognized as fundamentally important regulators of lipid metabolism, and they are also regulators of genes important in cell differentiation, and other metabolic processes, such as glucose homeostasis. There are three members of the *ppar* family on which the most important are *pparaa.* The latter is a key regulator of fatty acid oxidation, and *pparg*, which contributes to energy storage by enhancing adipogenesis (Chen and Yang 2014; Lee et al. 2003; Lodhi and Semenkovich 2014).

Cebpa was chosen due to his role in lipogenesis (Desvergne et al. 2006; Lefterova et al. 2008; Matsusue et al. 2004) while *fabp11a* was chosen since fatty acid binding proteins are the most abundant single proteins in the cytosol of cells which are most active in long chain fatty acid uptake and metabolism, oxidation and storage, exploiting their role of binding free fatty acids (Flynn et al. 2009; Karanth et al. 2009).

Agpat4 is the gene that catalyse the second acylation step in the glycerol phosphate pathway (Yamashita et al. 2014), while *dgat2* catalyse the final step in triacylglycerol (TAG) synthesis, playing a key role in determining the carbon flux into TAG (Harris et al. 2011; Liu et al. 2012).

After their synthesis TAGs can be stored in lipid droplets by the action of *fitm2* (Gross et al. 2011; Kadereit et al. 2008), or together with cholesterol, they can be stored and mobilized through lipoprotein particles. Beyond TAGs and cholesterol, in lipoprotein we can find also different proteins such as apoliproteins, which constitute the skeleton of these particles. There are different forms of apolipoproteins absolving specific functions (Otis et al. 2015). *ApoA4b.1,* which major role is to facilitate the packaging of additional lipid into the chylomicron

particle (Kohan et al. 2011; Otis et al. 2015), *apoBa,* which is mainly involved in the assembly of very low density lipoprotein (Haas et al. 2013) and *apoAla*, which major functions involve activation of lecithin/cholesterol acyltransferase and endowing high-density lipoprotein with multiple-atherogenic activities (Eckardstein and Kardassis 2015).

Given the role of the genes tested in the present study, we demonstrate that BPA affects energy homeostasis in different ways dependent upon its concentration. At a concentration of 5 µg/L, BPA increased the lipid production and storage as supported by the higher level of *fitm2*, *acat2*, *fabp11a*, *srebf1*, *cebpa* and *fitm2* (Table 2) which is in accord with increased HSI (Fig. 1A).

The results provide a support for the hypothesis that BPA activates fatty acid synthesis pathway that can lead to production of cholesterol esters (*acat2* mRNA level increase while *dgat2* decrease) and conversion to lipid droplets by increased expression of *fitm2*. The present results can be compared with those obtained by Hu and colleagues (Hu et al. 2013) who observed the capacity of parabens to exert their obesogenic effects through the increase of *pparg*, *cebpa*, *fabp* and *fasn* expression and with the discovery made by Somm and colleagues (Somm et al. 2009) that low BPA concentrations can stimulate adipogenesis in rat females. Higher concentrations of BPA caused reduction in *dgat2*, *acat2*, *srebf1* and *pparaa* (Table 2), suggesting a general decrease of the fatty acid synthesis and oxidation, as well as a decrease of cholesterologenesis, as suggested by the *acat2* decreases (Table 2). This can lead to increased transportation of fatty acid and production of chilomicrons due to higher expression of *fabp11a* and *apoA4*. A possible outcome could be decreased production of high density lipoproteins. Based on the results, at 20 µg/L, BPA exposure can promote de novo lipogenesis and cholesterologenesis (increasing mRNA levels of *fasn* and *srebf2*) (Table 2), the storage of fat into lipid droplets (increasing mRNA levels of *fitm2*) and production of lipoprotein (increasing mRNA levels of *apoA4b.1* and *apoAla*) (Table 2). Previous studies by Verhague and colleagues (Verhague et al. 2013) reported that in mice affected by hepatic steatosis the induction of *apoaA-IV* promotes lipoprotein expansion despite of a reduction in hepatic lipid burden. The present results in zebrafish suggests that BPA may be contributing factor in development of lipid steatosis.

The results on DGB revealed more significant effects at lower concentration on lipid metabolism than higher levels. The lower DGB concentrations (0,01 µg/L and 0,1 µg/L) increased the *de novo* lipogenesis, cholesterol

esters (up regulation of *fasn*, *srebf2* and *acat2*) and TAG production (up regulation of *agpat* and *dgat2*) (Table 3), and the lipids were presumably converted into apolipoprotein particles. It is important to note that *apoAla* decreases its expression, while there are increasing mRNA levels of the apoliproteins involved in very low density lipoprotein and chylomicron production. Higher DGB concentrations (10 and 100 µg/L) also increased *cebpa* levels, a gene involved in adipocyte differentiation (Table 3). The transcript results are consistent with changes in HSI which appear to be non-monotonic fashion (Fig. 1B).

FTIR imaging was used to elucidate the effects of the pollutants on the chemical composition and assess the functionality of liver. The spectra extracted from the acquired chemical maps were analyzed on well-known spectral ranges: lipid stretching vibrations, Amides I and II, phosphate groups of nucleic acids, C=O moieties stretching modes, -CH2 groups vibrations; these regions were chosen in order to quantitatively locate the main biomolecules in liver samples, and to evidence eventual alterations in their presence, characteristics and distribution. Results of FT-IR imaging are consistent with those obtained by Real-Time qPCR. Hence, this vibrational technique shows how both BPA (Fig. 2) and DGB (Fig. 3) alter the biochemical composition of livers, as a result of the modification in lipid metabolism.

In particular, it is clear that the lowest concentration of BPA has a strong effect on the amount and the characteristics of the lipids (Fig. 2A), together with the phosphate groups (Fig. 2C) which decreased following treatments. Previous studies reported that a decrease in the intensity of the band associated with the phosphate groups can be related to apoptotic mechanisms (Ahmed et al., 2009).

Exposure to DGB also increased the lipid content (Fig. 3A) together with increased length of the aliphatic chains (Fig. 3F) and the presence of ester groups (Fig. 3E). Unlike BPA, DGB exposure caused a deep alteration in the protein composition, while phosphate groups were not affected.

In conclusion, the present study supports the hypothesis that both BPA and DGB are contributing factor in increased obesity. Low environmentally relevant concentration of BPA increased the storage of TAG in lipid droplets and fatty acid synthesis. Exposure to DGB may not be a contributing cause of fatty liver, because it increased the production of lipids, both cholesterol esters, fatty acids and TAG as well as promoting lipid transport through blood. It is alarming that DGB increased *apoBa*, which is involved in very low density lipoprotein production and can be related with type-II diabetes and atherosclerosis (Ahn and Choi 2015; Bell

et al. 2006). Given the high synteny between zebrafish and human genome, our study can represent an important starting point for the understanding of traditional plasticizer and/or their substitute effects on lipid metabolism alterations also in human.

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Table 1.

Gene	Forward primer 5'-3'	Reverse primer 5'-3'	Acc number
18S rRNA	TCGAATGTCTGCCCTATCAACT	AGACTTGCCCTCCAATGGATC	AF308735
arp	CTGAACATCTCGCCCTTCTC	TAGCCGATCTGCAGACACAC	NM131580.2
Fasn	ATGAAACACACAGGGACTCAGG	TTCTTGAATCTGAACGCGGGTA	XM_001923608
agpat4	TGCTGAAAACTCAGTTGCTG	GACCATAAACGGTGCCAACT	NM_212992
dgat2	CCATACTTGCTGCATATTCC	ATGTCATGATAAACTGCAGC	NM_001030196
fitm2	CTGGTCTCCCTCCACAGCCGA	ACACCAGCTGCCCTCCGCTT	NM_001020498
acat2	GGGCTCAAGATCAACTGGCT	ACCTCGACTGGACCTTTCCT	NM_131370
apoA4b.1	GGCCTACTGGTGGACTAAGC	TGGTTTGGGCTCATCAGCAT	NM_001079861
apoBa	TGAGAATGGGGCTTTGGGTCTA	ATGTCGTGAGGGACGGGAAA	XM_689735
apoAIa	ATTCGTGGCTCTTGCACTGA	AAGCTGGAGTTTGTACTGCTCA	NM_131128
fabp11a	AATGACCACCAGCGACAACT	AGATTGGGTTTGGTCCGGTT	NM_001004682
srebf1	TCGGCTTCACCAATCCTGAC	GTCACGTCCGGTTTCAGAGT	NM_001105129
srebf2	CTGAGCTGTGATTGGTCGGT	AACTCCCCAGCATCCTCTCT	NM_001089466
pparaa	TCCACATGAACAAAGCCAAA	AGCGTACTGGCAGAAAAGGA	NM_001161333
pparg	CTGCCGCATACACAAGAAGA	TCACGTCACTGGAGAACTCG	NM_131467
rxraa	CGTCATTTTCGCACCGATCC	CAACTCCGTCAGCACTCTGT	NM_001161551
ceba	AACGGAGCGAGCTTGACTT	AAATCATGCCCATTAGCTGC	NM_131885
hnf4a	ACGGTTCGGCGAGCTGCTTC	TCCTGGACCAGATGGGGGTGT	NM_194368

Table 1. *arp*: acidic ribosomal protein; *fasn:* fatty acid synthase; *agpat4:* 1-acylglycerol-3-phosphate o-acyltransferase; *dgat2:* diglyceride acyltransferase; *fitm2:* fat storage-inducing transmembrane protein2; *acat2:* acyl-coenzime A cholesterol acyltransferase; *apoA4b.1:* apolipoprotein a-IV; *apoBa*: apolipoprotein B; *ApoAla:* apolipoprotein Ala; *fabp11a*: fatty acid binding protein 1A; *srebf1:* sterol regulatory element-binding protein 1; *srebf2:* sterol regulatory element-binding protein 2; *pparaa:* peroxisome proliferator-activated receptors α; *pparg:* peroxisome proliferator-activated receptors γ; *rxraa* retinoid X receptor α; *cebpa:* CCAAT/enhancer binding protein α; *hnf4a:* hepatocyte nuclear factor 4α.

Table 2.

Gene	Ctrl	BPA 5µg/L	BPA 10µg/L	BPA 20µg/L
fasn	2,29±0,25^{a}	2,33±0,29^{a}	2,25±0,74^{a}	4,17±0,56^{b}
agpat4	1,72±0,48^{a}	2±0,76^{a}	2,16±0,25^{a}	1,56±0,43^{a}
dgat2	4,27±0,32^{a}	2,59±0,41^{b}	2,88±0,39^{b}	1,80±0,55^{c}
fit2	1,4±0,38^{a}	2,51±0,24^{b}	1,65±0,66^{a}	4,92±0,2^{c}
acat2	3,39±0,32^{a}	8,07±0,46^{b}	1,98±0,65^{c}	2,58±0,34^{a,c}
apoA4b.1	1,68±0,62^{a}	2,52±0,24^{a}	4,56±0,56^{b}	4,37±0,36^{b}
apoBa	2,85±1,24^{a,b}	1,77±0,35^{a}	3,60±0,31^{b}	3,15±0,43^{b}
apoAla	4,17±0,37^{a}	3,30±0,18^{a,b}	2,51±1,02^{b}	6±0,54^{c}
fabp11a	1,95±0,66^{a}	4,37±0,43^{b}	3,91±0,24^{b}	4,52±0,22^{b}
srebp1	5,59±0,31^{a}	9,62±0,36^{b}	1,65±0,56^{c}	3,34±0,44^{d}
srebp2	1,71±0,35^{a}	1,47±0,48^{a}	2,81±0,42^{b}	3,24±0,41^{b}
pparaa	4,61±0,3^{a}	2,34±1,19^{b}	2,62±0,50^{b}	3,99±0,45^{a}
pparg	3,09±1,4^{a}	3,11±0,4^{a}	2,08±0,3^{a}	3,18±0,29^{a}
rxraa	2,30±0,9^{a}	2,55±0,4^{a}	1,88±0,5^{a}	1,99±0,5^{a}
ceba	8,53±0,3^{a}	10,46±0,2^{b}	1,36±0,5^{c}	1,89±0,5^{c}
hnf4a	1,66±0,5^{a}	1,68±0,4^{a}	1,74±0,4^{a}	2,09±0,3^{a}

Table 2: Transcriptional profiles of genes involved in fatty acid synthesis, storage, oxidation and transport in fish exposed to BPA. In yellow have been reported the genes whose expression does not change compared to the control. In red the ones that are down regulated compared to the control. Green ones that are up regulated compared to control. Data are expressed as mean ± standard deviation; letters indicate differences between treatments ($p < 0.05$ compared with untreated controls; ANOVA followed by Tukey's multiple comparison tests).

Table 3.

Gene	Ctrl	DGB 0,01 µg/L	DGB 0,1 µg/L	DGB 1 µg/L	DGB 10 µg/L	DGB 100 µg/L
fasn	2,6±0,6^{a}	4,55±0,3^{b}	1,56±0,4^{c}	3,18±0,3^{a,d}	3,65±0,5^{b,d}	1,34±0,3^{c}
agpat4	1,94±0,2^{a}	3,34±0,3^{b}	3,95±0,4^{b,c}	1,29±0,2^{a}	4,38±0,7^{c}	3,38±0,2^{b,d}
dgat2	3,15±0,2^{a}	4,15±0,6^{b}	6,19±0,4^{c}	3,24±0,4^{a}	1,89±0,6^{d}	3,02±0,4^{a}
fit2	2,94±0,3^{a}	2,81±0,4^{a}	3±0,4^{a}	3,56±0,3^{a}	1,82±0,6^{b}	3,23±0,5^{a}
acat2	4,31±0,3^{a}	19,53±0,5^{b}	24,53±0,5^{c}	4,82±0,4^{a}	2,62±1^{d}	4,78±0,5^{a}
apoA4b.1	1,89±0,6^{a}	6,65±0,3^{b}	6,63±0,3^{b}	1,7±0,5^{a}	2,35±0,4^{a}	6,69±0,2^{b}
apoBa	1,74±0,5^{a}	3,44±0,3^{b}	3,63±1,7^{b}	3,27±0,3^{a,b}	4,04±0,5^{b}	6,51±0,7^{c}
apoAla	3,9±0,6^{a}	2,24±1,1^{b}	4,20±0,4^{a}	2,65±0,6^{a,b}	2,25±0,5^{b}	10,83±0,8^{c}
fabp11a	1,65±0,6^{a}	3,81±0,4^{b,d}	2,57±0,4^{c}	4,55±0,3^{c}	1,48±0,3^{a}	3,25±0,5^{c,d}
srebp1	2,80±0,5^{a,c}	2,64±0,4^{a,c}	9,65±0,3^{b}	3,39±0,4^{a}	2,12±0,8^{c}	3,02±0,4^{a,c}
srebp2	2,56±0,3^{a}	5,75±0,4^{b}	4,67±0,4^{c}	2±0,7^{c}	5,16±0,3^{b,c}	7,17±0,3^{d}
pparaa	2,77±0,1^{a,b}	3,26±0,2^{a}	2,78±0,6^{a,b}	2,29±0,7^{b}	2,19±0,4^{b}	5,38±0,4^{c}
pparg	2,52±0,4^{a}	4,36±0,5^{b}	4,90±0,5^{b}	2,12±0,3^{a,c}	2,52±0,3^{a}	1,63±0,4^{c}
rxraa	1,79±0,5^{a}	2,16±0,3^{a,b}	2,42±0,3^{a,b}	2,51±0,3^{b}	3,97±0,4^{c}	3,29±0,4^{c}
ceba	5,39±0,4^{a}	1,37±0,3^{b}	2,30±0,6^{c}	3,65±0,4^{d}	3,68±0,3^{d}	10,46±0,2^{e}
hnf4a	3,10±0,6^{a}	3,81±0,6^{a}	3,77±0,5^{a}	3,04±0,6^{a}	2,73±1,0^{a}	3,87±0,5^{a}

Table 3: Transcriptional profiles of genes involved in fatty acid synthesis, storage, oxidation and transport in fish exposed to DGB. In yellow have been reported the genes whose expression does not change compared to the control. In red the ones that are down regulated compared to the control. Green ones that are up regulated compared to control. Data are expressed as mean ± standard deviation; letters indicate differences between treatments ($p < 0.05$ compared with untreated controls; ANOVA followed by Tukey's multiple comparison tests).

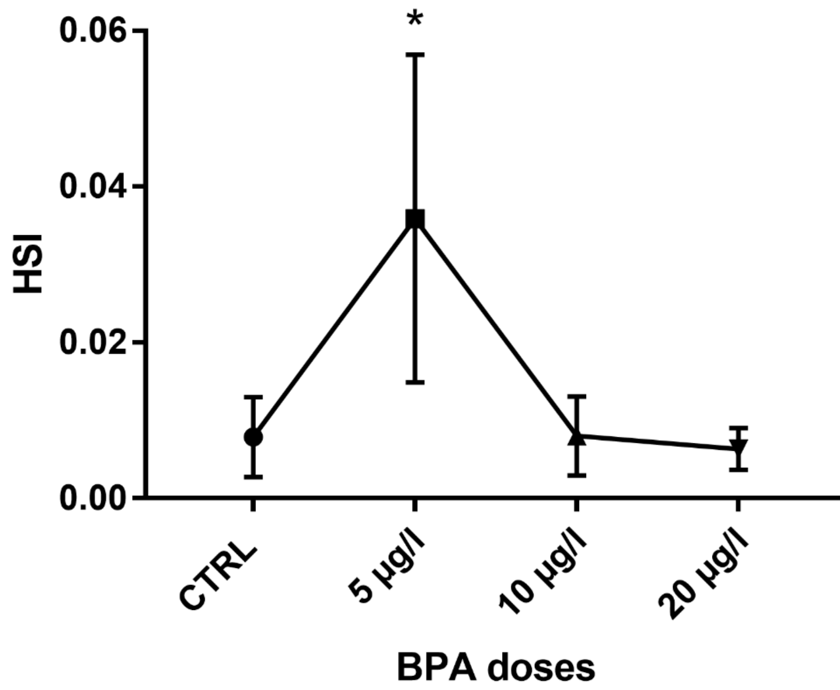
Figure Legends

Fig. 1: Hepatosomatic index (HSI; the ratio of liver weight to body weight) of fish treated with BPA (**A**) and fish treated with DBG (**B**). Data are expressed as mean ± standard deviation; asterisks indicate difference between treated groups and control group ($p < 0.05$ compared with untreated controls; ANOVA followed by Tukey's multiple comparison tests).

Fig. 2: Quantitative analysis of lipids (**A**), phosphate (**C**), proteins (**D**), C=O (**E**) and Ch2 (**F**) groups and chemicals map integrated under the lipids stretching region (3100–2800 cm^{-1}) (**B**) in fish exposed to BPA.

Fig.3: Quantitative analysis of lipids (**A**), phosphate (**C**), proteins (**D**), C=O (**E**) and Ch2 (**F**) groups and chemicals map integrated under the lipids stretching region (3100–2800 cm^{-1}) (**B**) in fish exposed to DGB.

Fig.1



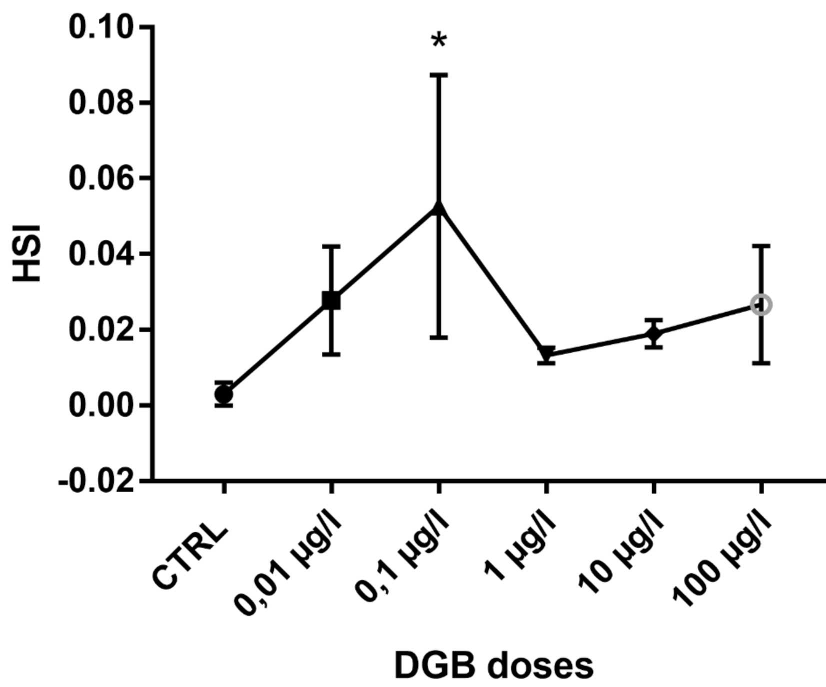


Fig.2

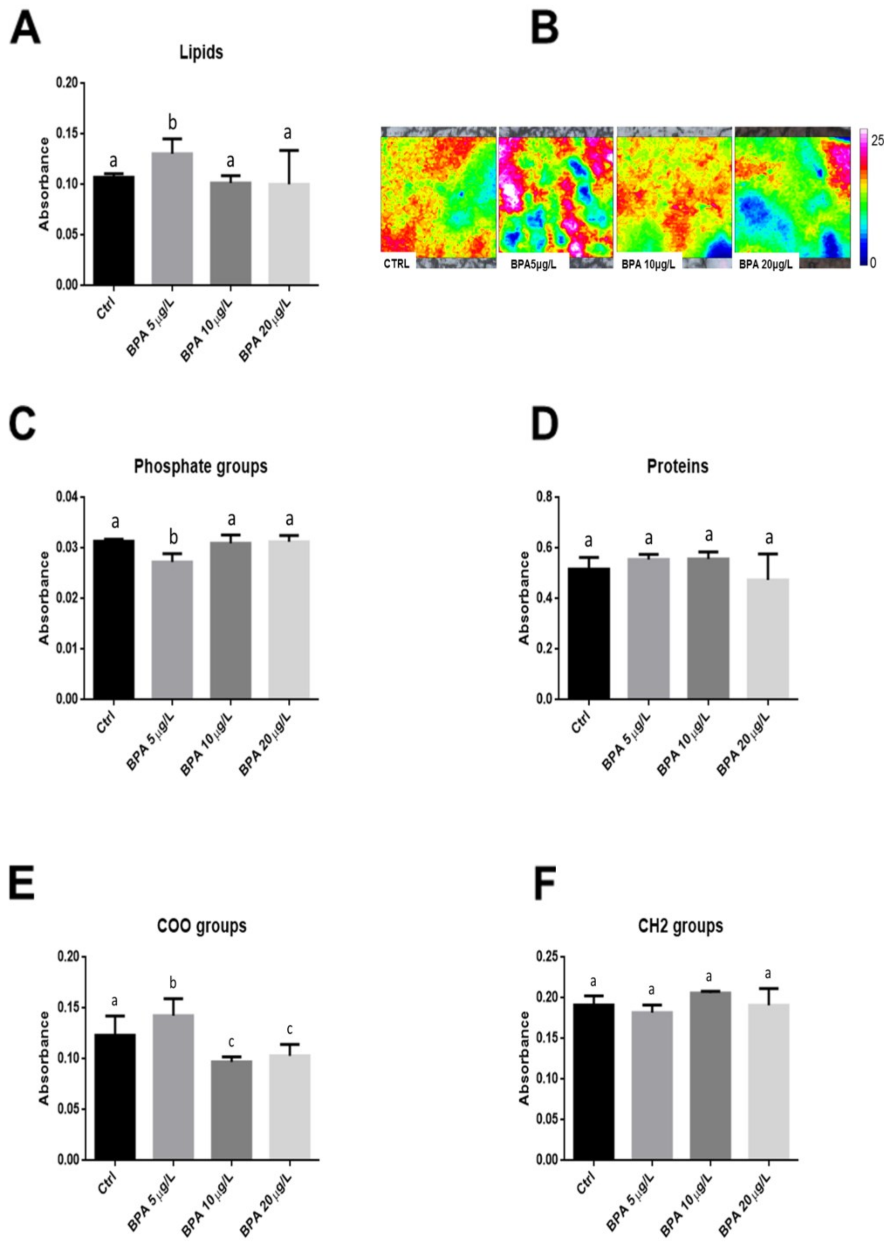
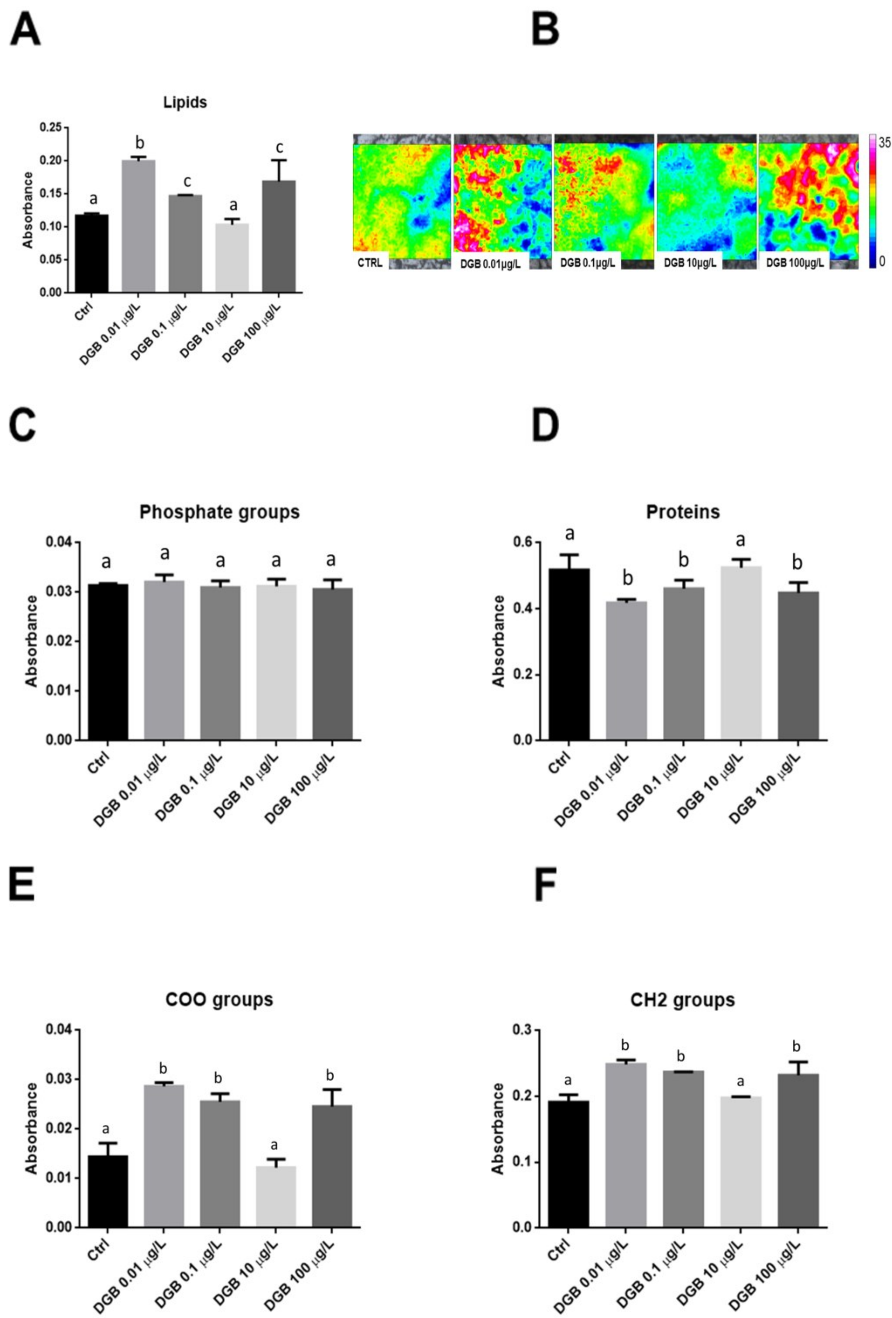


Fig.3



7. XENOBIOTIC-CONTAMINATED DIETS AFFECT HEPATIC LIPID METABOLISM: IMPLICATIONS FOR LIVER STEATOSIS IN *SPARUS AURATA* JUVENILES

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Abstract

The metabolic effects induced by feed contaminated with a lower or a higher concentration of -nonylpnenol (NP), -4-tert-octylphenol (t-OP), or -bisphenol A (BPA), three environmental endocrine disruptors, were assessed in juvenile sea bream liver. Histological analysis demonstrated that all these three xenobiotics induced hepatic lipid accumulation and steatosis. These findings prompted analysis of the expression of the major molecules involved in lipid metabolism: peroxisome proliferator activated receptors (which is encoded by *ppars*), fatty acid synthase (encoded by *fas*), lipoprotein lipase (encoded by *lpl*) and hormone-sensitive lipase (encoded by *hsl*). The enzymes encoded by *ppars* and *fas* are in fact responsible for lipid accumulation, whereas *lpl*- and *hsl*- encoded proteins play a pivotal role in fat mobilization. The three xenobiotics modulated *ppar* mRNA expression: *pparα* mRNA expression was induced by the higher dose of each contaminant; *pparβ* mRNA expression was upregulated by the lower doses and in BPA2 fish *ppary* mRNA overexpression was induced by all pollutants. These data agreed with the lipid accumulation profiles documented by histology. *Fas* mRNA levels were modulated by the two NP doses and the higher BPA concentration. *Lpl* mRNA was significantly upregulated in all experimental groups except for BPA1 fish while *hsl* mRNA was significantly downregulated in all groups except for t-OP2 and BPA1 fish. The plasma concentrations of cortisol, the primary stress biomarker, were correlated with the levels of *pepck* mRNA level. This gene encodes phosphoenolpyruvate carboxykinase which is one of the key enzymes of gluconeogenesis. *Pepck* mRNA was significantly overexpressed in fish exposed to NP2 and both t-OP doses. Finally, the genes encoding cyclooxygenase 2 (*cox2*) and 5-lipoxygenase (*5 lox*), the products of which are involved in the inflammatory response, transcriptions were significantly upregulated in NP and BPA fish, whereas they were unchanged in t-OP specimens. The present findings suggest that dietary xenobiotic contamination can give rise to metabolic disorders also in fish and highlight the potential for their vertical transfer through the trophic levels and ultimately to humans.

1. Introduction

There is mounting evidence for the adverse health effects of numerous EDCs, including BPA, NP, and t-OP. Concerns regard especially impairment of reproductive function and carcinogenicity, which have been

described both in humans and in wild species (Sharma et al., 2009). These compounds are widespread in the environment, owing to their use in the manufacture of a wide range of products and materials, including plastics (Thompson et al., 2009), medical devices, food packaging (Wilson et al., 2006), cosmetics, children's toys, industrial detergents, and emulsifiers (Ying et al., 2002). The main routes of human exposure are believed to be ingestion of contaminated foods, especially fish and some degree drinking water, and contact with some personal care products and detergents (Ferrara et al., 2001; Vandenberg et al., 2007).

An emerging research field assesses the contribution of "environmental obesogens" (Grün and Blumberg, 2009) to the dysregulation of lipid metabolism and, through it, to the development of obesity and other metabolic disorders. These compounds have the liver as the primary target organ (Pereira-Fernandes et al., 2013). The liver is the center of FA synthesis and lipid circulation. Several studies have found that BPA exposure through different routes induces alteration of lipid metabolism in rats (Marmugi et al., 2012), Drosophila (Williams et al., 2014), sea bream (Maradonna et al., 2014), and zebrafish (Riu et al., 2014), contributing to hepatic steatosis. Obesity has been induced in mice and sea bream by dietary exposure to alkylphenols (Hao et al., 2012; Traversi et al., 2014). There is no direct evidence of the obesogenic potential of t-OP in humans, even though higher serum concentrations have been described in young obese girls (Choi et al., 2014). Obesity is a complex metabolic disorder resulting from an imbalance between energy intake and expenditure that may have a genetic and/or behavioral origin (Ogden et al., 2012; Tang-Peronard et al., 2011). Obesity is the most significant risk factor for fatty liver or hepatic steatosis (Gonzales-Periz et al., 2009), which are characterized by an abnormal accumulation of lipid droplets in the hepatocyte cytoplasm. In vertebrates, histopathological liver alterations related to dietary exposure are important biomarkers to assess the impact of dietary manipulation (Wang et al., 2014; Zhang et al., 2014) or contamination (Maradonna et al., 2014; Traversi et al., 2014).

Several interdependent and/or cross-regulated pathways are responsible for lipid metabolism. FA are the most common forms of energy, both stored and circulating; they also accumulate in hepatocytes, where their content is regulated by the cellular signals that facilitate their uptake, synthesis, esterification, and oxidation. Several transcription factors are involved in FA regulation, including Ppars (Schoonjans et al., 1996) and *Rxr* (Dubuquoy et al., 2002). The three known *Ppar* isoforms, α, β and γ, have different roles in lipid and energy

dynamics, show differential expression patterns, and display distinct but overlapping expression and functions (Poulsen et al., 2012). Pparα and pparβ play a role in clearing circulating lipids via regulation of a set of enzymes responsible for FA oxidation; Pparα is found mainly in the liver, whereas Pparβ has a ubiquitous distribution (Lefterova et al., 2014). Pparγ controls lipid accumulation and regulates adipocyte differentiation to enhance blood glucose uptake (Singh et al., 2011). The finding that in several murine models of obesity and diabetes Ppary upregulation is associated with developed fatty liver (Matsusue et al., 2014) and hepatic steatosis (Yu et al., 2003) demonstrates that Ppars play a key role in lipid accumulation. Fish have three Pparγ isoforms that are similar to the mammalian ones; in sea bream, *pparα* is expressed in liver and heart, *pparβ* is found in all tissues (Leaver et al., 2005), whereas *ppar*γ transcripts have been detected in adipose tissue, liver and muscle (Kaneko et al., 2013).

Several other signals besides Ppars contribute to the complex mechanism of lipid turnover. Fas is a key multifunctional enzyme involved in FA synthesis that converts excess food into lipids for storage; when energy is needed, it provides it via *β*-oxidation. Subsequent lipid deposition and metabolism are regulated by Lpl (Albalat et al., 2006; Ibanez et al., 2008; Weil et al., 2013). When the body needs energy, Hsl mobilizes fat stored in adipocytes in the form of lipid droplets (Lampidonis et al., 2011). However, very little research on Hsl has been carried out in fish, and scant evidence is available about its function in the piscine liver (Khieokhajonkhet et al., 2014).

Pollutants have the potential to affect cortisol dynamics in fish (Mommsen et al., 1999). Contaminants in the diet can result in increased cortisol levels (Menezes et al., 2014) and overexpression of hepatic GR. The cortisol/GR complex binds to the GRE, stimulating the synthesis of Pepck, a key enzyme in glucose and lipid metabolism (Nash et al., 2013). A number of xenobiotics have been found to affect hepatic Pepck activity (Vijayan et al., 2006; Nash et al., 2013), and Pepck downregulation has been shown to induce fatty degeneration or hepatic steatosis (Hakimi et al., 2005; She et al., 2003). Steatosis is a key step in the progressive development of liver inflammation (Purushotham et al., 2009), and fat accumulation in hepatocytes increases liver vulnerability to secondary insults from cytokines or oxidative stress (Farrel and Larter, 2006). AA is released by Pla2 in response to various stimuli, including tissue damage; AA is further metabolized by two major enzyme pathways, Cox-2 and 5-Lox, leading to the release of prostanoids and leukotrienes, which are

implicated in several physiological processes including immune and inflammatory responses as well as reproduction (Serhan et al., 2015).

Based on the above considerations, the present study investigated the effects of feed contaminated with BPA, NP, or t-OP on the lipid metabolism of gilthead sea bream (*Sparus aurata*). Considering their broad diffusion and the high potential for human exposure, these EDCs may pose a concrete risk to human health.

2. Materials and Methods

2.1. Chemicals and reagents

The chemicals used to spike feed samples, BPA (99% analytical purity), NP (85-90% laboratory grade purity) and t-OP (97% laboratory grade purity), were supplied by Sigma Aldrich. Materials for LC/ESI-QTRAP-MS/MS analysis the analytical grade standards included: BPA (98% analytical purity), NP (99.9% analytical grade purity) and t-OP (98.6% analytical grade purity) were purchased from Sigma Aldrich (Milano, Italy). HPLC grade methanol (MeOH) and acetonitrile were provided by Romil (Cambridge, UK). Analytical grade ethyl acetate, glacial acetic acid, and sodium chloride were obtained from Carlo Erba (Milano, Italy). HPLC grade water was in-house produced using a MilliQ laboratory system (Millipore, Bedford, MA, USA). AFFINIMIP® SPE Bisphenol A cartridges (Polyntell, Val-de-Reuil, France) provided high purification selectivity of the analyte.

2.2. Fish breeding

Gilthead sea bream juveniles (initial weight, 10.6 ± 3.7 g) were purchased from a fish farm (Orbetello Pesca Lagunare, Grosseto, Italy). Acclimatization and rearing were carried out in a closed system equipped with biological, mechanical, and UV filtration under the following conditions: temperature 20 ± 1 °C, salinity 35 ± 1 ppt, oxygen 6 ± 1 mg/l, and photoperiod 12hL: 12hD. The water in the tanks was replaced up to 10 times a day through a dripping system. Ammonia and nitrite levels were kept below 0.01 mg/l. The initial stocking density was 10 individuals/100 l. Every day *ad libitum* feed, a commercial sea bream feed (INVE, Dendermonde, Belgium) with increasing pellet diameter to account for fish growth, was spread on the water by hand.

2.3. Animal treatment and ethical statement

After acclimatization for 30 days, fish were divided into groups of 20 specimens, in duplicates. They were fed an amount of feed equal to 2.5% of their body weight (bw) once a day for 21 days. The feed was carefully spread on the surface of each tank to ensure that all fish received approximately the same amount. All tanks were maintained in duplicate. All fish groups received the same commercial pelleted feed. Feed was prepared according to Maradonna et al., (2014); details are reported in Supplementary Material 1.

The feeding protocol was as follows: control (C) individuals received only the commercial feed; the BPA1 group was fed the commercial feed enriched with 5 mg/kg bw BPA; the BPA2 group was fed the commercial feed enriched with 50 mg/kg bw BPA; group NP1 received the commercial feed enriched with 5 mg/kg bw NP; group NP2 received the commercial feed enriched with 50 mg/kg bw NP; group t-OP1 was fed the feed enriched with 5 mg/kg bw t-OP; and group t-OP2 the feed enriched with 50 mg/kg bw t-OP. An even higher dose of each pollutant (100 mg/kg bw) was tried, but on day 14 all fish had died, as reported by Maradonna et al., (2014) and Traversi et al., (2014).

A sample of water (100 ml) was collected from each tank on day 21. All procedures involving animals were conducted in accordance with Italian laws on animal experimentation and were approved by the Ethics Committee of Università Politecnica delle Marche (Prot. #24/INT/CESA12-16). All efforts were made to minimize suffering. Fish were euthanized with an excess of anesthetic (MS222, Sigma-Aldrich). On day 21, fish were sacrificed and livers were stored at -80 °C until processing for biochemical and molecular biology analyses. Plasma samples were collected using heparinized syringes and stored at -20 °C (Maradonna et al., 2004).

2.4. Food intake analysis

On days 7, 14, and 20 the animals received pre-weighed food in excess (5% bw). FI was measured at each time point and was calculated as follows: $FI = Wi - (Wf \times F)$, where Wi is initial dry feed weight, Wf is remaining dry feed weight, and F is a correction factor. F was previously calculated in the absence of fish to determine the dissolving effect of water during feeding, and represents the reduction in feed weight after 5 h.

2.5. NP and t-OP cleanup and determination in water and feed by LC/ESI-QTRAP-MS/MS analysis

The BPA content of feed pellets was determined by chemical analysis as reported in Maradonna et al., (2014). NP and t-OP were purified from tank water and food samples prior to quantitative analysis by liquid chromatography-tandem mass spectrometry on a QTRAP 4000 triple quadrupole (LC/ESI-QTRAP-MS/MS). Details of the procedures are reported in Supplementary Material 2.

2.6. RNA extraction and cDNA synthesis

For Real Time PCR analysis, total RNA was extracted from 20 fish livers per group using RNAeasy® Minikit (Qiagen, Milano, Italy), following the manufacturer's instructions, and eluted in 50 µl of RNAse-free water. The final RNA concentration was determined using the Nanophotometer ™P-Class (Implem GmbH, Munich, Germany); RNA integrity was verified by ethidium bromide staining of 28S and 18S ribosomal RNA bands on 1% agarose gel. RNA was stored at -80 °C until use. Total RNA was treated with DNAse (10 IU at 37 °C for 10 min, MBI Fermentas, Milano, Italy). These analyses were performed on 10 fish from each duplicate tank (20 fish overall). RNA extracted from two livers was pooled to minimize differences in mRNA expression levels and obtain 5 cDNAs from each duplicate tank; the final data therefore came from 10 cDNA samples per group. A total amount of 1 µg RNA was used for cDNA synthesis with iScript cDNA Synthesis Kit (Bio-Rad, Milano, Italy).

2.7. Real-time PCR

PCRs were performed with SYBR green in an iQ5 iCycler thermal cycler (Bio-Rad) in triplicate as described previously (Maradonna et al., 2014). The genes encoding *β-actin (act)* and elongation factor1a (*ef1α*) were the housekeeping genes. The primer sequences for the genes analyzed are reported in Table 1.

2.8. Cortisol assay

Cortisol was extracted from 25 µl plasma from each fish. Analyses were performed with a Cortisol EIA kit (Cayman Chemical Company, Arcore, Italy) using a standard curve in the range of 7.8– 1000 pg/ml, according to the kit instructions. Assay sensitivity was 2 ng per tube; inter- and intra-assay coefficients of variation were

6.3% and 4.4%, respectively. To validate the cortisol assay, parallelism between the standard curve and the serial dilutions of the extracted solution was performed.

2.9. Liver histology

Small liver specimens were fixed in 4% paraformaldehyde for 24 h at 4 °C and served in 70 % ethyl alcohol. For general histological examination, sections 2-3 μm in thickness were embedded in Bio-Plast or Historesin (both from Bio-Optica, Milano, Italy) and stained with hematoxylin-eosin or toluidine blue and gentian violet, respectively. Nine fields randomly chosen from at least 3 sections were examined in each liver. Hepatocytes of control and treated fish were classified as "absent", "mild", "moderate" or "severe" based on the extent of lipid vacuole accumulation. If the classification interested more than the 50% of the tissue sample, the category was assigned to the whole liver (Fig.1)

2.10. Statistical analysis

One-way analysis of variance followed by Tukey's multiple comparison test was used to determine differences among groups. Statistical significance was set at $P < 0.05$.

A contingency table was drawn up and contingency coefficients were calculated for each of the four lipid accumulation categories. A contingency coefficient > 0.1 was used as the cut-off for significance. The χ^2 test was applied to the lipid accumulation data (Tanagra).

3. Results

3.1. Food Intake

A significant FI reduction was detected on day 7 in the t-OP groups, and on day 14 in the NP and t-OP groups. On day 20, FI was reduced in all experimental groups; the highest reduction was observed in fish treated with both doses of t-OP (Table 2).

3.2. LC/ESI-QTRAP-MS/MS confirmatory analysis

In preliminary MS tests conducted to choose quantifier and qualifier ions and identify optimal experimental

conditions, flow injection full scan mass spectra were recorded in negative ionization mode under LC conditions.

The chromatographic separation of NP and t-OP showed single sharp peaks at about 10.80 and 10.40 min, respectively; the high sensitivity of the QTRAP 4000 detector allowed to obtain optimum separation with only 5 µl of sample extract. Moreover, the sample extract was diluted, not concentrated as customary, to reduce possible matrix interference and ion suppression effects. NP and t-OP were quantified down to 0.05 ng/ml, corresponding to 0.001 mg/kg of feed, further demonstrating the high sensitivity of the method. Sample cleanup was rapid and simple; water was only diluted and centrifuged, whereas feed samples were extracted and then purified by affinity chromatography, to increase selectivity in separation. The NP and t-OP determination method was specific and sensitive, providing high mean recoveries from spiked samples (NP, 94.6%; t-OP, 72.5%), and unambiguous identification of both analytes based on two product ions. No significant matrix interference was observed in the MRM chromatograms of spiked samples or in the blank reagents. NP and t-OP qualifier ions were detected in all spiked samples, providing unambiguous confirmation. For NP and t-OP quantification in water and feed samples, calculated concentrations were corrected for mean recovery. Very low amounts of NP were also detected in some water samples collected from t-OP tanks, presumably due to contamination from the reference material added to water. Likely, cross-contamination between NP and *t*-OP occurred in feeds (Table 3). The laboratory grade purity of the NP and t-OP standards was responsible for the cross-contamination detected in the groups exposed to NP2 and t-OP2. t-OP cross-contamination in NP2 fish was about 1.2% compared with the nominal concentration, which is consistent with the 85-90% purity of the NP used to spike feed. NP cross-contamination in the t-OP2 group was probably around 4.5%, compared with the 97% purity of t-OP used to spike the feed, considering the uncertainty involved in the measurement. In either case, the cross-contamination was negligible, since it was detectable only in feed spiked with the higher concentration.

3.3. Lipid accumulation

In C fish, 83.3% and 16.7% of the livers analyzed were assigned to the moderate and the severe lipid accumulation categories, respectively (Table 4). NP1 exposure induced severe lipid accumulation in more than 40% of livers and halved those exhibiting moderate accumulation (41.7%). No lipid accumulation was detected

in 60% of NP2 livers and severe accumulation was detected in the remaining 40%.

In both t-OP groups, dietary exposure induced a significant increase in severe lipid accumulation even at the lowest concentration in that the values reported were 80% and 85.7%. A decrease in moderate lipid accumulation was observed in both t-OP treatments (20% and 14.3%, respectively).

The two BPA-exposed groups showed an increased percentage of livers with severe lipid accumulation and a decreased percentage of livers with moderate accumulation; in particular, moderate and severe lipid accumulation was detected in 60% and 40% of BPA1 fish, respectively, and in 14.3% and 85.7% of BPA2 fish, respectively. Data are reported in Table 4.

3.4. Molecular findings

3.4.1. Expression of biomarkers of FA synthesis and accumulation

In general terms, all xenobiotics affected the *ppar* system. The higher dose of each contaminant induced significant transcriptional *pparα* upregulation (Fig. 2A), whereas the lower doses did not induce significant changes (Fig. 2A). All the lower concentrations induced significant transcriptional *pparβ* overexpression. With regard to the lower doses, significant upregulation was observed in BPA1 fish, but not in NP1 and t-OP1 fish (Fig. 2B). All the diets induced significant upregulation of *pparγ* transcripts, which were highest in NP2 fish (Fig. 2C). *Rxr* mRNA expression rose significantly in NP2 and BPA1 fish, with trends that were similar to those of *pparγ* transcripts (Fig. 2D). *Fas* mRNA expression rose significantly in NP1, NP2, and BPA2 fish (Fig. 2E).

3.4.2. Expression of lipolysis biomarkers

Lpl transcripts were significantly and variably increased in all groups except BPA1 fish (Fig. 3A), whereas *hsl* gene transcripts decreased significantly in all groups except t-OP2 and BPA1 specimens, where levels were similar to those of C fish (Fig. 3B).

3.4.3. Cortisol and *gr* and *pepck* gene expression

Cortisol levels were significantly affected by NP and t-OP. A significant increase was induced in NP2, t-OP1, and t-OP2 fish (Fig. 4A). *Gr* mRNA levels were highest in t-OP1 and t-OP2 fish (Fig. 4B). *Pepck* mRNA was also significantly overexpressed in NP2, t-OP1, and t-OP2 fish (Fig. 4C).

3.4.4. Expression of biomarkers involved in inflammatory responses

Pla2 mRNA expression was significantly upregulated in NP2, BPA1, and BPA2 fish (Fig. 5A). All NP and BPA doses raised *cox-2* mRNA levels and both t-OP concentrations reduced them (Fig. 5B). *5-lox* gene expression increased significantly in NP2 and BPA fish, while changes in NP1, t-OP1, and t-OP2 fish were not significant (Fig. 5C).

4. Discussion

Earlier studies from our group found that a diet contaminated with BPA, NP, or t-OP induced estrogenic effects in sea bream juveniles, including vitellogenin and zona pellucida proteins, female reproductive molecules, as well as severe morphological and structural liver changes (Maradonna et al., 2014; Traversi et al., 2014). Understanding the potential impact of these chemicals on wildlife and human health requires elucidating their effects on a variety of physiological or pathophysiological processes besides reproduction.

Over the past few years, a number of pollutants, including several xenoestrogens, have been found to have the ability to induce obesity. The molecular mechanisms involved in the development of obesity are being extensively investigated, since obesity is the most significant single risk factor for the development of fatty liver in children and adults (Heideln, 2003; Lubrano et al., 2013). Although several human studies have demonstrated that low BPA concentrations can induce hepatic lipid accretion, leading to steatosis (Huc et al., 2012), little is known about the ability of NP and t-OP to induce lipid accumulation in hepatocytes (Hao et al., 2012). This study examined the hepatic effects of BPA, NP, and t-OP on sea bream, a widely consumed fish species, with emphasis on their impact on lipid metabolism. The biomarker panel investigated documented different effects of the three contaminants. Since we focused on mRNA level variations, our conclusions are based on the assumption that the mRNA changes observed in all the biomarkers analyzed reflect the activity

of the encoded proteins. In his case the modulation of *ppars* demonstrates their key role in the lipid uptake and distribution process, as previously described in mammals (Wang, 2010), and agree with the anti-inflammatory role proposed for Ppar in mammalian steatotic liver (Auguet et al., 2014). The similar modulation of the α and β isoforms found in the present study can be explained by the fact that the encoded protein share a role in preventing the liver from storing excessive amounts of fat, as reported in mice (Patsouris et al., 2006). The present molecular findings, in conjunction with the histological data, strongly suggest a steatogenic effect of BPA, NP, and t-OP. Moreover, as noted in mammals, BPA and t-OP fish exhibited *fas* downregulation possibly due to the high intracellular lipid content (Oleksiak, 2008) and the increase in *lpl* transcripts, whereas *fas* overexpression in NP fish may be due to the substantial number of individuals showing non-severe lipid accumulation and an increased demand for lipid synthesis. In NP fish, *fas* upregulation could be related to apoptotic process, as reported in other studies (Lodhi et al., 2015). The morphological liver changes suggesting lipid accumulation typical of steatosis, detected in all fish groups, were associated with downregulation of the transcription of *hsl* whit product mainly involved in lipid mobilization.

At the same time, the increased plasma cortisol detected in NP and t-OP fish strongly supports the stressor nature of the contaminants. In physiological conditions, cortisol directly modulates the expression of *pepck*, the product of which is one is one of the key enzymes for gluconeogenesis and glycerogenesis (Cassuto et al., 2005); the latter process generates glycerol 3 phosphate, which may be involved in FA re-esterification and subsequent fat accumulation (Franckhauser et al., 2002). Indeed, hepatic glycerogenesis is the major contributor to triglyceride production during starvation in humans and may have a large role in regulating very low-density lipoprotein triglyceride production (Kalhan et al., 2000). The *pepck* data strongly support our findings: despite the decreased FI, found in all experimental groups, the significant increase in steatotic liver tissue may be related to dysregulation of lipid metabolism due to increased glycogenesis signaling. Preliminary data from our laboratory also demonstrated the involvement of the neuroendocrine system in the FI reduction, with significant downregulation of *npy* and *cb1* messenger and protein in the brain of fish fed the xenobiotic-contaminated diets (data not shown).

Finally the excess of fat hepatic storage can stimulate inflammation (Arendt et al., 2015). As noted above, Pparα and Pparγ activation may protect against inflammation (Pedersen, 2015). Several aspects of

inflammation are regulated by signals from the COX and LOX cascades (Martel-Pelletier et al., 2003). Based on the assumed correlation between mRNA and protein levels, the upregulation of *cox-2* and *5-lox* described in our sea bream specimens suggests a proinflammatory effect of NP and BPA, as observed in rats (Liu et al., 2014). However, t-OP exposure at the concentrations used in our study might not induce inflammation, as documented by the low catalase and glutathione transferase activity detected in a similar study by our group (Traversi et al., 2014).

Studies of the presence of the three compounds in the aquatic environment (Ferrara et al., 2001; Mita et al., 2011; Staniszewska et al., 2014) have shown that the concentrations of some contaminants in fish can reach the order of magnitude of the doses tested in this study. Thus the effects on lipid metabolism reported in this study are likely to occur also in wildlife.

A crucial issue is whether current environmental human exposure to these pollutants through fish consumption is biologically active and can impact lipid metabolism. A thorough review of human exposure to BPA (Vandenberg et al., 2007) has found that most studies report exposure ranges from under 1 µg/kg bw/day to almost 5 µg/kg bw/day. The European Food Safety Authority (EFSA, 2015) recommends a BPA dietary intake up to 0.875 µg/kg bw/day for infants and toddlers and up to 0.388 µg/kg bw/day for adults.

Human exposure to NP and OP has been investigated less extensively. A study of human samples from the Italian population found concentration ranges of 10-266 and 0.8-20.5 ng/g for NP and OP, respectively (Ferrara et al., 2011). The same group estimated that strong fish consumers may be exposed to NP and OP doses of up to 0.5 and 0.02 µg/kg bw/day, respectively (Ferrara et al., 2008). Even though these concentrations are considerably lower than those tested in the present study, the risk of adverse biological effects in humans cannot be ruled out, particularly considering their cumulative exposure to multiple chemicals acting on the same pathways and the sensitivity windows of the developing fetus. According to some studies, the circulating levels of some of these pollutants measured in humans are probably explained by much higher actual oral intake (Shin et al., 2004).

The present findings document the negative effects of dietary exposure to BPA, NP, or t-OP on lipid metabolism. The histopathological changes described in liver highlight the toxic effects that may be induced

in wildlife and humans by consumption of contaminated food or direct exposure to these environmental pollutants.

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Table 1

Primer list.

Gene	Forward	Reverse	References
act	GGTACCCATCTCCTGCTCCA	GAGCGTGGCTACTCCTTCAC	Maradonna et al. (2014)
pparα	TGCAAGGGTTTCTTCAGGAG	GCAGTACTGCTTGTTGC	This work
pparβ	GCTTTCCAGAAGTGCCTGTC	TCTTCAGGTCAGAGCCACCT	This work
ppary	CGCCGTGGACCTGTCAGAGC	GGAATGGATGGAGGAGGAGGAGATGG	This work
ef1α	AGTCCACCTCCACCGGTCAT	AGGAGCCCTTGCCCATCTC	Maradonna et al. (2014)
pla2	CCAGACCATCTTCACCATCC	CACCCAATCCACAGGAGTTC	Alves Martins et al. (2012)
pepck	AGAGCCATCAACCCTGAGAA	CTCCCACCACACTCCTCCAT	Alves Martins et al. (2012)
hsl	CGGCTTTGCTTCAGTTTACC	ACCCTTCTGGATGATGTGGA	Alves Martins et al. (2012)
cox2	TGTCCCGTGGCATCCTTT	ATCACGGAGCCCGTCTCTT	Paredes et al. (2014)
5-lox	CCTGGCAGATGTGAACTTGA	CGTTCTCCTGATACTGGCTG	Alves Martins et al. (2012)
fas	TGGCAGCATACACACAGACC	CACACAGGGCTTCAGTTTCA	Paredes et al. (2014)
lpl	GAGCACGCAGACAACCAGAA	GGG GTAGATGTCGATGTCGC	Paredes et al. (2014)

Table 2

Effect of NP, t-OP and BPA administration on food intake in *S. aurata* juveniles. Data are expressed as mean feed intake (mg) ± SD per g of bw per 60 min of observation. Asterisks indicate significant differences evaluated by one-way ANOVA with Tukey's post-test, with significance set at $p < 0.05$. Values are tested against the respective control.

	C	NP1	NP2	t-OP1	t-OP2	BPA1	BPA2
7 days	475 ± 20	438.3 ± 25.1	469.7 ± 16.1	369 ± 21.4*	315 ± 8.5*	493.3 ± 7.6	456.3 ± 27.1
14 days	476.7 ± 22.5	387.3 ± 8.6*	371 ± 3.6*	307 ± 10.1*	206.3 ± 6.6*	476.7 ± 12.5	456.3 ± 9.6
20 days	490 ± 13.2	302 ± 4.6*	313.3 ± 12*	294 ± 14.2*	105 ± 20*	414.3 ± 7.8*	434 ± 10.1*

Table 3

Daily BPA, NP and t-OP concentrations provided with the feed.

Experimental groups [Nominal concentration]	Real concentration (Daily xenobiotic concentrations provided with the diet) BPA	NP	t-OP
Control [0]	nd	1.1×10^{-6} mg/kg bw	3×10^{-7} mg/kg bw
NP1 [5 mg/kg bw NP]	nd	2.8 mg/kg bw	nd
NP2 [50 mg/kg bw NP]	nd	12.7 mg/kg bw	0.6 mg/kg bw
t-OP1 [5 mg/kg bw t-OP]	nd	nd	1.5 mg/kg bw
t-OP2 [50 mg/kg bw t-OP]	nd	2.2 mg/kg bw	20.1 mg/kg bw
BPA1 [50 mg/kg bw BPA][a]	2.6 mg/kg bw	nd	nd
BPA2 [50 mg/kg bw BPA][a]	33.4 mg/kg bw	nd	nd

nd: not detected.

Table 4

Contingency table for lipid accumulation in control and exposed fish. A) NP2 fish show significantly different lipid accumulation both vs. C ($p = 0.0018$) and vs. NP1 ($p = 0.0304$) fish (contingency coefficient 0.50); B) t-OP1 ($p = 0.0134$) and vs. t-OP2 ($p = 0.0128$) fish show significantly different lipid accumulation vs. C fish (contingency coefficient 0.37);
C) BPA2 fish show significantly different lipid accumulation vs. C ($p = 0.0128$) and vs. BPA1 ($p = 0.0449$) fish (contingency coefficient 0.24). N = number of specimens per group. a: non significantly different vs. C; b: significantly different vs. C.

		% lipid accumulation					
		N	Absence	Mild	Moderate	Severe	Significance
A	C	6	0	0	83.3	16.7	a
	NP1	12	16.6	0	41.7	41.7	a
	NP2	10	60	0	0	40	b
B	C	6	0	0	83.3	16.7	a
	t-OP1	10	0	0	20	80	b
	t-OP2	7	0	0	14.3	85.7	b
C	C	6	0	0	83.3	16.7	a
	BPA1	15	0	0	60	40	a
	BPA2	7	0	0	14.3	85.7	b

Figure captions

Fig. 1 Representative images of the different degrees of hepatic steatosis in seabream.

Mild (A), moderate (B), and severe (C) lipid accumulation in seabream liver. (A): hematoxylin-Eosin, (B): gentian violet; (C): toluidine blue.

Fig. 2 *Pparα* (a)*, pparβ* (b), *ppary* (c), *rxr* (d), *and fas* (e) mRNA levels

Pparα (a), *pparβ* (b), *ppary* (c), *rxr* (d) and *fas* (e) mRNA levels normalized against *act* and *ef1α;* in C fish and in fish treated with NP1, NP2, t-OP1, t-OP2, BPA1, and BPA2. a.u.- arbitrary units-. Error bars indicate mean±S.D. Different letters denote significant differences among experimental groups ($p<0.05$), analyzed by ANOVA followed by Tukey's multiple comparison test. Multiplicity adjusted P values for comparison of C vs. experimental groups are shown. Family wise significance and 95% confidence intervals were set at 0.05.

Fig. 3 *Lpl (a), hsl* (b) mRNA levels

Lpl (a) and *hsl* (b) mRNA levels normalized against *act* and *ef1α* in C, NP1, NP2, t-OP1, t-OP2, BPA1, and BPA2 fish. a.u.- arbitrary units-. Error bars indicate mean±S.D. Different letters denote significant differences among experimental groups ($p<0.05$), analyzed by ANOVA followed by Tukey's multiple comparison test. Multiplicity adjusted P values for comparison of C vs. experimental groups are shown. Family wise significance and 95% confidence intervals were **set** at 0.05.

Fig. 4 Plasma levels of cortisol (a), *gr* (b) and *pepck* (c) mRNA levels.

Plasma levels of cortisol (ng/ml) (a) and *gr* (b) and *pepck* (c) RNA levels normalized against *act* and *ef1α* in C, NP1, NP2, t-OP1, t-OP2, BPA1, and BPA2 fish. a.u.- arbitrary units-. Error bars indicate mean±S.D. Different letters denote statistical significant differences among experimental groups ($p<0.05$), analyzed by ANOVA followed by Tukey's multiple comparison test. Multiplicity adjusted P values for comparison of C vs. experimental groups are shown. Family wise significance and 95% confidence intervals were set at 0.05.

Fig. 5 ***Pla2*** **(a),** ***cox-2*** **(b),** ***5 lox*** **(c) mRNA levels**

Pla2 (a), *cox-2* (b), *5-lox* (c), and *rxr* (d) mRNA levels normalized against *act* and *ef1α* in C, NP1, NP2, t-OP1, t-OP2, BPA1, and BPA2 fish. a.u.- arbitrary units-. Error bars indicate mean±S.D. Different letters denote significant differences among experimental groups ($p<0.05$), analyzed by ANOVA followed by Tukey's multiple comparison test. Multiplicity adjusted P values for comparison of C vs. experimental groups are shown. Family wise significance and 95% confidence intervals were set at 0.05.

Fig.1

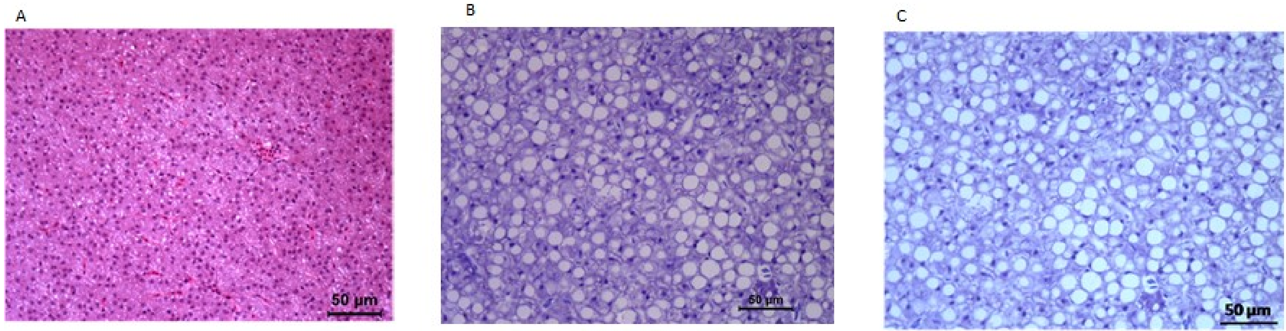


Fig.2

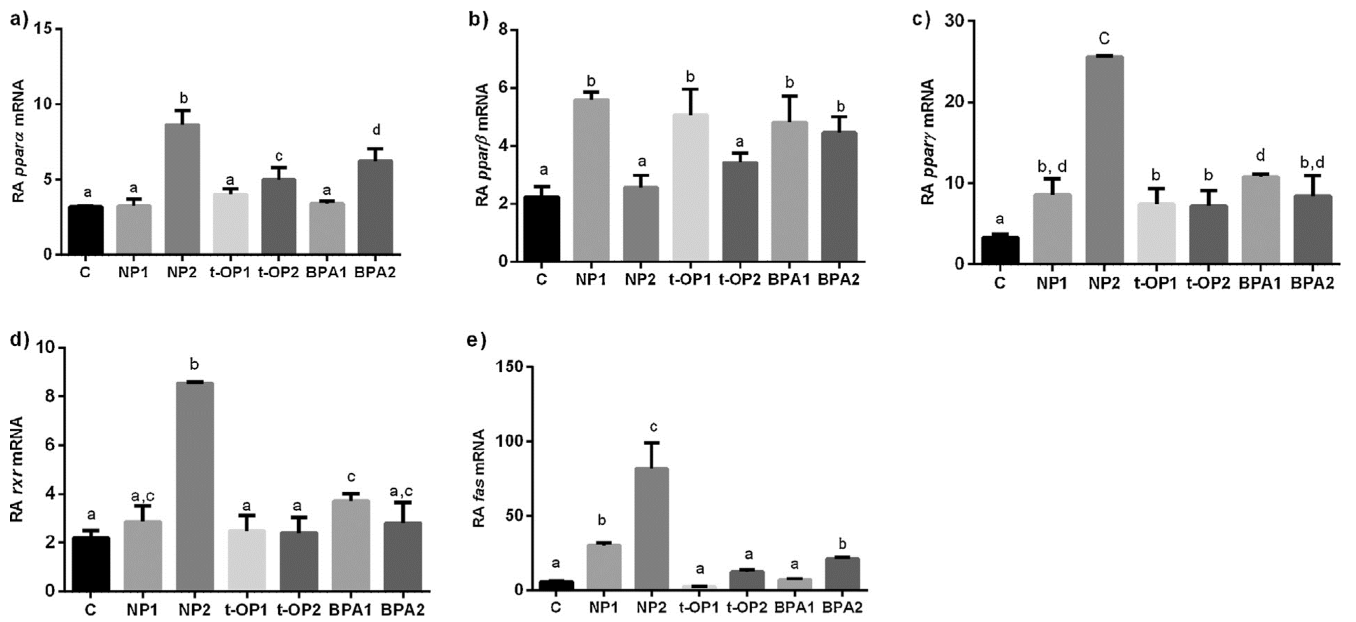


Fig.3

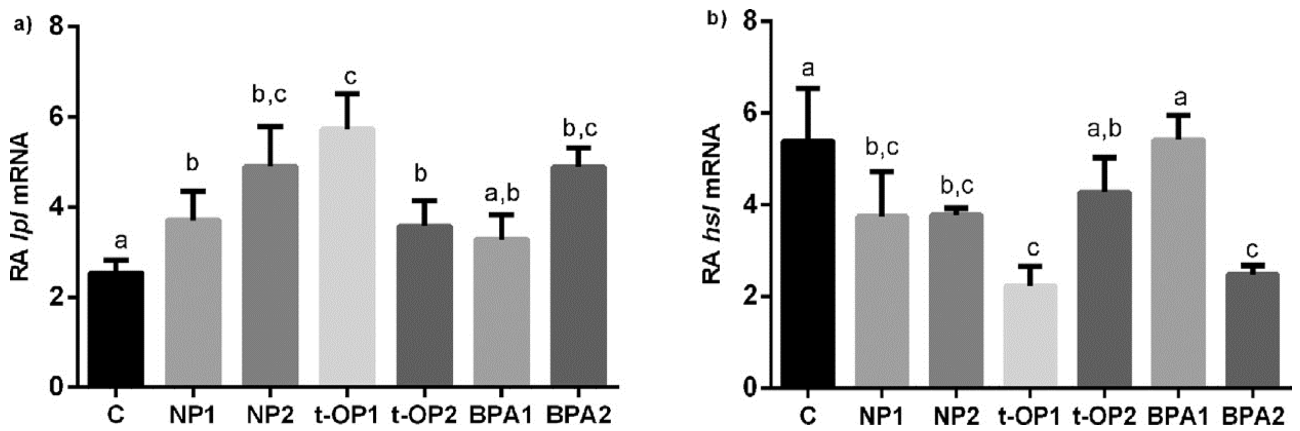


Fig.4

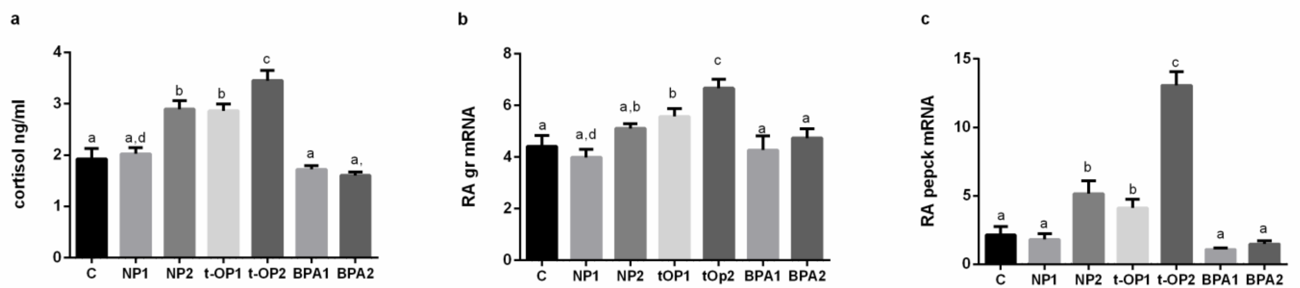
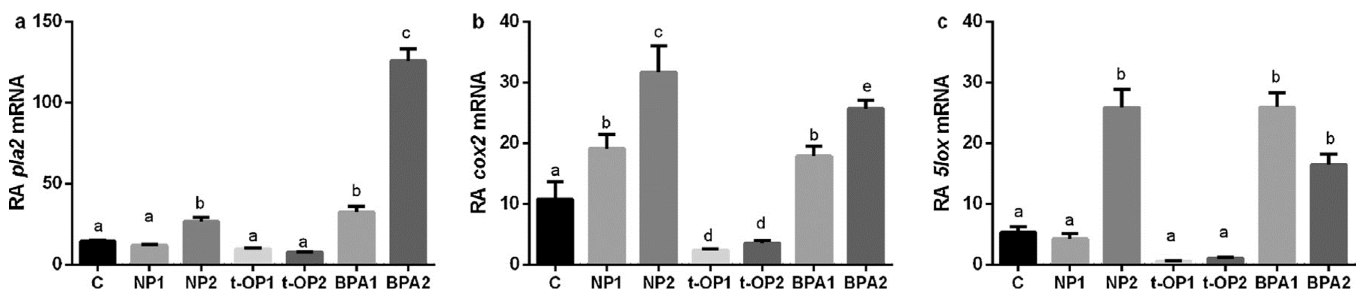


Fig.5



8. EFFECTS OF BPA ON FEMALE REPRODUCTIVE FUNCTION: THE INVOLVEMENT OF EPIGENETIC MECHANISM

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Abstract

Epigenetic modifications are classified as heritable and reversible chemical modifications of chromatin that do not cause changes in DNA sequence. Changes in epigenetic modifications can be caused by exposure to certain environmental factors, such as contaminants like bisphenol A (BPA). Bisphenol A is ubiquitous in the environment and produced in large quantities, and known to have hormone-like activity, whereby disrupting endocrine function. Because of evidence for disruption of sex steroid mediated pathways, there is a concern that BPA could have adverse effects on female reproduction. The purpose of this review is to summarize the effects of BPA on adult female reproduction with focus on epigenetic changes that can be heritable.

1. Introduction

There is a decreasing trend in human fertility, even in young woman, especially in western countries (Brannian and Hansen, 2006; Crain et al., 2008; Diamanti-Kandarakis et al., 2009; Frey and Patel, 2004; Nyboe Andersen and Erb, 2006; Zama and Uzumcu, 2010). There may be several reasons for this decrease, likely possibility is the exposure to environmental endocrine disrupting compounds (EDCs) that can interfere with the reproductive system in vertebrates (Patisaul and Adewale, 2009). It is alarming that reproductive changes may involve epigenetic changes that can be heritable (Bollati and Baccarelli, 2010; Jirtle and Skinner, 2007; Zama and Uzumcu, 2010).

EDCs are both synthetic and natural compounds, present in the environment, that have the ability to interfere with the actions of endocrine system, by mimicking or antagonizing the actions of endogenous hormones (Bhandari et al., 2014). One of the most aboundant EDCs is bisphenol A (BPA), which is used in the production of plastics polycarbonate and epoxy resins. BPA has been detected in the urine of 93% of the US population (Schug et al., 2011) and there is a growing concern regarding the ability of this pollutant to interfere with epigenetic mechanisms (Bhandari et al., 2014; Kundakovic and Champagne, 2011; Manikkam et al., 2013; Singh and Li, 2012; Wolstenholme et al., 2011; Zama and Uzumcu, 2010).

The goal here is to review studies concerning the effects of BPA on the reproductive system of adult female and its ability to interfere with epigenetic mechanisms. The epigenetic effects are heritable, and modifications

of chromatin leads to adjustments in its activity without changing the underlying DNA sequence (Jirtle and Skinner, 2007; Tollesfsbol, 2011).

2. Bisphenol A – a widespread toxin

BPA is a phenol formed by a hydroxyl residue directly bound to an aromatic ring (Flint et al., 2012). It was synthesized firstly in 1891 by the Russian chemist Alexander P. Dianin. Later in 1930 its estrogenic effects were discovered (Rubin, 2011). The use of BPA in the plastics industry started in 1940 because of its property to increase heat resistance and elasticity. Use of BPA worldwide has increased progressively bringing its production currently to more than 10 millions of tons per year (Fenichel et al., 2013).

BPA is present in 95% of products containing epoxy resins and polycarbonates such as food-containers, bottles, toys, dental products, CD and DVD and water pipes. It is also used in the vinyl chloride and thermal paper production which are used for the manufacture of slips, books, booklets, ticket, kitchen rolls, toilet paper and food paperboard (Nam et al., 2010) and is released mainly in landfill or in plastic degradation process (Kang et al., 2006).

There is evidence that people are exposed to BPA continuously, mainly through skin contact and food ingestion. The daily exposure of humans to BPA through food is estimated at between 0.48 to 1.6 μg/Kg/body weight/day (Vandenberg et al., 2007).

Some studies conducted in Europe showed that Norwegian people have a concentration of BPA in their urine of 4.5 μg/dm^3 which his very high compared to that of Netherlands (2.5 μg/dm^3) and Belgium (2.55 μg/dm^3) inhabitants and similar to that found in the citizens of US which is of 3.9 μg/dm^3 (Michałowicz, 2014; Pirard et al., 2012).

Once ingested BPA is rapidly metabolized in the liver into bisphenol A-glucuronide, which has higher solubility and can be excreted in urine (Mileva et al., 2014). While BPA metabolism limits the amount of “free” unconjugated BPA to bind to ERs, the exposure to this pollutant has been shown to have lasting consequences for human health. There is evidence that BPA can act as a potent EDC, and may have overlap in specificity with both estrogen (Lapensee et al., 2009) and androgen (Kinch et al., 2015) receptor system at very low doses (Mileva et al., 2014).

3. BPA and female reproduction

BPA exerts its action by binding both estrogen receptors, estrogen receptor α (ERα) and estrogen receptor β (ERβ), and, therefore, changes the gene expression by activation or repression. It has dual mechanism of action, and can exert pro- and anti-estrogenic effects depending of tissue (Kundakovic and Champagne, 2011; Rubin, 2011). It was initially classified as a weak environmental estrogen and more recent studies demonstrated its ability to affect the physiological functions of cells and tissues, binding of nuclear receptors, even at very low (pico- and nanomolar) concentrations (Wetherill et al., 2007). By binding to the non-classical membrane estrogen receptors (mERs), it can trigger rapid non-genomic mediated changes (Mileva et al., 2014).

In a study conducted on mice it was shown that BPA has a non-linear dose response curve, called non-monotonic curve. Therefore, study of BPA at high doses may not provide accurate information on its potential adverse mechanism of action (Miyawaki et al., 2007; Vandenberg et al., 2012).

In the ovary, Lee and colleagues (Lee et al., 2013) have tested two doses of BPA (0.001 and 0.1 mg/kg body weight) on adult female rats for 90 days to determine its effects on steroidogenesis and pathophysiological effects. They found a reduction in the levels of 17β-estradiol (E2) for both BPA concentrations, despite an increase in apoptotic caspase 3-positive cells which can lead to follicular atresia and luteal regression. They have also found a reduction in expression of steroidogenic acute regulatory protein (*StAR)* (involved in the transfer of cholesterol from the outer to the inner mitochondrial membrane), and *P450* aromatase (catalyze the biosynthesis of estrogen). Changes in the expression of *StAR* and *P450* aromatase are of fundamental importance for steroidogenesis and, the authors conclude that the long-term exposure to BPA can lead to prolonged reduction in E2 levels, which in turn, increases apoptosis of ovarian cells.

A study by Ziv-Gal and colleagues (Ziv-Gal et al., 2013) examined the effects of a wide range of BPA concentrations (0.004-435 μM) on ovarian follicle function of mice, and demonstrated its ability to alter the endocrine system by interacting with the aryl hydrocarbon receptor (AHR). They have demonstrated dose-related effects of BPA on decreased E2 levels and inhibition of follicular growth. They also found that BPA can alter the expression of the anti-apoptotic factor *Bcl2*, which is a factor involved in follicular atresia, and

concluded that contributing adverse effects of BPA include disruption of follicular growth and AHR signaling pathway to a lesser extent.

These studies confirm the work conducted by Peretz and colleagues (Peretz et al., 2011) that demonstrated the ability of BPA to inhibit both *StAR* and *P450* aromatase mRNA expression in isolated antral follicles obtained from 32 days old mice. They have also demonstrated the ability of this pollutant to decrease the production of progesterone, dehydroepiandrosterone, androstenedione, estrone, testosterone and E2.

In fish, BPA was shown to decrease the ovarian *cyp19a1* transcript in adult rare minnow after 35 day exposure at 15 µg/L (Qin et al., 2013). Furthermore, three months exposure to BPA (5 µg/L) delayed ovulation or inhibited ovulation in brown trout (Lahnsteiner et al., 2005). In female carp, exposure to BPA (1 µg/L) resulted in atretic follicles (Mandich et al., 2007). These results were confirmed by other investigators who demonstrated the ability of BPA to increase follicular atresia (Molina et al., 2013; Santangeli et al., 2016).

In addition to these studies, BPA was shown to reduce the concentration of E2 in plasma and expression of genes involved in the growth of oocytes and absorption of cholesterol in zebrafish and fathead minnow (Villeneuve et al., 2012).

4. Epigenetic mechanisms

The term *epigenetics* was described for the first time in 1942 by Conrad Waddington as "…the interaction of genes with their environment which bring the phenotype into being" (Waddington, 1942). Epigenetic modifications are now described as heritable and reversible chemical modifications of chromatin, resulting in adjustment of its activity without change in the underlying DNA sequence. Epigenetic plays an important role in cellular differentiation, growth, metabolism, and regulating of gene expression by silencing and activating specific genes (Bernstein et al., 2007; Esteller, 2007; Jirtle and Skinner, 2007). In addition, it was shown that epigenetic mechanisms facilitate interaction between gene transcription and environment (Esteller, 2007).

The main types of epigenetic modifications include DNA methylation, histone modification and changes associated with non-coding RNAs (ncRNAs) (Tammen et al., 2013).

4.1. DNA methylation

DNA methylation has been widely studied and is believed to be one of the main causes of epigenetic changes involving addition of methyl group (CH_3) to 5th ring carbon cytosine, mediated by a S-Adenosylmethionine (SAM) donor, and occurs mainly at locations rich in cytosine and guanine bases known as CpG islands (Bernstein et al., 2007; Tammen et al., 2013). CpG dinucleotides are present within the promoter region and account for only 1-2% of the entire genome (Zhang and Ho, 2011). This process, catalyzed by the DNA methyltranferases (DNMTs), leads to the formation of 5-methyl-cytosine. The DNA methylation correlates with gene silencing, and thus, inactivation of their transcription. This process is important in many cellular processes, such as aging, cell differentiation, and X chromosome inactivation (Jirtle and Skinner, 2007).

4.2. Histone modification

The histone modifications are another important type of epigenetic modifications. Histones are proteins that form the nucleosome. Nucleosomes are the main component of chromatin and allow the DNA to be carefully packed inside a cell. Each nucleosome is built by about 146 base pairs of DNA wrapped around a histone core to form an octamer. The histone modification occurs on the histone tail (Turner, 2002). The combination of histone which is positively charged, and DNA which is negatively charged, give rise to heterochromatin, in which the transcription is not possible. Enzyme-catalyzed modifications to histone, changes the structure of chromatin, leading to activation or suppression of transcription. These changes occur on residues of specific amino acids and the most well studied are the acetylation of lysine, methylation of lysine (levels 1-3) and arginine, and phosphorylation of serine and threonine on different histone proteins.

Generally, the acetylation of lysine residues is associated with relaxation of euchromatin, granting access to transcription factors and initiation of gene transcription. Similarly, methylation of lysine 4 of histone 3 (H3K4) and H3K79 are associated with changes in eucrhromatin and activation of transcription. In contrast, various levels of methylation of H3K9, H3K27 and H4K20 leads to changes in the density of chromatin (heterochromatin) and silencing of transcription (Kouzarides, 2007; Zama and Uzumcu, 2010). The silencing of transcription may be reversible or irreversible, depending on further changes. A typical example is that some

of histone methylation events, such as H3K9me3, occur in combination with the DNA methylation to permanently silence specific genes (Zama and Uzumcu, 2010).

4.3. Non-coding RNAs

Non-coding RNAs, such as miRNA (micro RNA), piRNA (piwi-interacting RNA) and others have been linked to epigenetic mechanisms. miRNA control gene expression and are involved in the control of cell development, cell programmed death, and cell growth (Malumbres, 2013), while piRNA regulate transcription by epigenetic mechanism of *de novo* methylation in germline (Landry et al., 2013). The ncRNAs also control the long-term memory cell processes through epigenetic mechanism, and an interruption in their function can lead to abnormal cellular states (Landry et al., 2013; Malumbres, 2013).

5. BPA and epigenetic process

Epigenetic mechanisms is one of the most plausible ways through which environmental toxins, such as BPA, may exert their long-term effects.

The first convincing study of epigenetic effects of BPA was provided by Dolinoy and her colleagues (Dolinoy et al., 2007) which used Agouti viable (A^{vy}) yellow mouse as a model. These mice have a metastable DNA methylation sensitive in A^{vy} allele that is involved in determining the coat's color. The expression of the *Agouti* gene is regulated by the degree of DNA methylation in the intracisternal A particle (IAP) retrotransposon, which is located upstream of the gene *Agouti*. The coat color of A^{vy} mice is therefore pure yellow associated with hypomethylation of the A^{vy} IAP, compared to brown pseudoagouti associated with hypermethylation of the A^{vy} IAP. Using this mouse, Dolinoy et al. shows that exposure to BPA during pregnancy causes hypomethylation of DNA on the epigenome of the offspring. Maternal exposure to BPA led to redistribution of the offspring of the coat to yellow color through the decrease of methylation at specific CpG sites of the A^{vy} allele.

In human breast cancer cell line as well as mammary gland of a 6-week old mice, exposure to BPA in uterus increased histone H3 trimethylation at lysine 27 which is usually associated with the repression of the gene

transcription (Doherty et al., 2010). The observed histone modification is generally catalyzed by histone methyltransferase EZH2 (Enhancer of Zeste Homolog 2) which was also increased (Doherty et al., 2010).

In utero exposure of BPA was also found to demethylate specific CpG sites in both the promoter and intron of *Hoxa10* gene*,* leading to up-regulation of gene transcription in the uterus of two weeks old female offspring (Bromer et al., 2010).

Furthermore, exposure to BPA during pregnancy was found to alter methyltransferase 1 and 3A (DNMT1 and DNMT3A) mRNA levels which are epigenetic regulators in the offspring leading to changes in expression of genes encoding estrogen receptors (Kundakovic et al., 2013).

A neonatal exposure to BPA has also been shown to induce hypermethylation of estrogen promoter regions in rat testis. This result suggests a possible adverse effect of BPA on spermatogenesis and male fertility mediated by epigenetic changes (Doshi et al., 2011).

In late gestation mouse, exposure to BPA (50 µg/Kg) was shown to decrease genome-wide DNA methylation on placenta, leading to its abnormal development (Susiarjo et al., 2013), indication adverse effects on female fertility. In a study performed in our lab, BPA was shown to interfere with the process of oogenesis in adult female zebrafish, acting through deregulation of epigenetic mechanisms (Santangeli et al., 2016).

The study demonstrated that BPA (5 µg/L) can increase apoptosis in mature follicle and interfere with the process of oogenesis through deregulation of genes that are critical as estrogen receptors (*esr1*, *esr2a)*, luteinizing hormone/choriogonadotropin receptor (*lhcgr)* and progesterone membrane receptor component 1 (*pgrmc1)* (Santangeli et al., 2016). The study also demonstrated down-regulation of *lhcgr* concomitant with respective decrease and increase of two specific histone modification marks, H3K4me3 and H3K27me3, that mark the transcription start site of this gene. In addition, we demonstrated that changes in histone modification were probably associated with the deregulation of total methylation due to the increase of mRNA expression of *dnmt1* (involved in maintaining methylation), and *dnmt3* (involved in de novo methylation). This study has therefore provided the first strong evidence that BPA can interfere with the oogenesis process through the deregulation of epigenetic mechanism (Fig.1) and in turn with fecundity. As a result, a total block of eggs production was observed by crossing BPA exposed female with control male (Santangeli et al., 2016).

6. Conclusions

There is convincing experimental evidence supporting the hypothesis that BPA can adversely affect female reproductive physiology. BPA seems to interfere with the steroidogenesis process by reducing *StAR* and *P450* aromatase, whereby the production of E2 is blocked (Lee et al., 2013; Peretz et al., 2011; Ziv-Gal et al., 2013). These changes are correlated with an increase in follicular atresia (Lee et al., 2013; Santangeli et al., 2016; Ziv-Gal et al., 2013) and seen in human diseases that lead to infertility, polycystic ovary syndrome, and endometriosis (Caserta et al., 2014, 2013; Cobellis et al., 2009; Kandaraki et al., 2011; Tarantino et al., 2013).

In addition there is evidence that BPA interfere with epigenetic mechanisms. At the level of ovary, BPA interfere with histone modification, leading to the downward adjustment of *lhcgr* mRNA levels, and probably with global methylation because of its ability to interfere with the dnmt expression (Santangeli et al., 2016).

Further investigation is necessary to elucidate the exact mechanism by which BPA is interfering with the epigenetic pattern that leads to infertility. It will be particularly important to study the genes involved in the process of oogenesis which are affected through BPA-related epigenetic mechanisms and to evaluate whether the presence of both histone modifications and DNA methylation can lead to stable shutdown of the transcription and whether this is reversible.

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Figure 1.

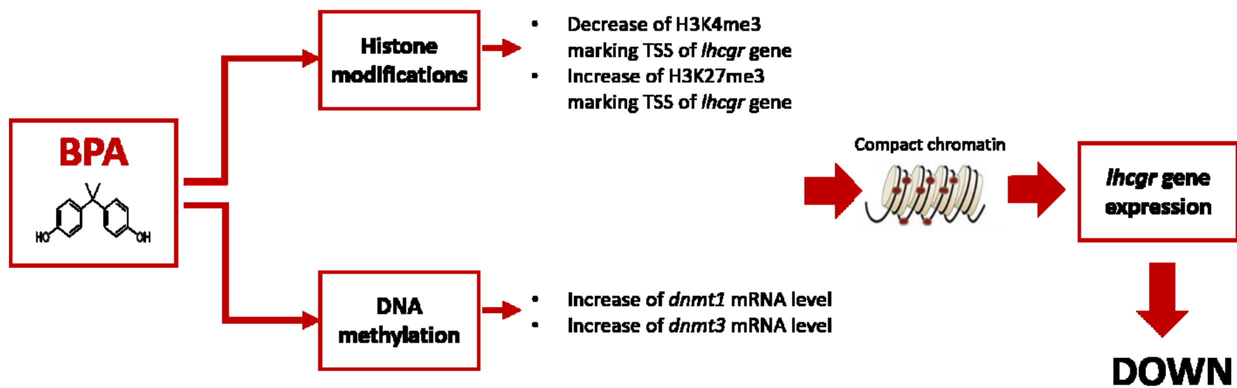

Fig. 1: The proposed mechanism of inhibitory action of BPA in the ovary.

BPA is able to down-regulate gene expression of *lhcgr* (luteinizing hormone/choriogonadotropin receptor) both decreasing and increasing respectively of H3K4me3 and H3K27me3 (trimethylation of lysine 4 (K4) and 27 (K27) and interfering with expression on DNMTs (DNA (cytosine-5-)-methyltransferases) mRNA.

9. CONCLUSIONS

The challenge with understanding the effects of EDCs, and the mechanism behind them is that EDCs involve many different classes of compounds, affecting different systems and pathways, and acting through multiple mechanism of action, making the picture of the effects of EDCs very complex. The results of the studies presented in this PhD thesis have contributed to the still existing need for more information regarding the effects of EDCs and mechanism behind the observed effects.

Much focus of this PhD project has been to investigate the effects of BPA on female's reproductive physiology and investigate whether, changes in the expression levels of genes related to reproduction are caused by histone modifications. With this work, we demonstrated that the lowest dose of BPA analyzed (5 μg/L) down-regulated oocyte maturation-promoting signals, likely through changes in the chromatin structure mediated by histone modifications, and promoted apoptosis in mature follicles. These data indicate that the negative effects of BPA on the female reproductive system may be due to its upstream ability to deregulate epigenetic mechanism.

To better elucidate epigenetic effects of BPA we focused our attention on the effects on F1 embryos obtained crossing BPA's treated females with control males. From this work we were able to demonstrate that both BPA doses tested (10^{-7} M and 10^{-5} M), interfere with gonadal formation in embryos, also by interfering with the methylation level of CpG island, marking the promoter regions of *amh;* while, only the lowest BPA's dose interfere with DNA methyltransferases gene expression. These data indicate that the negative effects of BPA on adult females can be inherited in the subsequent F1 generation, interfering with gonadal formation and the general methylation status of embryos.

Once we have understood the effects of BPA, we decided to investigate the effects of DiNP, a high molecular weight phthalate commonly used as a plasticizer, to elucidate his effects on zebrafish reproductive physiology. The results we obtained demonstrate a non-monotonic dose response relationship, with greater differences at the lower and higher doses. These findings provide evidences that exposure to DiNP significantly alter steroidogenesis, oocytes growth and maturation leading to disruption of reproduction in zebrafish.

Afterwards, we moved our attention on the effects of EDCs on metabolic system and thus we have investigated the effects of two EDCs, BPA and DGB, on lipid metabolism in *Danio rerio*'s liver. The results we have

obtained have shown detrimental effects on lipid metabolism for both the pollutants. The lower BPA dose (5 μg/L), has the ability to increase the storage of triglycerides and fatty acid synthesis, while DGB is acting following a non-monotonic dose response curve with lower and higher doses that increase lipid production and their mobilization.

Because of the need to test a different range of concentration in various plastic pollutants, it was necessary an experimental model easy to reproduce, treat and analyse; hence the choice of introducing zebrafish as experimental model for these studies.

Concomitantly, the effects of NP, t-OP and BPA on lipid metabolism were studied in sea bream juveniles. From this work we could find that dietary xenobiotic's contamination can give rise to metabolic disorders also in marine fish and highlight the potential for their vertical transfer through the trophic levels and ultimately to humans.

In conclusion, the present PhD project, demonstrates that endocrine disruptors compounds, used in the manufacturing of plastics, are able to interfere with reproductive and metabolic system of teleost fish, acting in a non-monotonic fashion. Furthermore we demonstrates the capacity of BPA to affects gene expression through the deregulation of epigenetic patterns.

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