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The Influence of Environmental Estrogens on Early-life Stage Zebrafish Development

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A thesis submitted to the School of Medicine, National University of Ireland, Galway, in fulfilment of the degree of Doctor of Philosophy

Supervisors: Prof. Peter Dockery
            Dr. Brendan Wilkins

February 2013
To my husband Darek, without whose love, constant support and understanding this chapter of my life would have not been started, and certainly, never completed...

***
“The task is...not so much to see what no one has yet seen, but to think what nobody has yet thought, about that which everybody sees.”

Erwin Schrödinger
Declaration

I certify that the work presented in this thesis is my own and that it has not been submitted for any other award at this or any other university.

Katarzyna Zielinska
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Abstract

Endocrine disrupting compounds (EDCs) including bisphenol A (BPA) and genistein are thought to induce oestrogenic effects in developing organisms. Genistein is a naturally occurring substance found in many plants but particularly in soy products. BPA is a chemical used extensively in the production of plastics and resins. Given the widespread environmental presence of both compounds, exposure to them and their mixtures is conceivable.

This study aimed to investigate the effects of BPA and genistein, individually and in mixtures, on the early development of zebrafish. It is generally understood that vertebrates are the most vulnerable to chemical exposure during early developmental stages.

Zebrafish is a widely used model for developmental biology and toxicology as it has many conserved developmental programs and processes that are also found in mammals.

In the present study high concentrations of BPA or/and genistein were tested for their toxic and teratogenic potential. These tests were based on the observations of survival, hatching, heart rate and morphology of the embryos and larvae up to 120 hpf (hours post fertilisation). Lower concentrations, including environmentally relevant doses, were tested for their potential to disturb the ultrastructure of the developing liver and their effect on vitellogenin 1 mRNA (vtg1) expression.

The present study demonstrated that BPA or/and genistein clearly influence the early development of zebrafish. Survival, heart rate, hatching success and morphology of the embryos and larvae were affected by the high concentrations of these compounds, individually and in mixtures. Decreased head size, heart oedema, enlarged yolk sac/yolk extension and changes in the skin pigmentation level were often observed. Most of the mixtures showed enhanced toxicity when compared to the corresponding treatments with individual compounds indicating additivity of BPA and genistein. Some observations revealed that synergistic and antagonistic interactions between these compounds may also occur when acting on zebrafish during the early stages of development.

Previous studies have demonstrated cytopathological alterations in the hepatocytes of fish exposed to xenobiotics. There was no study to date, however, investigating the effects of environmental oestrogens, and particularly their mixtures, on the fine structure of the liver. In this study, the ultrastructural alterations in the hepatocytes of the developing zebrafish treated with BPA or/and genistein were investigated. Stereological methods were used to objectively describe the phenotype of the organ and enable quantitative and statistical analysis of the data. Several cytological alterations were observed following exposure to either BPA or genistein and included the alterations in the volume of hepatocytes, mitochondria, as well as glycogen. Particularly affected were the size or/and shape of individual mitochondria. Considerably fewer morphological changes were seen in the hepatocytes of the larvae treated with the mixtures than in the corresponding treatment groups with individual EDCs. Such observations may indicate antagonistic interactions between BPA and genistein when acting on the liver of zebrafish larvae. The synergistic interactions of these EDCs could however also be inferred based on the significant alterations in the amount of RER.
Vitellogenin is a yolk precursor protein produced in the liver of oviparous vertebrates in response to the activation of oestrogen receptors (ERs). Many environmental compounds are able to bind ERs and thus induce vitellogenesis. In this work the expression level of vtg1 in the larvae treated with BPA or/and genistein has been investigated. Genistein showed visibly higher potential to induce vtg1 expression than BPA. The estrogenicity of the mixtures was enhanced, although not significantly, when compared to the treatments with individual EDCs.

This study was the first to investigate the effects of BPA and genistein mixtures on the early development of zebrafish. The present study has identified a number of possible chemical interactions, namely additivity, antagonism and synergism between these EDCs depending on their concentrations, stage of development and the observed endpoints.

The multidisciplinary approach, adopted in the present study, provides a toolset to help unravel the complex interactions encountered when studying complex chemical exposure to sensitive developmental processes.
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First and foremost, I would like to thank Professor Peter Dockery for his supervision, great support, patience and amazing ‘Baba Jaga vs. snowmen’ attitude which made the whole process of working on this project so enjoyable!

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Moreover, I want to sincerely thank Dr. Brendan Wilkins for every encouraging word, advice, support and help during my College years.

Furthermore, I would like to express my thanks to Dr. Lucy Byrnes for letting me use the zebrafish facility and all PCR-related equipment in the Biochemistry Department. I am also grateful to Dr. Byrnes and Dr. Grealy for including me in their regular lab meetings.

There is not a single person in the Anatomy Department whom I would not wish to include on my ‘thank-you’ list: Thank you, Mark and Pierce, for all your technical support, patience, and kindness during all these years, and for your sense of humour which made the whole process of learning more interesting. Sincere thanks to all the academics and staff in the Department, particularly Alex, Fabio, Fidelma, Helen, Peter, Siobhan and Yolanda for their kindness, willingness to help and every word of encouragement. Last, but never least, heartfelt thanks to Alanna, Bridget, Eva, Kerry, Laurena and Stef for their friendship, understanding, support, and sharing all the ‘ups and downs’ of the PhD adventure!

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List of Abbreviations

BPA – bisphenol A
bpm – beats per minute
DMSO – dimethyl sulfoxide
dpf – days post-fertilization
dph – days post hatch
E1– oestrone
E2 – oestradiol
EC50 – effective concentration 50%
EDC – endocrine disrupting compounds/chemicals
EE2 – ethinylestradiol
ER– oestrogen receptor α, β and γ
HSI – hepatosomatic index
hpf – hours post-fertilization
hph – hours post-hatch
Hsp – heat shock protein
LC50 – lethal concentration 50%
nER – nuclear oestrogen receptor
NFW – nuclease free water
NOEC – no-observed effect concentration
pmER – plasma membrane oestrogen receptor
ROS - reactive oxygen species
rt PCR – real time polymerase chain reaction
Vtg/vtg - vitellogenin protein/mRNA
1 General Introduction

1.1 Endocrine disrupting compounds

Humans are thought to be exposed to dozens if not hundreds of compounds with hormonal activity which can disturb hormonal homeostasis. Endocrine disruptors were the main subject of the Wingspread conference held in Wisconsin in 1991 (Markey et al., 2002; Vandenberg et al., 2009) where alarming reports regarding the disturbances in the morphology and physiology of wildlife were discussed. Reported observations included the alterations in the reproductive abilities and development of animals living in the areas contaminated by some industrial and agricultural substances. These alterations were correlated with modifications in the endocrine system and as a result of the discussion the term of endocrine disruptor was coined. This conference could be described as the beginning of the big scientific debate on the risk posed by substances with hormonal activity for wildlife and human beings. Public attention to the problem of endocrine disruptors was drawn by Colborn et al. (1996) who published their famous book entitled “Our stolen future”.

There are many definitions of the term “endocrine disruptor”. One of the definitions has been given by the US Environmental Protection Agency and it says that endocrine disruptors are “exogenous agents that interfere with the synthesis, secretion, transport, binding, action, or elimination of natural hormones in the body that are responsible for the maintenance of homeostasis, reproduction, development, and/or behaviour” (Markey et al., 2002). A somewhat complementary definition was established at the European Workshop on the Impact of Endocrine Disrupters on Human Health and the Environment (EUR, 1997) which states that “an endocrine disrupter is an exogenous substance that causes adverse health effects in an intact organism, or its progeny, subsequent to changes in endocrine function”.

Currently there are hundreds of substances, which have been shown to disrupt hormonal balance of both living organisms and in vitro systems. There are many sources of endocrine disrupting substances in our environment but the main and biggest one is human activity. The rapid development of industry and technology in the last centuries has caused the appearance of hundreds of new chemicals many of which have been observed to have endocrine disrupting potential. Although an increasing body of national and international regulations has considerably reduced the amount of chemicals released into the environment, at least in developed countries, concealed but insidious contamination at low doses of a vast number of environmental contaminants still pose a threat to living organisms. As Braunbeck noticed, in the light of the fact that most environmental pollutants are low in concentration but chronic in character, a shift from the relatively crude methods used for the determination of acute toxicity to the development of more refined methods is necessary (Braunbeck, 1998).
Endocrine disruptors are a structurally heterogeneous group of compounds and consequently it is difficult to estimate hormonal activity of a given substance based solely on its chemical structure. Some of these compounds may share literally no similarity apart from having relatively small molecular mass not exceeding 1000 Daltons (Diamanti-Kandarakis et al., 2009). Some structural features of the compounds, however, may increase suspicion of endocrine disrupting potential. The presence of a phenolic moiety – the feature characteristic for the structure of the natural steroid hormones, and the presence of halogen group substitutions are good examples of such characteristics (Diamanti-Kandarakis et al., 2009; Santodonato, 1997).

In order to find whether a given substance possesses endocrine disrupting potential, however, more complicated and sophisticated methods, including in vitro and in vivo hormonal assays, are required (Markey et al., 2002). The process of risk assessment is particularly difficult as several factors constitute an additional load and further complicate such tasks. The existence of multiple receptors through which endocrine disrupting chemicals (EDCs) can act, different outcome of EDCs’ action dependent on the receptors, sex and stage of development of the organisms, differential plasma protein binding and the influence of other oestrogenic compounds are just examples of the factors making the process of establishing endocrine regulations particularly hard (Degen and Bolt, 2000).

Endocrine disruptors can interfere with any known natural hormone. Because of the great influence of oestrogens and androgens on normal sex development, compounds which disrupt the balance of sex hormones constitute a particular hazard for living organisms and have been of great interest. Much attention has particularly been paid to the substances with potential to disturb living organism’s oestrogenic homeostasis which have been defined as xenoestrogens.

1.2 Xenoestrogens

Xenoestrogens are a group of chemicals including both man-made and naturally occurring substances. Most of the scientific statements to date agree that xenoestrogens in high doses can evoke developmental and reproductive disturbances. Much discussion has been devoted to the question of whether xenoestrogens at realistic, i.e. environmentally relevant concentrations can pose risk for living organisms. While some scientists claim that there is no threat, others seem to be convinced that xenoestrogens pose great danger for living organisms, including humans. The lack of unanimity in this matter may result from the fact that there are no methods which, with a hundred percent certainty, would allow assessing the interactions of environmental oestrogens with the endocrine system (Degen and Bolt, 2000).

Some observations suggest that there is a real problem concerning excessive oestrogenic exposure. A somewhat drastic example of endocrine disruption resulting from the exposure to xenoestrogens is the case curiously entitled: “The mortician’s mystery: gynecomastia and reversible hypogonadotropic hypogonadism in an embalmer” (Finkelstein et al., 1988). In this study a 50-year old man was presented with a progressive loss of libido, a decrease in testicular
size and beard growth, and marked breast development. When the presence of oestrogen producing tumour was excluded, the existence of an external source of disturbing factor was concluded. Further study revealed that a hormonally active substance which was contained in the patient’s serum could effectively displace radio-labelled oestradiol from its receptor. The source of this substance was identified as the embalming cream used by the patient in his work. In addition, after removing of the source of environmental oestrogen a significant restoration of libido, testes size and sperm count, as well as reduction in breast size was observed.

The disturbances in male reproductive system associated with the exposure to hormonally disrupting substances have also been reported elsewhere (Main et al., 2010; Petrelli and Mantovani, 2002; Rozati et al., 2002; Toppari et al., 1996). The most common of the reported disorders included testicular cancer, cryptorchidism (maldescent of the testes) and hypospadias (the defect of the urethra that involves an abnormally placed urinary meatus). The opinion that a substantial decrease in the male fertility occurred in the last half a century has also been expressed (Carlsen et al., 1992; Carlsen et al., 1993; Swan et al., 1997). Moreover, the drop in the semen volume and sperm counts was recognized to be associated with foetal exposure to EDCs (Carlsen et al., 1992; Giwercman, 1997). Foetal development has been suggested to be the period of particular importance in the genesis of male tract malformations (Giwercman, 1997). This observation was confirmed by Sharpe et al. (1993) who have reported similar anomalies in the sons of women treated with diethylstilbestrol (DES) during pregnancy as well as in animals exposed to EDCs during foetal life.

Health adverse effects related to the exposure to oestrogenic environmental compounds have also been reported in women. The exposure to hormonally active environmental compounds has been suggested as the reason behind increased occurrence of breast cancer. Both epidemiologic and experimental observations support this hypothesis (Ardies and Dees, 1998; Davis et al., 1993; Nikaido et al., 2004). Similarly to the situation observed in males, foetal exposure to EDCs has been observed to result in the adverse effects on the reproduction related organs. For instance, the development of vaginal adenocarcinoma has been reported in so called DES-daughters, i.e. daughters of women exposed to DES while pregnant (Herbst et al., 1999). The detrimental influence of foetal exposure to xenoestrogens on the development of the reproductive tract and the mammary gland in females has been repeatedly proved experimentally (Cabaton et al., 2010; Markey et al., 2005; Newbold et al., 2007a; Nikaido et al., 2004).

1.3 Xenoestrogens and oestrogen receptors

Substances with endocrine disruptive potential, including xenoestrogens, can exert their deteriorating effect on living organisms through a few identified pathways. One of the modes of action is mimicking of endogenous hormones and evoking oestrogen-typical responses and this pathway is described as agonistic action of EDCs. Another way to influence the endocrine
system is by antagonizing natural hormones which takes place when EDCs bind to the hormones’ receptors and, by doing so, prevent endogenous hormonal action. Moreover, hormonally active compounds can alter the pattern of synthesis and metabolism of endogenous hormones thereby affecting the levels of natural hormones in the blood. In addition, EDCs can modify the levels of natural hormones’ receptors and thus affect the action of hormones (Markey et al., 2002; Soto et al., 1995). EDCs may also interfere with the binding proteins and, subsequently, have an effect on the transport of endogenous hormones to their destination.

Environmental oestrogens have received relatively more attention than other EDCs and consequently the mechanism of action of xenoestrogens is one of the better understood. In general, xenoestrogens are able to mimic the activity of natural hormones (i.e. oestradiol) and depending on their action can be classified as either oestrogenic or oestrogen-like compounds (Gillesby and Zacharewski, 1998). The compounds which, due to binding to the specific proteins defined as oestrogen receptors (ERs), are able to initiate cellular and tissue effects similar to those evoked by oestradiol, are defined as oestrogenic. Oestrogen-like substances, in turn, evoke responses similar to those of oestrogen but without binding to ER (Gray et al., 1997) but through other, very diverse signalling pathways which makes the study of EDCs a particularly challenging and complex practice.

ERs belong to the group of nuclear proteins which share some structural similarities and are called the nuclear receptor super-family. Nuclear receptors (NRs) may act as ligand-dependent and ligand-independent transcription factors and are highly conserved in the animal world, ranging from invertebrates to higher organisms (Margolis and Christakos, 2010). It has been widely studied that nuclear receptors control a wide variety of developmental, physiological and metabolic processes. That is why exploration of the knowledge on the modulation of the activity of these important transcription factors is of considerable importance. The fact that chemically distinct natural ligands interact with the structurally related receptors underpins the ability of xenohormones to elicit physiological responses.

Depending on the kind of activating molecules, several subfamilies can be discerned within the NR superfamily. Steroid receptors responding to the steroid hormones make up one of NR subfamilies and are represented by ER, progesterone receptor (PR), androgen receptor (AR), glucocorticoid receptor (GR), thyroid receptor (TR), vitamin D receptor (VDR) and mineralocorticoid receptor (MR). Another group within NRs is constituted by peroxisomes proliferator activated receptors (PPARs), the bile acid receptor (FXR) and oxysterol receptor (LXR). These receptors are activated by well and less well characterized natural ligands to which very often belong endogenous metabolites derived from nutrients, xenobiotics and lipids (Margolis and Christakos, 2010). Orphan nuclear receptors (ONRs) represent another subfamily of NRs for which the natural ligands are not known. These receptors, however, play important roles in a variety of developmental and metabolic actions and can be also involved in disease (Horard and Vanacker, 2003).
As already mentioned, nuclear receptors act as transcription factors activated, in most cases, by their ligand. At a molecular level, the action of NRs is carried out by the binding of the receptor-ligand complexes to the specific DNA fragments called the hormone response elements (HREs) and affecting the expression of downstream genes. Nuclear receptors may also bind to DNA even in the absence of the ligand and sometimes function as suppressors of the gene expression. The hormone’s action is further complicated by the conformational changes in the hormone/receptor complexes which can form homo- or heterodimers and engage in their action different coregulatory proteins and RNA (Margolis and Christakos, 2010).

The diagrammatic representation of the structure of a nuclear receptor is presented in Figure 1.1. The N terminal region (A/B domain), referred also to as a modulator domain, is highly variable and usually contains a transcriptional activation function (AF-1). The most conserved region of the NR is DNA binding domain (DBD, C domain). DBD contains two highly conserved zinc fingers, each finger having four cysteines chelating one zinc ion. Zinc fingers are engaged in recognizing and binding to the specific response elements on the DNA strands. In the case of the ERs, these specific chromatin fragments are called the oestrogen response elements (EREs). Response elements are short palindromic sequences in DNA located near the target genes. DBD is also involved in the dimerization of NRs. The next region in the NR’s structure - D domain, is less conserved and constitutes a flexible hinge in the NR structure. The hinge contains a nuclear localization signal (NLS) which can overlap with the C domain. Its main function is to serve as a hinge between DBD and LBD, hence its name. LBD (ligand binding domain, E domain) is located in the C-terminal half of the receptor. It is a moderately conserved domain and the largest region in the NR. LBD possess a specific property of ligand recognition and upon binding of the ligand it shifts the receptor to a transcriptionally active state. A ligand dependent transactivation is dependent on the presence of the activation function 2 (AF-2) which is a highly conserved motif localized in the carboxy-terminal end of the LBD. AF-2 plays the obligatory role in the recruitment of coactivators required for the ligand dependent transactivation. Ligand binding domain ensures both specificity and selectivity of the physiological responses. LBD is also employed in the change of conformation, dimerization, the recruitment of coactivators, nuclear localization and often in a repression function (Giguere, 1999; Mangelsdorff et al., 1995; Margolis and Christakos, 2010; Robinson-Rechavi et al., 2003). The last domain in the structure of the NR is named F domain and it constitutes a variable C-terminal region.
ERs are classical nuclear receptors and follow all the structural characteristics typical for the superfamily. Due to a whole genome duplication event early in the vertebrate evolution, ERs are present in mammals as a duplicate gene pair and are termed alpha and beta (ERα and ERβ). Further gene duplication has taken place in the evolution of bony fishes and resulted in additional ER gene isoforms. The data concerning this issue is, however, not consistent. While some authors report the presence of a third type of receptor called ERγ, arising as the result of ERβ gene duplication (Hawkins et al., 2000; McLachlan, 2001), the existence of four different receptor isoforms is mentioned elsewhere (Meyer and Schartl, 1999; Nagler et al., 2007). Regardless of the classifications, all ERs belong to the steroid receptor subfamily and are engaged in the regulation of many biological processes such as development, behaviour and reproduction. Because of the oestrogenic character of the biological responses evoked by xenoestrogens, molecular pathways including ERs are of the most likely mechanisms of action for this group of EDCs.

ERs are ligand induced transcription factors and elicit their cellular effects by working in a characteristic fashion. Figure 1.2 depicts the schematic presentation of the ER action upon the ligand binding. In this model ER is kept in an inactive conformation through the interaction with other proteins, primarily heat shock proteins, such as Hsp 59, 70, 90. Ligand, in this case oestradiol which is produced by the ovaries and transported in the blood, is passively taken up by the cell. Next, oestradiol crosses the nuclear membrane and binds to ER causing heat shock proteins to dissociate. This leads to the change in the ER conformation from an inactive to an active form. Once activated, ligand-bound receptors form homodimers which in turn bind to the chromatin at the specific DNA sequence - the oestrogen response element (ERE). As a consequence of the homodimer binding, the chromatin undergoes rearrangement and next, the transcription of target, oestrogen dependent genes, occurs (Gillesby and Zacharewski, 1998).
The following steps involve transport of the transcribed mRNA to the cell cytoplasm and translation to the specific protein, such as, for instance, vitellogenin in the liver cells. In general, the transcription resulting from the binding of a given compound to the ER may affect numerous functions and have pleiotropic responses in the cells. The possibility of binding different chemical substances such as xenoestrogens to the ER further increases the range of possible biological effects.

Closely related to ERs, and thus suspected of mediating the action of xenoestrogens, are ER related receptors (ERRs). ERRs belong to the orphan nuclear receptor subfamily and share the sequence similarity and identical domain organization with other nuclear receptors (Giguere, 1999). The action of some xenoestrogens, for example bisphenol A (BPA) and genistein, may also be mediated by these receptors.

1.4 Non-genomic mechanisms of xenoestrogens action

For many years the prevailing dogma was that ERs are present in the cytosol and/or nucleus and function as the transcription factors upon binding of their ligands. Subsequently, the estimation of oestrogenic properties of exogenous compounds was mainly based on the ability of xenoestrogens to bind to the ERs and to act as the transcription factors through binding to EREs (Nilsson et al., 2001). The observations of oestrogenic effects of exogenous substances, while experimental systems testing the classical nuclear transcriptional activity showed weak
or no activity, led to the conjecture that other molecular mechanisms are activated by these compounds. Moreover, the observations of the fast cellular responses excluded the more time consuming processes related to genomic pathway (of the order of hours) and stimulated the search for new, non-genomic cellular mechanisms triggered by oestrogenic stimuli (Szego and Davis, 1967). This has led to the understanding that ERs can be associated with the plasma membrane (Levin, 2009; Pietras and Szego, 1977). With time, a variety of approaches has shown that the plasma membrane ERs (pmERs) might contribute greatly to the actions of oestradiol in both traditional and nontraditional target cells, and that mechanisms other than transcriptional may be involved in exerting cellular actions. In general, steroid action on the cell surface includes the activation of second messenger signalling systems inside the cell. For instance, triggering an intracellular spike of calcium (Tesarik and Mendoza, 1995), stimulating adenyl cyclase (Aronica et al., 1994; Zivadinovic et al., 2005), the activation of phospholipase C (LeMellay et al., 1997) and mitogen-activated protein kinases (Zivadinovic and Watson, 2005) have been identified as rapid (within a few seconds to minutes), nongenomic effects of oestradiol exerted via pmER. Moreover, membrane receptors, like classical ERs, may also be engaged in the positive or negative transcriptional modulation caused by oestadiol. The modification of cytosolic signal transduction pathways involving the activation of kinases, such as the ERK (extracellular-signal-regulated kinase) or MAPK (mitogen-activated protein kinase), have been identified to take part in conducting the genomic actions of ligand-bound pmERs (Pages et al., 1993; Watters et al., 1997). These and other signals lead to the phosphorylation of many proteins and affect biological responses such as cell migration, survival and proliferation.

Membrane associated ERs, which seem to play a crucial role in transmitting molecular signals from the ligands that do not penetrate the cell, show structural diversity. Depending on the similarity of the structure of pmERs to the classical nuclear ERs (nERs), Nadal et al. (2001), have classified pmERs into three groups, namely 1) a pmER identical to the nER, 2) a pmER sharing some of the domains of the nER, particularly the ligand binding domain, and 3) a pmER unrelated to the nER. Elsewhere, however, the classification of the membrane receptors is limited to membrane versions of nuclear receptors ERα and ERβ and a novel transmembrane receptor GPR30 (Watson et al., 2007a). In general, it is speculated that a post-translational modification of nERs is an important way of producing pmERs (Levin, 1999).

As already mentioned, the vast majority of the research has been devoted to the problem of xenoestrogens’ potencies in nuclear transcription. Several recent studies, however, have shown that oestrogenic mimics are capable of exerting cellular reactions in doses much lower than those required to affect nuclear transcription responses. Moreover, the obtained data indicate that xenoestrogens appear to be selective pmER modulators (Watson et al., 2010). The selectivity of the effects could result from tissue-selective profiles dependent on partnering with different signalling proteins in different tissues (Watson et al., 2007b). In addition, the possibility of binding different oestrogenic mimics to the ERs increases the diversity of potential intracellular mechanisms. This “promiscuity” of ERs in accepting so many diverse
ligands may be explained by their status as the most evolutionary primitive versions of the ligand-activated regulatory proteins (Baker, 2004). This occurred probably as a result of responding to a diverse set of molecules in the cell’s environment and contributed to the fact that so many different, exogenous substances can serve as oestrogenic ligands in an inappropriate way (Watson et al., 2007b).

The evidence that a range of environmental compounds can evoke non-genomic cellular response has been given in a series of studies carried out by the Watson group in the University of Texas. In this research several environmental compounds, which have been previously reported as weak oestrogenic, such as bisphenol A, have been shown to be quite robust in evoking non-genomic responses. The observed signalling pathways included the activation of extracellular signal-regulated kinases (Bulayeva et al., 2004; Bulayeva and Watson, 2004) and the influx of calcium and secretion and release of prolactin by tumour pituitary cells (Watson et al., 2007b; Wozniak et al., 2005). The rapid signalling actions due to exposure to some oestrogenic pesticides and manifested by changes in the intracellular calcium content were also reported for other cell types such as endocrine (Wu et al., 2006) and endothelial (Younglai et al., 2006). Another study which has confirmed the non-genomic, rapid effects of xenoestrogens was carried out by Thomas and Dong (2006). In their study, several xenoestrogens including bisphenol A and genistein, and already known to exert oestrogenic activities through nERs, were found to bind to a novel oestrogen membrane receptor GPR30 and, consequently, have potential to interfere with oestrogen signalling. Up-regulation of adenylyl cyclase activity constituted a molecular effect exerted by the studied oestrogen mimics. These results suggested that despite considerable differences in the structures of the classical ERs and GPR30, there are substantial similarities in the ligand binding domains of these two ER classes. All of these examples show that xenoestrogens are able to evoke fast, non-genomic responses with the use of membrane associated receptors. It is important, however, to remember that, apart from having an impact on the second messenger system, liganded membrane receptors may ultimately activate nuclear signalling pathway via post-translational modifications of transcription factors including nuclear receptors themselves (Watson et al., 2007b).

The wide variety of possible structural combinations of ERs, and especially the existence of novel, unrelated to nERs, membrane receptors, seems to underpin the diversity of action of estrogens. Repeating after Watson et al. (2007b), it can be stated that “Steroid action, regardless of where in the cell it takes place, is likely to result from a very complex sequence of events that can assemble a different repertoire of proteins to function in different cell types, and under different circumstances. The job is unlikely to be carried out by a simple protein making all response decisions. The existence of multiple types of steroid-binding proteins (receptors, enzymes, transporters, blood and cellular binding globulins and their receptors) and multiple examples of each type, contribute to the diversity of situations to which cells can respond”. The diversity of the effects evoked by the oestrogenic chemicals is further complicated by the possibility of activating, at once, many parallel signalling pathways while
acting selectively with respect to timing and predominant use of different signalling avenues (Bulayeva and Watson, 2004). Moreover, when the abundance of xenoestrogens able to interfere with the pathways of the natural estrogens is taken under consideration, the diversity of biological responses is further increased.

1.5 Non-monotonic dose response patterns

Important factors for the explanation how xenoestrogens may disrupt oestrogenic responses are the broad tissue distribution of multiple types of ERs and the existence of different molecular pathways following ligand binding (i.e. transcriptional vs. membrane-initiated second messengers and kinase cascades). Additional aspects, however, need to be taken under consideration when the effects of estrogens and oestrogenic mimics are studied, namely nonconventional/non-monotonic dose response patterns.

Looking for the effects of the compounds’ concentrations higher than the maximum tolerated dose, or, in other words, higher than the lowest observed adverse effect level (LOAEL) is often a characteristic of toxicological studies. It is so because the view expressed simply as ‘the higher the exposure the greater the harm’ seems to prevail in toxicology. This paradigm, however, is very often misleading as xenobiotic estrogens have been repeatedly observed to evoke actions with non-monotonic course, a feature typical for endogenous hormones. The stimulation of responses by low doses of endogenous hormones, while inhibiting a given response by the higher doses, is a phenomenon well known to endocrinologists and very often used in clinical endocrinology to treat diseases (Welshons et al., 2003).

Nonconventional dose-responses are those effects which do not follow typical threshold dose response model in which the low-dose effects rise from a threshold and plateau at higher concentrations. A non-monotonic dose response curve has a bimodal character and its shape reverses as the level of the contaminant increases, as simply presented on Figure 1.3. Large effects from small exposures can be explained by non-monotonic dose response models which can be presented as U-shaped or inverted U-shaped curves. U-shaped curve reflects the situation when a stimulation of a given performance is observed at low and at high doses of contaminant, while inverted U-shaped curve shows that the greatest response occurs at the intermediate doses of an investigated substance. The result of such a course of the response curve is somewhat puzzling because it indicates that greater impacts are observed at lower doses of contaminant than at the higher ones. Interference with an increasing number of endocrine-response systems as dose increases (e.g., due to binding or cross talk of an EDC with other nuclear receptors) (Weltje et al., 2005), activation or inhibition of different genes at different doses (Coser et al., 2003) and reaching toxic levels by a given oestrogen (Welshons et al., 2003) may underpin high dose inhibition observed for xenoestrogens.
A good example of non-monotonic way of action of xenoestrogens is the effect of foetal exposure to some oestrogenic substances (including bisphenol A) on the size of prostate later in life. The studies have shown that while low doses of oestrogen mimics cause enlargement of the gland, higher doses do not cause such alterations (Gupta, 2000; Welshons et al., 1997). Moreover, high doses were observed to lead to the decrease in the size of prostate in comparison to the controls. An attempt to explain this trend has been made by Welshones et al. (2003) who suggested that high doses of contaminant become overly toxic and lead to the poisoning of the system manifested in the shrinkage of the gland.

Taking into account that xenoestrogens may evoke non-monotonic responses it seems important to realize that linear extrapolation from experiments using only high doses of the hormone-mimicking chemicals cannot be used to predict effects at low doses (Welshons et al., 2003). That is why studies ought not to be limited only to the high doses of EDCs and the effects exerted by lower doses, including those below no observed effect concentrations (NOEC), should be explored.

1.6 Mixtures of endocrine disruptors

Very low oestrogenic potency of most of the so far identified hormonally active compounds is a somewhat problematic factor in the assessment of the health risk posed by these substances. Although there is an evidence that some of the xenoestrogens can cause adverse effects in wildlife, the question whether it would be possible for these weak estrogens to lead to such serious adverse effects as breast cancer or male reproductive problems still remains unresolved.
Some attempts to unravel this issue have been undertaken. One of the views is the possibility of interaction between individual environmental hormones which could potentially lead to the enhanced effects. The likelihood of interactions between EDCs seems quite plausible especially when the fact of the ubiquitous existence of EDCs (water, air, food) is taken into consideration. This and the copious quantity of the compounds classified as endocrine disrupters make it reasonable to think that the organisms living in today’s world are exposed to the mixtures of endocrine disruptors, rather than to the individual EDCs.

The idea that the effect of a given substance may be modified (enhanced or diminished) in the presence of another compound is not strange, especially in the pharmacological and toxicological fields of studies. The existence of such interactions between the compounds makes the assessment of the results difficult, as it is not simply the sum of individual substances’ effects. For instance, some studies have shown that individual oestrogenic substances, both natural and exogenous, differ in their potencies and temporal response patterns. As a consequence, when exposure to several oestrogenic ligands with different temporal, oscillating time course patterns takes place, sustained oestrogenic responses could be observed in the cell/organism.

The subject of xenoestrogen mixtures is a relatively new and there are comparably fewer reports on the effects exerted by the combined EDCs than on the influences of the individual oestrogenic xenobiotics. One of the debated issues is the character of the interactions occurring between xenoestrogens and whether they lead to antagonistic, additive or synergistic (above additivity) combinational effects. Synergistic interactions between oestrogenic EDCs present the worst case scenario when the effects of individual compounds are enhanced when applied simultaneously. Could synergistic interactions between EDCs of weak oestrogenic potential lead to the adverse effects in human and wildlife? This question although undoubtedly of great importance, seems to remain unresolved as reports both proving and disproving synergistic interactions between oestrogenic xenoestrogens can be found in the literature. Synergistic effects for the binary combinations of xenoestrogens have been reported by Arnold et al. (1996a). As an explanation for synergistic relations between compounds the authors suggested that two chemicals could combine in the oestrogen-binding site on the ER and mimic one molecule of natural hormone. Consequently, the transcription of oestrogen related genes would be increased. Another possibility proposed by the proponents of a synergistic action of environmental hormones is the presence of at least two oestrogen binding sites in the ER which, when both occupied by ligands, would lead to the synergistic transcriptional activity (Arnold et al., 1996b). This theory of synergistic relations between oestrogenic EDCs has, however, been debunked as a result of unsuccessful attempts to replicate these results by others (Ashby et al., 1997; Ramamoorthy et al., 1997), as well as by the authors themselves, and consequently the study has been withdrawn (McLachlan, 1997). This, however, does not preclude the possibility of synergistic interactions between xenoestrogens as they have been reported elsewhere (vanBirgelen et al., 1996). To further complicate the case of synergistic interactions, conflicting results and, subsequently,
contradictory conclusions have been obtained by Bergeron et al. (1994) who found synergistic effects and Arcaro et al. (1998) who did not, using a mixture of several weakly oestrogenic compounds. The lack of conformity in the matter of synergistic interactions between oestrogenic EDCs warrants further research. In addition, the exploration of the additive and antagonistic effects of the mixtures is of no lesser importance.

Not only interactions between xenoestrogens but also the reciprocal actions between the exogenous compounds and the endogenous estrogens are possible. There is a risk that the environmental estrogens action could be additive with that of the endogenous estrogens and lead to the increased oestrogenic effects. Consistent with this view is the study of Rajapakse et al. (2002) who using a yeast reporter gene assay (human ERα) observed that the effect of 17β-oestradiol was more than doubled when combined with a mixture of xenoestrogens, each at the below no-observed-effect concentration. This report has confirmed that the responsiveness of a system to endogenous oestrogen can be dramatically increased in the presence of a mixture of environmental estrogens at very low concentrations. Such a phenomenon could underpin the inappropriate oestrogenic activities and, consequently, lead to the adverse health effects (Watson et al., 2007b).

In their review on human health and chemical mixtures, Carpenter et al. (1998) conclude that although people are rarely exposed to individual chemicals, most of the available information on adverse human effects regards single compounds. As the authors further notice, for two or more chemicals additive, antagonistic or synergistic interactions may occur. Chemical compounds may also act on different systems and thus not interact with each other. In addition, even a single compound may evoke various effects on different organs simultaneously. Moreover, the chemicals effects may vary with age (Carpenter et al., 1998). Following this reasoning one might say that there are still many gaps in the knowledge regarding the effects exerted by EDCs in mixtures. That is why new studies, scrutinizing effects of not previously explored mixtures should be undertaken. Considering the amount of existing oestrogenic EDCs and even the greater amount of their possible combinations, there is still much to investigate in the field of xenoestrogen mixtures.

1.7 “Sensitive windows” of the exposure to endocrine disruptors

Another important biological issue when studying the effects of xenoestrogens is the matter of exposure during different developmental windows. The possibility of the combined effects of xenoestrogens and endogenous estrogens already present in the living organism should be especially considered when developmental windows of particular sensitivity to hormonal imbalance are taken into account. Foetal period belongs to these sensitive windows in the development of the living organisms. During this time endogenous hormones regulate important processes such as cell proliferation, differentiation and growth, involving complex patterns of cell signalling and cell migration, which are exquisitely sensitive to the changes in
hormone concentrations. Due to such sensitivity during the foetal life, small fluctuations in endogenous hormone signals may lead to permanent changes in the course of differentiation of cells. These cellular alterations may lead to the permanent changes in the gene activity and, consequently, in organ function. This phenomenon has become the basis of the relatively new scientific view called epigenetics which seeks the source of the inter-individual variety in signals that cells are exposed to, rather than due to a fixed genetic program. The alterations occurring during developmental stages through the interfering with organogenesis and histogenesis are considered as organizational and irreversible (McLachlan, 2001; Vandenberg et al., 2009). Therefore, exposure to oestrogenic chemicals, as well as to other EDCs, poses a particular threat when introduced during foetal and neonatal stages of the organism's development. In contrast, post-developmental changes are considered to have activational and reversible character, although, as Maffini et al. (2006) notice, it cannot be excluded that exposure to deleterious factors later in life does not cause additional negative effects.

An unfortunate example of the unique sensitivity of the developing foetus to the disturbance by compounds with hormone-like activity is adverse health effects observed in the offspring of women treated with diethylstilbestrol (DES) during pregnancy. DES is a synthetic oestrogen which was prescribed to more than five million women in the late 1940s-1970s to prevent abortions and pregnancy complications (Newbold, 2004; Palmlund et al., 1993). Whereas women exposed to DES remained without obvious health problems, their exposed in utero daughters showed significant increase in uterine, cervical and vaginal malformations, such as, for instance, clear cell adenocarcinoma - a very rare cancer of the vagina (Herbst and Anderson, 1990; Herbst et al., 1971). It was only when the so called ‘DES daughters’ reached puberty, or when they attempted to become pregnant, that those severe abnormalities in the reproductive organs were observed. Soon the cause of adverse health effects was identified as the exposure to DES during foetal development and subsequently the administration of drug during pregnancy was banned (Toppari et al., 1996). The importance of the time of exposure to the generation of disorders was further revealed when DES-daughters exposed during the first 7 weeks of gestation were compared with those exposed at week 15 or later. This comparison showed that women exposed earlier in the foetal development had a higher risk of reproductive organ malformations (Hatch et al., 2001).

The sensitivity of the foetus to endocrine disruptors was also revealed in the case of the ‘DES-sons’. Structural anomalies of the reproductive organs such as hypospadias, epididymal cysts, testicular abnormalities, cryptorchidism and pathological semen were significantly more frequent in DES exposed men than in controls (Gill et al., 1977; Gill et al., 1979). As in the case of women, the importance of the timing of exposure (time of organogenesis) was indicated when men exposed to DES before week 11 of gestation showed twice as high frequency of genital anomalies than those who were exposed later in prenatal life (Wilcox et al., 1995).

The relatively new hypothesis entitled “the developmental origin of adult disease” is focused around unique sensitivity to the surrounding environment during foetal development. Foetal developmental plasticity which allows the foetus to respond to poor conditions in the uterus is
the basis for this hypothesis, often called “Barker’s hypothesis” because its foundations were laid by Barker (De Boo and Harding, 2006). This theory states that adverse environmental influences early in development, and particularly during intrauterine life, can result in permanent alterations in organ structure and physiology leading to the increased susceptibility to disease in adulthood (Barker, 2004a, b).

The environmental compounds with hormone-like activity can interfere with the programming of endocrine signalling pathways which are established during prenatal life and lead to adverse health effects that may not be apparent until much later in life. This new insight into the foetal origin of adult disease has prompted scientists to reason that prenatal exposure to oestrogen-like chemicals may underpin the increased incidence of breast cancer in the last 50 years. Several animal experiments in which rodents were exposed in utero to low doses of EDC (including bisphenol A), have revealed altered development and morphology of the mammary gland, and thus gave scientific support of this assumption (Markey et al., 2001; Munoz-de-Toro et al., 2005; Soto et al., 2008). Foetal exposure to xenoestrogens may also underpin the development of obesity and obesity-associated diseases in adult life, as have been suggested elsewhere (Newbold et al., 2008; Newbold et al., 2007b). This assumption was supported by the experimental studies in which mice exposed early in development to diethylstilbestrol (DES) showed increased body weight resulting from an increase in the percentage of body fat, later in life. Foetal exposure to xenoestrogens has also been correlated with the alteration in the histoarchitecture (in the periductal stromal compartment) of the prostate in rats (Ramos et al., 2001). This observation is significant in the light of the data on human prostate cancers which shows that changes in the stroma compartment may lead to the invasive and/or malignant potential of the nascent tumour. Increased prostate size in adult mice was also observed following foetal exposure to environmentally relevant doses of xenobiotic estrogens, including BPA (Welshons et al., 1999).

To summarize, when all the above mentioned factors such as different ligands and their diverse doses, different times of exposure, the types of ERs, and different molecular pathways involving activation of diverse repertoires of proteins (i.e. co-regulators in the case of transcription factors, and signalling molecules in the case of the membrane steroid receptors) which are different in each cell type (Watson et al., 2007a) are taken into consideration, it may be concluded that each oestrogen, as well as an oestrogenic mimic, will have a different effect in the different tissues.
1.8 Bisphenol A

![Molecular structure of bisphenol A](image)

Figure 1.4 The molecular structure of bisphenol A. Adapted from Gillesby and Zacharewski (1998).

1.8.1 Introduction

Bisphenol A (BPA) is “one of the most ubiquitous endocrine disruptors” (Maffini et al., 2006). This compound has received a great amount of attention mainly because of widespread human exposure and public concern for possible health effects caused by its oestrogenic character. It is encountered by an average person of the developed world on a regular basis since it has been found in the air, water and food. BPA is a monomer that is polymerized to produce polycarbonate plastic and resins used to line metal cans, consequently the sources of BPA include water and baby bottles (Cao and Corriveau, 2008; Kubwabo et al., 2009) and food and beverages cans (Thomson and Grounds, 2005). BPA is also used as an additive in other types of plastic such as polyvinyl chloride (PVC) used in medical tubing, toys, and water pipes, the latter leading to concerns about transdermal BPA exposure through bathing in BPA-containing water (Welshons et al., 2006). Some polymers used as dental materials also contain BPA (Fleisch et al., 2010). Human exposure to BPA takes place mostly through oral route as a result of drinking liquids and eating food contaminated with it (Chapin et al., 2008). In addition, BPA may also contaminate organisms through respiratory system because it has been found in the air (Rudel et al., 2001).

Human exposure to BPA takes place due to hydrolysis of ester bonds by which BPA molecules are held together in plastic polymers. The process of hydrolysis is accelerated by high temperature and physical contact with acidic and basic substances and can take place during heating plastic containers and metal cans containing food (Brotons et al., 1995; Howdeshell et al., 2003; Krishnan et al., 1993). For instance, whereas the amount of BPA (mean ± SD) released from new polycarbonate baby bottles was 0.23 ± 0.12 μg/L, dishwashing, boiling and brushing simulation liberated much higher amounts of BPA, namely 8.4 ± 4 μg/L (Brede et al., 2003). High temperature treatment is, however, not necessary to cause plastic to release BPA molecules. The study of Carwile et al. (2009) demonstrated that drinking cold beverages from polycarbonate bottles increased urinary BPA concentrations by two-thirds compared to BPA concentrations detected in the urine after a washout phase of one week. The study by Goodson et al. (2002) who examined the BPA contents in 62 different canned foods (fish in aqueous media, vegetables, beverages, soups, desserts, fruit, infant formulas, pasta, and meat...
products) in the UK detected BPA in the 38 samples. Moreover, in 37 out of 38 canned foods this xenoestrogen was present at levels above 7 μg/kg and one sample of meat had a mean level of BPA equal to 380 μg/kg.

Based on the in vitro studies in which oestrogenic potential of BPA was compared to oestradiol, BPA was characterized as a weak environmental oestrogen. This opinion was based on low affinity of BPA for the nERs relative to oestradiol controls with EC50 equal to 2-7 x 10⁻⁷ M versus 1-6 x 10⁻¹³ M for oestradiol (Andersen et al., 1999; Fang et al., 2000). This view, however, has changed in the light of studies showing that BPA may also act via mechanisms independent of nERs.

Hundreds of studies have been conducted to unravel the mechanisms and effects of BPA action. Even today, however, there is controversy between scientists as to whether BPA poses a health risk for human and animal health. The problem is crucial not only from a scientific, but also from an industrial point of view. BPA production exceeds 6 billion pounds (2.7 x 10⁹ kg) per year which renders it one of the highest volume chemicals produced worldwide (Vandenberg et al., 2009).

1.8.2  Mechanisms of BPA action

The molecular mechanisms through which endocrine disrupting compounds act as hormone-mimics are still only partially resolved. BPA can exert its action through both traditional genomic pathways as well as through the non-genomic ways with much faster than genomic cellular responses (Welshons et al., 2006). The first mode of action involves binding of BPA to nERs, both form α and β, and, consequently, affecting the transcription of particular oestrogen-related genes. Biochemical assays have shown that BPA binds 10 times stronger with ERβ than ERα (Gould et al., 1998; Kuiper et al., 1998). Moreover, different co-activators can be recruited when BPA is bound to both ERα and ERβ which may contribute to the complex tissue-specific responses to BPA exposure (Wetherill et al., 2007). In the second mechanism BPA exerts its action through the membrane bound receptors and in this case cellular changes can be observed much sooner and at the clearly lower concentrations than those required to act through the classical nuclear receptors. The receptors involved in the non-genomic action of BPA include membrane-bound form of ERα and a transmembrane ER called G protein-coupled receptor 30 (GPR30; Watson et al., 2007b; Welshons et al., 2006). The binding of BPA to GPR30 has been found to be 8-50 times higher than its affinity to the ERs (Thomas and Dong, 2006).

The complexity of the possible BPA molecular pathways arising from existence of different receptors, their diverse localization dependent on the cell type, tissue, organ, and different expression at individual stages of development, may underpin diverse effects of low doses of BPA (Welshons et al., 2006). The low-dose effects of BPA may also be caused by the additivity of BPA with other endogenous or exogenous estrogens already present in the living organism, as discussed in more detail in Section 1.6. The hypothesis that many BPA studies missed its
effects simply by choosing too high doses of the compound has been presented by some scientists (vom Saal and Hughes, 2005). Many studies have reported a wide range of adverse effects at doses of BPA below the current reference dose of 50 μg/kg/day (vom Saal and Hughes, 2005). This reference dose has been set by the U.S. Environmental Protection Agency (EPA) as the daily dose which is safe for humans over the lifetime based on the threshold dose response model. Non–monotonic dose response theory is able to explain the effects of BPA at the concentrations lower than a reference dose. The term ‘low dose’ refers also to the doses within the range of typical human exposure (vom Saal et al., 2007) which is detailed in Section 1.8.3.

1.8.3 BPA human exposure levels

The presence of BPA in the adult human tissues has been well documented. A large scale test involving 394 people in the United States demonstrated the presence of BPA in 95% of the urine samples tested (Calafat et al., 2005). A similar study was carried out on human population of several Asian countries (296 people) and revealed that 94.3% of analyzed urine samples demonstrated the content of BPA at concentrations ranging from < 0.1 to 30.1 ng/ml (Zhang et al., 2011b). Takeuchi and Tsutsumi (2002) have shown that the concentrations of BPA in adults’ serum (mean ± SEM) are 1.49 ± 0.11 ng/ml in men and 0.64 ± 0.10 ng/ml in women. Considerably higher concentrations of BPA ranging from 0.79 to 7.12 ng/ml (mean ± SD equal to 2.91 ± 1.74 ng/ml) were detected by Cobellis et al. (2009) in the blood serum of endometriotic women. This widely demonstrated presence of BPA in human tissues is a cause for concern when potential health damage is taken under consideration, especially as there are studies which present an association between BPA content and some diseases. For example, the study of Wang et al. (2012) who examined over 3300 middle-aged and elderly Chinese people demonstrated that BPA was positively correlated with generalized obesity, abdominal obesity and insulin resistance. Furthermore, cross-sectional analysis of BPA concentrations and health status in the general adult population of the United Sates carried out by Lang et al. (2008) revealed that higher urinary BPA concentrations were associated with cardiovascular diagnoses, diabetes and clinically abnormal concentrations of the liver enzymes γ-glutamyltransferase and alkaline phosphatase.

In the light of these facts it is not unexpected that BPA was also found in the plasma of pregnant women as well as in the foetal plasma and in the placental tissue (Ikezuki et al., 2002; Schonfelder et al., 2002a). Ikezuki et al. (2002) detected 8.3 ± 8.7 ng/ml BPA in the amniotic fluid at 15-18 weeks gestation, whereas concentrations of BPA in other studied fluids (serum, ovarian follicular fluid) were about 5-fold lower. In this study BPA in the foetal serum was estimated to reach 2.2 ± 1.8 ng/ml. Yamada et al. (2002), who also studied the content of BPA in the amniotic fluid, observed 2.80 – 5.62 ng/ml BPA concentrations in several women in the early second trimester of pregnancy. A study of BPA exposure in Korean pregnant women and their foetuses revealed concentrations from non-detectable to 66.48 μg/L BPA in the pregnant
women's blood and from non-detectable to 8.86 μg/L BPA in the umbilical cords (Lee et al., 2008). These facts indicate that it is possible to be born with BPA already present in the blood. This exposure may be further increased when babies are fed with the use of bottles from which BPA is leaching. In addition, BPA was detected in the milk of nursing mothers at the mean concentration of 0.61 ± 0.20 ng/ml (Sun et al., 2004). In special cases, such as in premature infants from neonatal intensive care units, who were exposed to phenols in association with the use of specific polyvinyl chloride plastic medical devices, reported BPA geometric mean urine concentration was as high as 30.3 μg/L (Moors et al., 2007). All these examples demonstrate that human exposure to BPA may begin before birth and continue throughout life. Early exposure to BPA may be particularly dangerous as animal studies have shown that organisms at perinatal stages of development are subject to a much higher degree of deleterious effects than adults (Richter et al., 2007). This is in accordance with the opinion of vom Saal et al. (2007) who stated that sensitivity to endocrine disruptors, including BPA, varies extensively with life stage which points to existence of specific windows of increased sensitivity at multiple life stages. In the light of these facts it seems that exposure to xenoestrogens, including BPA, early in life is of particular interest and takes a special place in the field of the endocrine disruption. Particularly that some studies revealed that perinatal exposure to low doses of BPA may increase susceptibility to mammary gland neoplasia in adult life, as it was observed in rats (Durando et al., 2007; Murray et al., 2007; Soto and Sonnenschein, 2010). The ban of the production of baby bottles containing BPA in Canada in 2008 followed by a similar ban in the European Union (EC, 2010) could be interpreted as the expressions of this concern.

Whether low doses of BPA are harmless for human and animal health has been the subject of hot debate in the last few years. The review of the existing literature clearly shows that there is controversy over the threat posed by BPA for living organisms. On one side there are those who claim that BPA in the amounts available in the environment pose no risk for human development and health in general. On the other side there are scientists who express a great concern in relation to human exposure to BPA. Interesting fact concerning different opinions about BPA has been noticed by vom Saal and Hughes (2005). In their review of available studies on BPA, authors report that one of the predictors of the results was the source of funding. While the majority of studies funded by government showed significant deleterious influence of low doses of BPA, the research financed from industrial sources showed lack of such effects. The question asked by the authors was whether any of these two groups of scientists is under pressure to find data showing only negative effects of BPA or their lack.

Although the debate over BPA's potential for harm is still unresolved, the appearance of reports on significant effects evoked by low doses of BPA attracts special attention. Noteworthy, for instance, is the report of the group of the world leading experts on BPA who in their consensus in 2007 stated that concentrations of unconjugated BPA detected in tissues and fluids in some foetuses, children and adults (parts per billion) are higher than levels observed in the blood of animals exposed to current reference dose of BPA (vom Saal et al.,
In addition, in the study of Welshons et al. (1999) an enlarged prostate was found in male offspring of mice exposed during pregnancy to 2 or 20 μg/kg/day BPA which is far below the current reference dose of 50 μg/kg/day. Moreover, changes in the function of pituitary tumour cells were observed under the influence of BPA at very low concentration of 1 pM, i.e. 0.33 pg/ml of cell culture medium (Wozniak et al., 2005). The number of studies which show no deleterious effects of BPA is, however, equally substantial. Thus there is no consensus on the influence of BPA on human health, and as long as the reports on the deleterious effects of this compound appear, the issue of BPA remains one of those requiring further investigation.

1.9 Genistein

1.9.1 Introduction

Not only compounds originating from the human activity but also chemicals produced naturally by plants can mimic oestrogenic effects in vertebrates. These biologically-active plant-derived substances which structurally and functionally mimic estrogens are named phytoestrogens (Dixon, 2004; Setchell, 1998). Phytoestrogens do not have any reproduction related functions in plants but instead are involved in the defence against herbivores and protection against radicals or reactive oxygen forms (Ibarreta et al., 2000). Phytoestrogens are practically unavoidable for people because they are present in the normal human diet although in different amounts. Legumes show particularly high content of phytoestrogens. Other plants, for instance those known to be rich in fibre, also contain substantial amounts of these oestrogenic substances.

Phytoestrogens are categorized according to their chemical structure into three main groups: lignans, coumestans and isoflavones (Ibarreta et al., 2000). Isoflavones are low molecular weight antimicrobial substances synthesized de novo in plants in response to exposure to bacterial pathogens (Burton and Wells, 2002). Isoflavones are found almost exclusively in plants of Fabaceae family, commonly known as legume family, such as soybeans, chickpeas, green peas, lupin, kudzu or fava beans. Genistein (4',5,7-Trihydroxyisoflavone) is a
predominant phytoestrogen in soy and soy derived products and accounts for approximately two-thirds of soy isoflavone content (Setchell et al., 1997). Consequently, genistein is a major contributor to the total oestrogenic effects of soy.

A handful of studies have associated phytoestrogen consumption with positive effects on human health. Lower incidence in hormone associated cancers such as breast or prostate cancer has been correlated with diets rich in soy (Dixon, 2004). There may also be a link between an isoflavone-rich diet and reduced risk of thyroid cancer (Horn-Ross et al., 2002), and colon cancer (Adlercreutz, 2002). Genistein and related isoflavones can also inhibit cell growth and/or development of chemically induced cancers in stomach, bladder, lung and blood (Dixon, 2004). For example, the growth of human stomach cancer cell lines was observed to be strongly inhibited by genistein through the activation of a signal transduction pathway leading to apoptosis (Yanagihara et al., 1993). In addition, reports on the positive effects of the phytoestrogens on glucose and lipid metabolism are also found in the literature (Cederroth and Nef, 2009). Moreover, lower frequency in post-menopausal oestrogen withdrawal symptoms (hot flashes, bone loss, cardiovascular events) has been linked with Asian diets containing high quantities of phytoestrogens (Adlercreutz and Mazur, 1997). The reports on the positive influences of phytoestrogen are presumably the reasons behind the rise in the consumption of isoflavone rich food and soy-based food supplements in recent times. Particular attention was given to phytoestrogens by women of peri-menopausal and menopausal age as the soy-derived extracts found their application as the alternative for hormone replacement therapy (HRT) to alleviate the oestrogen deficiency symptoms (Wutte et al., 2003). There has also been a substantial increase in the use of soy-based baby milk formulas. (Polack et al., 1999).

It was not phytoestrogens’ positive health effects, however, which attracted the attention of scientists over 60 years ago. Adverse effects on sheep and cattle fertility in consequence to consuming phytoestrogen-rich pastures were noticed by veterinary physicians in Australia in 1946 (Bennetts et al., 1946). Since the observation of this so-called ‘clover disease’ scientific reports on the potentially harmful effects of phytoestrogens have emerged. An interesting observation, for example, was reported for captive cheetahs which showed hepatic venooclusive disease and reproductive failure due to feeding on soy-based diets (Setchell et al., 1987). In addition, when the soy-based diet was replaced with meat, the function of the liver improved. The occurrence of the phytoestrogen related fertility inhibition was also observed in California quail feeding on isoflavone-rich annuals growing in a dry year (Leopold et al., 1976). The reports on phytoestrogens’ adverse effects on hormonally-related functions resulted in an inclusion of these compounds in the group of endocrine disruptors.

All these facts make the subject of phytoestrogens’ influence on human health very controversial. For instance, despite significant research in the field of breast cancer - the leading cause of cancer deaths in women (Jemal, 2011), there still is no consensus on the role of phytoestrogens in this disease. On the one hand, consumption of soy-rich foods is thought to be associated with lower breast cancer. A handful of studies showed reduced breast cancer risk for women with higher phytoestrogen levels (Dai et al., 2001; Hirose et al., 1995; Ingram et
al., 1997; Linseisen et al., 2004; McCann et al., 2006; Shu et al., 2001; Thanos et al., 2006). Some speculate that it is precisely exposure to soy bean products containing genistein that leads to the breast cancer protection in Asian women (Lamartiniere, 2000). In addition, meta-analysis of studies on correlation between soy intake and breast cancer risk led authors to conclude that soy intake may be associated with a small reduction in breast cancer risk (Trock et al., 2006). On the other hand, reports presenting no association between phytoestrogen exposure and breast cancer prevention, and, what’s more, studies showing harmful effects of these compounds on women’ health, can also be found in the literature. For example, the indicators of an increased risk to develop a mammary cancer, such as breast density, mammary gland fluid production and progesterone receptor formation, were observed in women eating typical western diet under isofalvone treatment (McMichael-Phillips et al., 1998; Petrakis et al., 1996). Another example is the study of Grace et al. (2004) in which the association between exposure to isoflavone (as assessed based on their level in the serum and in the urine) and increased breast cancer risk was observed. Reports of this type may lead to concerns that the consumptions of isofalvones by peri- or post-menopausal women could increase rather than decrease the risk of developing a mammary cancer (Wutte et al., 2002).

One of the hypotheses aiming to reconcile contradictory observations from the studies on breast cancer is that cancer preventing effects of phytoestrogens may occur when soy nutrition takes place from the beginning of life onwards (Knight and Sorensen, 2001; Lamartiniere, 2000). Increased vulnerability during the early developmental stages, hormonal activity of phytoestrogens and wide ranges of phytoestrogens’ levels to which babies may be exposed may, however, bring more harm than the potential breast cancer-preventing effects.

### 1.9.2 Mechanisms of genistein’s action

There are many different mechanisms through which phytoestrogens such as genistein may trigger their action. Oestrogenic effects may result from the ability of phytoestrogens to bind to ERs. In general, isoflavone compounds have lower binding affinity to ERs when compared to the mammalian estrogens (Benassayag et al., 2002; Routledge et al., 2000). Genistein has been found to be a highly effective competitor for oestradiol at ERs. The study of Fioravanti et al. (1998) showed that genistein’s binding affinity to cytoplasmic ER was 50-fold lower than that of endogenous oestradiol in the ER-positive cell line (MCF-7). It was demonstrated elsewhere that binding affinity of genistein is preferential as it binds about 20-fold stronger with ERβ than ERα (Kuiper et al., 1998; Routledge et al., 2000). Genistein can also act through GPR30 towards which it has higher binding affinity than several other environmental estrogens including BPA (Thomas and Dong, 2006).

Genistein may also act through other mechanisms. One of the wider studied subjects is genistein’s potential to inhibit tyrosine specific protein kinases (Akiyama et al., 1987; Kim et al., 1998). Genistein has also been demonstrated to inhibit DNA topoisomerase II, angiogenesis
and the production of reactive oxygen species (which may lead to tissue damage and DNA modification) and to modulate cell cycle progression (Barnes and Peterson, 1995; Peterson, 1995). Moreover, the study of Pino et al. (2000) showed that genistein displaces bound oestrogen and testosterone from human sex hormone binding globulin (SHBG) and can increase plasma levels of this globulin in postmenopausal women. Increased synthesis and secretion of SHBG was also observed in an in vitro study with the use of human HepG2 hepatocarcinoma cell line (Mousavi and Adlercreutz, 1993).

1.9.3 Genistein’s human exposure levels

Although genistein’s oestrogenic activity is weak compared to 17β oestradiol, levels of phytoestrogens in the body can reach 100 to 1000-fold higher levels than peak concentrations of the endogenous oestrogen in premenopausal women (Limer and Speirs, 2004; Zava and Duwe, 1997). This leads to concerns about influence of plant-originating estrogens on human health, particularly during vulnerable stages of development.

There are significant differences in the concentrations of isoflavonoids between different human populations. In general, concentrations of phytoestrogens are relatively low in subjects consuming an omnivorous diet without soy foods as opposed to higher levels observed in populations for which soy rich foods constitute a staple (Morton et al., 2002). For example, Adlercreutz et al. (1993) compared the geometric mean plasma concentrations of four isoflavonoids in men of similar age from Finland and Japan and saw 7 to 110 times higher concentrations in the Japanese than in the Finns. The plasma levels of studied isoflavones were generally very high in the Japanese men although there was also a significant inter-individual variation. Although the mean total genistein concentration in the Japanese was estimated as 276 nM, one subject had a genistein concentration exceeding 2.4 µM. When the size of the human population studied is taken under consideration (n=14) it can be concluded that genistein levels of micro molar range in Japanese male population are not exceptional.

The exposure to phytoestrogens in food may, as already mentioned, begin early in life due to infant formulas based on soy available on the grocery market. Soy-based formulas were initially developed for infants with lactose intolerance or allergies to cow’s milk (Polack et al., 1999). In recent years, however, substantial increase in the use of soy protein-based formulae has taken place and the number of infants fed with this kind of food far surpasses the percentage of infants requiring it for medically justified reasons. In the United States, for example, approximately 25% of all formula-fed infants are fed with soy-based formulas (Bhatia et al., 2008).

The blood concentrations of total genistein in infants fed soy-based formulas are an order of magnitude greater compared with concentrations observed in adults consuming high soy diets and several hundred-fold higher than adults eating Western diets (Adlercreutz et al., 1993; Setchell et al., 1997). Moreover, the plasma concentrations of isoflavones able to disrupt the
menstrual cycle in premenopausal women are approximately 10-fold lower than concentrations of total genistein found in the plasma of soy-based infant formulas (Cassidy et al., 1994). Such data leads to concerns that early exposure to relatively high doses of phytoestrogens could cause reproductive and other problems later in life. The study of Cimafranca et al. (2010) is one of the reports which justify an existing concern. In this study neonatal mice were orally exposed to genistein to mimic the route of infants’ exposure and the total plasma genistein concentrations reached were of 2-3 µM. This range of genistein concentrations corresponds to the levels of 3.36 µM (median) and 684 ± 443 ng/ml (mean ± SD) reported for the soy formula-fed infants by Cao et al. (2009) and Setchell et al. (1997), respectively. Such levels of genistein were observed to cause an increase in the relative uterine weight, decrease in the expression of progesterone receptor in the uterine epithelium, a decrease in the thymic weight relative to body weight, increased incidence in multioocyte follicles in the ovary and disturbances in the length of oestrus cycle later in life. These results suggest that genistein exposure seen in the soy formula-fed infants may be sufficient to result in oestrogenic alterations in reproductive and other organs (Cimafranca et al., 2010). This implication is further justified by the fact that mice pups were exposed to the phytoestrogens for the first five days of life which constitutes only a quarter of the pre-weaning period, whereas human infants can be exposed to genistein through the entire nursing period. In addition, animals were fed with genistein only once a day which was reflected in serum concentrations which decreased with time, while babies who are fed regularly during 24 hour period can be exposed to higher and more stable concentrations of this phytoestrogen.

1.10 Endocrine disruption in fish

The field of endocrine disruption is a relatively new discipline of environmental research which has rapidly grown in the last few decades. Many aspects related to this topic are studied and presented in hundreds of reports each year. A large proportion of these studies are dedicated to the problem of endocrine disruption in aquatic organisms. This interest is largely due to the fact that the aquatic environment has increasingly been the depository for pollutants of different origins and aquatic organisms are subjected to continuous exposure of different chemicals throughout their lives (Sumpter, 2005).

Aquatic environments can be polluted by different substances, of both natural and synthetic character and through both intentional and accidental release. The intentional discharge of chemicals occurs mainly through release of effluents from sewage treatment plants and some industries, while accidental release takes place when spills, run-offs and atmospheric deposition take place. The fate of pollutants can vary and while some substances can be degraded to harmless compounds, others cannot be decomposed or can even be degraded to more harmful substances. In either case these compounds may be absorbed by the organisms living in the water and in the case of fish this occurs mainly through the skin, gills and with food.
As a consequence these chemicals may get into the food chain to higher vertebrates (Sumpter, 2005).

The whole story of endocrine disruption in fish started in the late seventies of the last century when English anglers observed hermaphrodite fish in the lagoons of some sewage treatment plants. These observations attracted scientific attention and subsequent studies confirmed that a chemical constituent found in the sewage treatment plant effluents might be the causative agent of unusually high frequency of intersexuality observed in some fish species. Even though the issue of endocrine disruption in fish was at that time discussed, albeit not openly, a public debate of a widespread sexual disruption in vertebrates began nearly twenty years later (Jobling et al., 1998; Sumpter and Johnson, 2008).

Since the evidence for endocrine disruption in wildlife populations has been mainly derived from the studies on aquatic vertebrates, fish have been recommended for the development of the tests for EDCs (Fenner-Crisp et al., 2000; OECD, 1999). Biological effects in fish that have been attributed to the outcomes of endocrine disruption include the inappropriate production of the blood protein vitellogenin in male and juvenile fish, disturbed ovarian or testicular development, intersexuality and/or masculinisation or feminization of the internal or external genitalia, abnormal blood steroid concentrations, increased ovarian atresia, reduced spawning success, impaired reproductive output, precocious male and/or female maturation, reduced hatching success and/or larval survival and altered growth and development (Vos et al., 2000).

EDCs present in the aquatic environment include a diverse group of synthetic industrial and agricultural chemicals and even some naturally occurring compounds. Sewage treatment plants and pulp and paper mills have been recognized to be one of the major sources of endocrine disrupting chemicals for both fresh and salt water inhabitants (Jobling and Tyler, 2003; Pait and Nelson, 2002). The study of Desbrow et al. (1998) have shown that natural steroid estrogens 17β-oestradiol (E2) and oestrone (E1), and the synthetic steroid oestrogen ethinyl oestradiol (EE2) can be the causative agents of feminization of the fish living downstream of sewage treatment plants. These oestrogenic compounds have been reported to be incompletely broken down in sewage treatment plants (Johnson and Sumpter, 2001; Johnson and Williams, 2004).

The estrogenicity of sewage treatment plant effluents may result also from the presence of naturally extracted female hormones or contraceptive pill constituents. In some specific locations xenoestrogens like alkylphenolic chemicals have been diagnosed as a significant factor in oestrogenic character of sewage treatment plant effluents (Sheahan et al., 2002a; Sheahan et al., 2002b). Both BPA and genistein have been identified as aquatic pollutants and can further increase estrogenicity of waterways. Discharge of BPA into aquatic environment occurs from the migration of BPA-based products into rivers and seas and also through effluent from sewage treatment plants and landfill sites (Kang et al., 2008; Spengler et al., 2001). Genistein’s presence, in turn, has been detected in 60% of the sewage treatment plant effluents sampled in Germany (Spengler et al., 2001) and in hardwood pulp and mill effluent.
Oestrogenic activity resulting from the presence of this phytoestrogen (143.4 µg/L) has also been diagnosed in the Japanese river from a primarily residential area with some industrial development (Kawanishi et al., 2004).

Although sewage treatment plants and pulp and paper mills are the major supply of EDCs for the aquatic environment, other sources of hormonally active pollutants have also been found. Manufacturing processes related to textile, plastic, polyvinyl and electrical equipment production are other sources of EDCs. In addition, agricultural run-offs also contain substances able to evoke hormonal disruption such as pesticides and herbicides. Furthermore, oil spills, recreational boating and shipping, and atmospheric deposition of the products of fossil fuel combustion are other sources of hormonally active substances in waters. All these facts show that there is a wide variety of human activities which can introduce EDCs into the environment, especially into waterways, which causes the aquatic species to be more vulnerable to endocrine disruption than terrestrial organisms. This is why it seems justified to look for effects of endocrine disrupting substances in aquatic species.

There is little evidence to suggest that fish are more susceptible to EDCs than other wildlife (Jobling and Tyler, 2003) which further justifies the interest in fish and recognition of this species as an appropriate model for biomedical research focused on endocrine disruption. Moreover, the evidence available on receptor binding affinities for compounds able to mimic hormones, including xenoestrogens, suggests that vertebrates are likely to be similarly sensitive to environmental EDCs. In addition, there are many similarities with respect to the nature of the hormones, their receptors and in the regulatory control of endocrine system between fish and higher vertebrates (Ankley and Johnson, 2004). Another reason is the need to search for alternative animal models for different fields of biomedical research. To date, small mammals like mice and rats have been the most common animal models used in biological studies. These vertebrates, although proven to be very advantageous animal models, are expensive to maintain, difficult to work with at embryonic stages and limited for large- scale genetic studies. Another reason for searching for alternatives is the principle of the three Rs, namely, reduction, refinement and replacement. The replacement rule requires the limitation of animal use to situations in which they are absolutely necessary. It also demands of the researcher to consider if a different animal species, lower in the phylogenetic scale, could be used (Wolfensohn and Lloyd, 1994). In general, although mammals constitute the most attractive animal models, other models are researched to complement deficiencies in the mammalian studies.
1.11 Why zebrafish?

Zebrafish (*Danio rerio*) is a small tropical freshwater fish belonging to *Cyprinidae* and native to the rivers of northern India, northern Pakistan, Nepal and Bhutan in South Asia. This species has received a great amount of interest in many scientific fields and increasing number of the publications in which zebrafish constitutes the main topic is an indicator of growing scientific fame of this small vertebrate (Figure 1.6).

![Figure 1.6 Graphic presentation of the increasing interest in zebrafish based on the number of scientific articles published in the last half century. Data based on the search using ‘zebrafish’ as a ‘topic’ on the Web of science database on 27.08.2012.](image)

There are many advantages of using zebrafish as a model species for various scientific disciplines. As it was observed by Segner (2009), this fish has become so popular in science because it offers both technical and practical advantages. The technical aspect is true because of ease and relatively low costs of this species maintenance, as well as its high fecundity. Rapid development (Figure 1.7) and ease of observation at early developmental stages due to transparency are also important merits of zebrafish-based studies.
Figure 1.7 A schematic presentation of rapid development of zebrafish. First embryonic divisions start before 1 hpf and at 24 hpf the embryos have an established body plan and major organs visible. Adapted from Wolpert et al. (2006).

The practical advantages result from the fact that this organism can provide conceptual insights into many aspects of vertebrate biology, toxicology, and disease. An additional advantage of working with this species is a rich source of information concerning different aspects of its biology, which often enables defining standard/normal values and consequently enables recognition of the changes caused experimentally (Segner, 2009).

All of these virtues of zebrafish have been appreciated by a wide variety of scientific disciplines. Some have even labelled it the “vertebrate Drosophila” as it offers many of the same advantages as invertebrate models with few of the disadvantages (Lessman, 2011; Nagel, 2001). Genetics is undoubtedly one of the disciplines in which potential of this species was used to the fullest and one of the reasons for this is the fact that zebrafish orthologs of human genes can be identified due to the high genetic conservation among vertebrates (Chu and Sadler, 2009). Zebrafish is also widely used as a model in developmental biology mainly due to the fact that fundamental developmental programs are well shared among vertebrates (Chu and Sadler, 2009; Ting and Peng, 2009). Moreover, genomic and molecular similarities between this organism and higher vertebrates make many important discoveries from zebrafish development applicable to humans (Veldman and Lin, 2008). For example, zebrafish has been shown to be an excellent model system to study the development of the liver. Studies on liver development in mammals (mice) are made difficult by the fact that liver development is
intertwined with vasculogenesis and hematopoiesis and dysfunction of one of these processes prevents the proper development of the others (Chu and Sadler, 2009). Moreover, in mammals the liver is an early hematopoietic organ and mutations affecting its development lead to death by causing anaemia (Reimold et al., 2000). In contrast, embryonic hematopoiesis does not take place in the zebrafish liver and that is why embryonic anomalies of this organ do not lead to the death due to hematopoietic dysfunction. Moreover, liver development can be investigated even in zebrafish embryos with mutations affecting the cardiovascular system. Even in the absence of blood circulation, embryonic zebrafish can survive and develop relatively normally for at least first several days of its development because oxygen can diffuse passively through body surface and nutrients come mainly from the yolk (Chu and Sadler, 2009; Stainier, 2001). Additionally, zebrafish embryogenesis occurs extrauterinely which makes the embryonic liver accessible for the study of development through a direct genetic approach (Ting and Peng, 2009).

The zebrafish attributes have also been appreciated by toxicologists and pharmacologists (Hill et al., 2005; Langheinrich, 2003). The size of a model animal for the studies dedicated to chemical testing is important as it is directly related to the costs of husbandry, the amounts of chemicals used for testing and the volumes of potentially hazardous wastes produced. For toxicological studies where the objective is to identify adverse effects of chemical exposure, particularly important is the abundance of information on ‘what is normal’ for this species. Moreover, in contrast to embryos of other vertebrates, zebrafish development and its disorders can be assessed continually due to external development. These and other characteristics contributed to the appreciation of zebrafish embryos as the test system for the studies focused on effects of chemical compounds, both drugs and pollutants. The embryo test with Danio rerio, called DarT, was proposed by Nagel (2001) as the substitution of the commonly used acute fish test in ecotoxicological and toxicological studies. This test is based on the usage of embryos which, following brief exposure to the tested compounds, are carefully analyzed for several parameters such as pigmentation, oedema formation or heart beat. Strong scientific support that the fish embryo toxicity test (FET) is a good surrogate for the acute fish toxicity was also provided by Lammer et al. (2009). Furthermore, zebrafish embryo-based tests might also be used to predict the teratogenic potential of chemicals in mammals as 88% of 41 substances known to be mammalian teratogens showed results in agreement with findings in mammals when tested with DarT (Nagel, 2001). For pharmacological studies a particular advantage of this species is the possibility of testing the drugs in an in vivo context, with the drugs affecting a number of different cells in their natural environment (Langheinrich, 2003).

A contributing factor in the popularity of zebrafish embryos and larvae as a model organism in the studies from many scientific disciplines may also be the drive to implement the 3Rs which are enshrined in the UK Animals Scientific Procedures Act 1986 (ASPA). The 3Rs are three main guiding principles for animal research, namely: replacement, reduction and refinement. According to ASPA a licence is required to conduct regulated procedures on fish from the time
at which they become capable of independent feeding which in the zebrafish is accepted to be at 5 dpf. Life stages before this time are considered to be not sufficiently aware that they will suffer when a procedure is carried out. Moreover, their use enables generating in vivo data in certain organs which is in accordance with replacement of mammals at more sentient and licensed stages (Fleming, 2007).

1.12 Zebrafish as a model for endocrine disruption studies

The values of zebrafish as an experimental model have also been recognized in the field of endocrine disruption. This fish was one of the species recognized by Ankley and Johnson (2004) as a vertebrate model for identifying and assessing the effects of endocrine-disrupting chemicals (EDCs). A significant degree of conservation of basic aspects of endocrine systems across vertebrates is of great importance as provides a basis for using results from fish tests to predict likely mechanisms of action of EDCs in other vertebrates (Ankley and Johnson, 2004). Both full life cycle (Nakari and Erkomaa, 2003; Nash et al., 2004; Schafers et al., 2007) and partial life cycle tests (Schafers et al., 2007; Segner et al., 2003a) have been used in the studies of EDCs using zebrafish. In both types of studies the endpoints related to sexual development and reproductive parameters such as start of spawning, mating behaviour, number of eggs per female, fertilization success of embryos and embryo survival are of the greatest interest. Gonad histology and vitellogenin levels have also been observed in fish exposed to EDCs (Brion et al., 2004; Van den Belt et al., 2001; van der Ven et al., 2007). In general, xenoestrogens have been observed to cause increased vitellogenin synthesis, impaired growth, delayed onset of maturation, altered sexual differentiation and sex ratio, and decrease in egg production and fertilization success (Nakari and Erkomaa, 2003; Nash et al., 2004; Schafers et al., 2007; Segner et al., 2003a; Segner et al., 2003b).

The relatively short life cycle of zebrafish enables full life cycle testing of EDCs easier than with other fish species. These tests, however are very challenging, time consuming and expensive. For these reasons partial life cycle tests seem more pragmatic. An important issue for the partial life cycle tests is to determine the critical period of sensitivity to tested EDC. Petersen et al. (2001) who studied the suitability of zebrafish as a test organism to detect EDCs observed that 40 day-test starting from 20 days post hatch is suitable for detection of both oestrogenic and androgenic effects. In another study, “sensitive window” for endocrine disruption with oestrogenic compounds has been determined as the period of final gonad differentiation which in the authors’ system occurred between 42 to 75 dpf (Segner et al., 2003a). Zebrafish belongs to the group of animals identified as undifferentiated gonochorists which means that in their early development all zebrafish individuals develop non-functional ovaries and then go through the phase when these organs will either proceed to develop mature ovaries or degenerate, leading to the development of normal, functional testes (Maack and Segner, 2003). Gonad histopathology, sex ratio and vitellogenin levels have been often observed in the partial
life cycle tests of EDCs with the use of zebrafish (Andersen et al., 2003; Brion et al., 2004; Hill and Janz, 2003; Holbech et al., 2006; Orn et al., 2003).

This species has been proposed as a model organism to identify targets as well as modes of action of endocrine disrupting compounds by Segner (2009). One of the insights expressed by Segner was that despite all the characteristics which render the zebrafish embryos so popular in toxicological and pharmacological studies, they have been rather rarely used for screening of EDCs. This justifies the need for further studies in which endocrine disruption could be explored in new ways with the use of zebrafish at early stages of development. Moreover, the majority of studies on EDCs with the use of zebrafish have addressed disruption of sexual differentiation and reproduction. Notwithstanding, as rightly pointed out by Segner (2009), other targets of EDCs action must not be overlooked.

1.13 Why liver?

The liver is an accessory digestive organ. It is the major metabolic organ engaged in the metabolism of carbohydrates, lipids and amino acids. It is also responsible for the storage of vitamins and minerals, as well as for the synthesis and secretion of the bile and the plasma proteins including albumins, clotting factors and complement proteins.

Several functions of the liver are particularly important when considering the effects of EDCs on the vertebrates. Firstly, the liver has a principal role in the detoxification of the blood which is constantly filtered through the hepatic capillaries. Consequently all substances carried by blood, including xenobiotics, encounter this organ on their way. Furthermore, the liver is a major site of accumulation, biotransformation and excretion of xenobiotics, including EDCs (Mortensen and Arukwe, 2007).

The liver is also the primary site for the absorption and recycling of hormones including estrogens and therefore takes part in the regulation of the plasma concentrations of these hormones. To study the effects of xenoestrogens on the hormonal homeostasis is hence correlated with the study of EDCs’ effects on the liver function. There have been several reports of the EDCs’ potential to affect the enzymes involved in the endogenous metabolism of native hormones (Kirk et al., 2003). For example, BPA and dioxin down-regulated CYP1A1 – an oestrogen metabolizing monooxygenase, in mouse hepatoma cells (Jeong et al., 2000).

The level of free and hence active native oestradiol is dependent on the amount of sex hormone binding globulin (SHBG). This protein, which by binding oestradiol renders it inactive, is synthesized and released to the bloodstream through the liver. Some studies have reported that environmental estrogens may affect the levels of this globulin (Mousavi and Adlercreutz, 1993; Pino et al., 2000) which further reveals engagement of the liver in the regulation of the hormonal environment.
Endocrine functions of the liver are clearly seen in the oviparous organisms in which vitellogenesis takes place. As will be discussed in more detail in Section 1.17, vitellogenesis is the production and release by the hepatocytes of the egg yolk precursor protein called vitellogenin. This process is completely hormone dependent and occurs normally in the females hepatocytes in response to the activation with the estrogens such as 17β oestradiol. Released by the hepatocytes vitellogenin is then transported with the blood to the ovaries where it is built in the developing oocytes as the yolk necessary for the development of the future embryos. The schematic illustration of this process is presented in Figure 1.8.

![Figure 1.8 A schematic presentation of the vitellogenesis. The liver responds to the ovary-derived oestradiol synthesizing and secreting vitellogenin. This protein is then transferred via blood back to the ovaries where it will be incorporated into the maturing oocytes.](image)

The importance of the liver in the studies related to endocrine disruption has been also recognized whenever liver size was taken under consideration. The liver size has been observed to be sensitive to oestrogenic exposure. In fact, hepatosomatic index (HSI) which is the ratio of liver weight to the total body weight, has been determined as a biomarker of oestrogen exposure (Lye et al., 1997). Fish exposed to the oestrogenic compounds have been repeatedly observed to have increased HSIs (Lye et al., 1997; Nimrod and Benson, 1996; Thorpe et al., 2000). Moreover, HSI was used as one of the main indicators of oestrogenic disruptors in the studies aiming to assess the status of the Irish aquatic environment (Tarrant et al., 2005).

There is an additional reason for which the liver seems a justified organ when studying endocrine disruption in the zebrafish, particularly at early stages of the development. The majority of the histopathological studies on EDCs have, for understandable reasons, focused on the organs of well known dependency on the sex hormones, such as gonads and the sexual organs. To study the effects of xenoestrogens on zebrafish reproductive parameters, such as the gonads architecture or function, animals older than 5 dpf need to be used. Zebrafish is a species which goes through a phase of juvenile hermaphroditism during its early development (Maack and Segner, 2003, 2004). This means that initially all the individuals, irrespective of genetic sex, display the gonads of undifferentiated ovary-like morphology (Maack and Segner, 2003; Uchida et al., 2002). Around 23 dpf these undifferentiated ovaries start to degenerate in
fish which will develop testes while in the remaining larvae they develop and mature as ovaries. This process is completed approximately 40 days post hatching. The fact that the final gonadal sex of zebrafish is dependent on hormonal balance during this sexually decisive period of development (Maack and Segner, 2004) has been the basis of many previous studies. Such studies, however, cannot be conducted on the early zebrafish larvae. For this reason, studies on the influence of sub-lethal concentrations of xenoestrogens on histological and cytological structure of embryonic/larval zebrafish, ought to be focused on the organs other than gonads. Liver seems to be a perfect candidate for this purpose since it appears in zebrafish development as early as 24-28 hours post fertilization (hpf) and is clearly recognized by 50 hpf (Field et al., 2003b; Ober et al., 2003).

1.14 Liver as a biomarker in phenotypic studies

The phenotype of an organ is correlated with its function and any disturbance in its physiology can alter the morphology of the cells and tissues. The use of stereological methods allows quantitative, unbiased and objective assessment of the morphology of the tissue phenotype. This, in turn, allows for the statistical analysis and comparisons of the phenotype descriptors between different treatment groups. It also helps to create a three-dimensional framework upon which to lay the physiological and molecular (biochemical) information. Including this objectivity in phenotypic studies is in line with the key principles of the animals’ welfare legislation and contributes to refinement, one of the 3R’s (Braunbeck, 1998).

Ultrastructural studies suggest that fish liver is extremely sensitive to xenobiotic compounds in the environment which makes this organ very attractive to explore the toxicity of these compounds on the animal. Cytopathological alterations in the livers of fish exposed to xenobiotics can reveal sub lethal effects of chemicals and elucidate underlying modes of their action (Braunbeck, 1998). Moreover, the alterations in the ultrastructure of the liver have been observed to be both species-specific and substance dependent (Braunbeck, 1998; Braunbeck et al., 1990) as well as dependent on the time of exposition (the stage of development) and the dose of the substance (Burkhardt-Holm et al., 1999).

The great potential of the fish liver phenotype for the toxicological studies has been highlighted by the studies of Braunbeck (Braunbeck, 1998; Braunbeck et al., 1990), Burkhardt-Holm (Burkhardt-Holm et al., 1999) and Oulmi (Oulmi and Braunbeck, 1996). In these studies the identification of “biomarkers” was of particular interest. Biomarkers were defined by these authors as contaminant induced- physiological and/or biochemical changes leading to the formation of altered structure in the cells, tissues or organs of the not-too sensitive organisms. Their studies showed species- and substance-specific alterations in the fish liver following treatments with several xenobiotics which contrast with the opinion that morphological changes are not specific and hence not representative. In his study Braunbeck (1998) observed both non-specific ultrastructural reactions as well as toxicant specific-phenomena in zebrafish
liver following the treatments with several chemical compounds. The disturbance of the cellular compartmentation, proliferation or reduction and fenestration and/or vesiculation within the endoplasmic reticulum, myelin formation and invasion of macrophages were assessed as the non-specific alterations in the zebrafish liver. In turn, the toxicant specific changes included steatosis (in response to lindane treatment), stratified inclusions within mitochondria (due to exposure to 4-chloroaniline) or the formation of crystalloid structures within mitochondria and peroxisomal proliferation (following the 3,4-dichloroaniline treatment).

The reports of high sensitivity of zebrafish liver architecture and specificity of the occurring alterations stimulated the present study, which examines the disruptive potential of endocrine disruptors, including xenoestrogens and phytoestrogens.

The usefulness of this model was noticed by Van der Ven et al. (2003) who wrote the article titled “Histopathology as a tool for the evaluation of endocrine disruption in zebrafish”. Although the authors focused on the histological alterations in the ovaries and testes and not the liver, the advantages of examining multiple organ systems was highlighted. The inclusion of morphometric techniques was proposed in this report as a way to further uncover insidious effects of endocrine disruptors.

Relatively few studies have been performed to date to investigate the effects of EDCs on fish liver. Volz et al. (2006) showed that 2,3,7,8-tetrachlorodibenzo-p-dioxin treatment caused alterations in gene expression patterns followed by histological changes in the liver of medaka (Oryzias latipes). In another study, Weber et al. (2003) found no clear concentration dependent effects of early exposure (2 to 60 dph) to 4-nonylphenol and 17α-ethinylestradiol on the morphology of zebrafish liver at 60 dph. The analysis of histopathological changes was, however, based only on counting the number of the cells with pyknotic nuclei per field of view and, as the authors concluded, the results might have been confounded by a concurrent, concentration dependent increase in the hepatocyte size. This illustrates the importance of adequate mensuration and utilization of appropriate, e.g. stereological, techniques in such studies.

Most studies on the effects of EDCs on zebrafish liver have used juvenile or adult zebrafish. There has been no study to date on the EDCs effects on the liver ultrastructure of zebrafish at early developmental stages. The ultrastructure of the liver of young larvae has, however, been examined in other toxicology studies. In the study of Oulmi and Braunbeck (1996), for example, dose- and time-dependent cytological changes were observed in the liver of 4 and 6 day old larvae due to the treatment with 4-chloroaniline – this compound has no reported endocrine disrupting properties. A similar study, but with the use of 10 day old larvae, was performed by Burkhardt-Holm et al. (1999) and showed alterations in the hepatocyte nuclei, mitochondria, peroxisomes, endoplasmic reticulum, Golgi apparatus, lysosomes, glycogen and lipid stores following exposure to 4-chloroaniline. Such observations suggest the potential of the use of the liver in phenotypic studies caused by EDCs even at the early developmental stages. The
potential usefulness of using such young animals in phenotypic studies is further increased because of the implementation of the animal welfare legislation principles, namely the 3Rs (Fleming, 2007).

1.15 Zebrasfish liver development

As in other vertebrates, zebrasfish liver arises from endoderm and develops as an outgrowth of the anterior intestine. In the studies of Field et al. (2003b) a novel zebrasfish transgenic line which express GFP throughout the digestive system was used to investigate liver development. In this report liver morphogenesis was divided to two phases: budding and growth. The budding phase takes place between 24 and 28 hpf and is further subdivided into three stages. First stage of budding corresponds to the appearance of a disorganized but cohesive mass of pre-hepatic cells in the anterior, ventral part of the intestinal rod. Intestinal rod is a solid rod of midline cells or, in other words, endoderm posterior to the constricted caudal end of the pharynx. The thickening is situated slightly left of the midline and projects from the ventral side of the intestinal rod at the level of the first somite. At the same time posterior thickening located dorsally on the intestinal rod starts the development of the pancreas. The second stage of the budding phase defines the period by 30 hpf when intestinal bulb primordium undergoes a leftward bend. In the meantime, the liver increases in size leading to the appearance of smooth, thickened area along the outer curvature of the intestinal bulb primordium. The formation of a furrow between the liver bud and the adjacent oesophagus begins the third stage of budding. This process starts at approximately 34 hpf. The enlargement of the furrow takes place over time. Eventually, by 50 hpf, the connection between the liver and the intestinal bulb primordium is restricted to a single tubular duct. This is a hepatic duct and its formation determines the end of the third stage of budding and the end of the whole budding phase. Next begins the stage of growth during which the liver, which is already a well defined structure, increases in size and changes its shape and placement. The liver is in the growth phase by 96 hpf and during this time it is expanding to the left and occupies the substantial portion of the abdominal cavity on the left side of the embryo and spread across the midline (Field et al., 2003b).

There are two main hypotheses regarding the origin of hepatoblasts. Korzh and coworkers claim that liver progenitor cells might be differentiated before the formation of endoderm rod and that liver forms later by migration and gathering of these liver progenitor cells. This hypothesis is based on the fact that the liver-specific marker ceruloplasmin can be detected in the dorsal endoderm at 16 hpf, which is prior to the liver development (Korzh et al., 2001). This theory, however, lacks sufficient amount of scientific proof according to others (Ting and Peng, 2009). Another hypothesis claims that liver progenitor cells are differentiated from the endoderm cells to form in situ liver bud after the alimentary canal morphogenesis. It is supported by the observations that two key transcription factors hhex and prox1, which are
markers for hepatoblasts, are detected in the endoderm region prior to liver budding (Shin et al., 2007; Wallace et al., 2001). Anatomical study of Field and coworkers also support the second theory (Field et al., 2003b).

1.16 Histology and cytology of the normal zebrafish liver

Given the great variety of fish it is not unexpected that histological architecture of the liver differs between different piscine species. The fish hepatocytes can be organized into tubules made up of five to eight hepatocytes whose basal surface face the sinusoids and apical surfaces line the centrally located biliary passages, as it was observed in several species including Atlantic salmon (Robertson and Bradley, 1992) and brown trout (Rocha et al., 1994). A different type of tubules, consisting of 8-10 hepatocytes with sinusoids and not biliary passages at the centre were, however, observed in the Atlantic croaker (Eurell and Haensly, 1982). The piscine liver can also be composed of anastomosing bilayered hepatocyte laminae separated by sinusoids as observed in tigerfish, *Hydrocynus forskahlii* (Geyer et al., 1996) or golden ide, *Leuciscus idus melanotus* L. (Braunbeck et al., 1987). The interconnected bilayered hepatocyte cords surrounding the central vein and separated from each other by sinusoids have been observed by Hardman et al. (2007) who studied the liver of medaka (*Oryzias latipes*) using three-dimensional reconstructions.

When it comes to the zebrafish liver morphology there has been some degree of contention in previous studies. Some have reported tubular arrangements of zebrafish hepatocytes with sinusoids located in the centre of the tubules (Paris-Palacios et al., 2000). Others, however, claim that the biliary passages, not sinusoids, lay in the centre of the tubules (Hinton and Couch, 1998). Yao et al. (2012) who recently studied the liver architecture in the healthy adult zebrafish believe, based on their observations, that zebrafish hepatocytes are arranged in bilayered cords which are distributed radially around a central vein. The cord-like arrangement of zebrafish hepatocytes was also observed by Rodrigues and Fanta (1998).

In zebrafish liver, in contrast to mammals, the portal veins, hepatic arteries and large biliary ducts are not grouped into portal triads but rather distributed randomly within the hepatic parenchyma (Ting and Peng, 2009). The system of biliary channels starts from the bile canaliculi in which intrahepatic bile ducts have their origin (Pack et al., 1996). There is some discrepancy in the literature regarding the bile canaliculi in fish belonging to *Cyprinade*, i.e. the family to which zebrafish belongs. Some authors report that there are both intra- and intercellular bile canaliculi (Byczkowska-Smyk, 1971; Peute et al., 1978). In this case the intracellular bile canaliculi were described as the ramifications of the intercellular bile canaliculi ending in the nuclear region and making possible transport from the cytoplasm to the lumen of bile canaliculi (Byczkowska-Smyk, 1971). Others report, however, that cyprinid fishes have only intracellular or, in other words, unicellular bile canaliculi (Kalashnikova and Kazanskaya, 1986; Vogt and Segner, 1997). These unicellular bile canaliculi have been described as being formed
by deep invagination of a cell membrane of one hepatocyte and running from the centre of hepatocytes to the cell membrane (Kalashnikova and Kazanskaya, 1986). The latest observation of the zebrafish liver by Yao et al. (2012) revealed that zebrafish biliary system starts from the intracellular canaliculi which extend through the intercellular canaliculi to the bile preductules, the latter being formed by bile preductule cells and the hepatocytes. In addition, based on their observations, Yao et al. suggest that zebrafish liver lobes resemble mammalian liver lobules in function, which is in agreement with previously expressed view on fish liver (Hardman et al., 2007).

Both male and female zebrafish liver cytology have been repeatedly studied in the past (Braunbeck et al., 1990; Paris-Palacios et al., 2000; Peute et al., 1985; Rodrigues and Fanta, 1998). In general, the hepatocytes of sexually mature zebrafish contain single, round nuclei which are mostly euchromatic and posses single conspicuous nucleoli. The nuclei are surrounded by the rough endoplasmic reticulum (RER) which forms stacks of about 20 parallel lamellae in the females, but in the males is limited to a few discontinuous cisternae. Golgi apparatus is observed in the females’ hepatocytes in the number of 3-4, but in the males this structure is poorly developed. The perinuclear regions of the hepatocytes are rich in mitochondria of both spherical and elongated shapes. These organelles are often seen in association with the membranes of RER. In addition, roundish or ellipsoid peroxisomes are observed in groups associated with mitochondria and RER cisternae. The major storage product in the female zebrafish liver is lipid. The amount and size of lipid droplets in females depend on the gonadal cycle and can vary between individuals. In turn, in the males the major storage product is glycogen which forms fields in the periphery of the hepatocytes. Smooth endoplasmic reticulum (SER) and lysosomes are rarely observed in the hepatocytes of either male or female zebrafish.

The youngest zebrafish for which the liver ultrastructure has been described is 4 dpf (Oulmi and Braunbeck, 1996; Strmac and Braunbeck, 1999). In general, at this early stage of development the zebrafish liver was described to closely resemble that of male adult zebrafish. The hepatocytes were of hexagonal shape and had single, centrally located nucleus. The nuclei exhibited a small amount of heterochromatin and mostly one conspicuous nucleolus. Spherical mitochondria surrounded the nucleus and were enclosed by cisternae of RER which also formed small piles in the cell periphery. Rare SER and Golgi fields were restricted to the peribiliary areas. Scattered in the cytoplasm were few peroxisomes which lacked a crystalline core. While the glycogen fields could be observed in the periphery of 4-day-old liver cells, lipid droplets were rarely present. Intracellular compartmentation takes place within hepatocytes as the time of the zebrafish development progresses (in the 8-, 10- and 31-day-old larvae). The compartmentation leads to the formation of a central, organelle containing portion and peripheral storage areas mainly comprising glycogen fields (Braunbeck et al., 1990; Burkhardt-Holm et al., 1999).
1.17 Vitellogenin – an oestrogen-specific endpoint

Vitellogenin is the precursor of the egg yolk proteins phosvitin and lipovitelline. Vitellogenesis occurs in the liver of female fish and is under multihormonal control with estrogens, particularly 17β-oestradiol, playing the dominant role (Sumpter and Jobling, 1995). The amount of vitellogenin produced is positively correlated with plasma levels of oestradiol (Kime et al., 1999). Thus the plasma levels of Vtg can increase over 1 million-fold during the time required to attain sexual maturity by rainbow trout females (Copeland et al., 1986). Fish ovaries are stimulated to synthesize oestradiol by gonadotropin (GTH) released form the pituary which, in turn, is stimulated by the hypothalamic gonadotropin releasing hormone - GnRH (Figure 1.9). The synthesized estradiol is then released into blood and reaches the liver where it can bind to the hepatocytes’ ERs initiating transcription and translation of Vtg (Kime et al., 1999; Wheeler et al., 2005). Vitellogenin protein is then transported via blood to the ovary where it is incorporated into the oocytes to form the yolk which will subsequently serve as food reserve for the developing embryos.

Figure 1.9 A schematic diagram of the hormonally regulated processes leading to the synthesis of vitellogenin in the fish hepatocytes. Oestradiol (E2) synthesized in the follicular cells under the influence of gonadothropin (GTH) is carried with steroid binding protein to the hepatocytes in which it initiates vitellogenesis. Adapted from Dréan et al. (1994).
Both male and juvenile fish also carry the vitellogenin gene but because oestradiol levels in the plasma are normally much lower compared to females (often undetectable), the vitellogenin protein is not greatly expressed. However, in the mid 1980s very high levels of vitellogenin were detected in the male trout maintained on an experimental fish farm which was supplied with water from a local river (Sumpter and Johnson, 2008). Soon the reason of this puzzling observation was identified – contamination of the water with an oestrogenic chemical, or more likely with a mixture of chemicals, originating from the sewage treatment plant effluent entering the river upstream of the fish farm. The potential of this discovery was very soon realized and vitellogenin has been suggested as a biomarker of the exposure to oestrogenic substances (Sumpter and Jobling, 1995). Since then, numerous studies demonstrating the usefulness and sensitivity of the vitellogenin-based tests have been carried out. The study of vitellogenin induction or suppression in adult and/or juvenile fish was often implemented in the studies aimed to monitor the effluents from sewage treatment plants, pulp and paper mills or in general surveys that covered a defined geographic area (Harries et al., 1997; Kirby et al., 2004; Sepulveda et al., 2002; Sumpter and Jobling, 1995). Numerous laboratory studies have also used this bioassay to screen for oestrogenic properties of specific chemicals (Ortiz-Zarragoitia and Cajaraville, 2005). Andersen et al. (2003), for example, have studied the effects of early exposure to 17α-ethinylestradiol on zebrafish vitellogenesis and observed that exposure from fertilization to hatching is sufficient to cause a significant increase in the vitellogenin concentration when measured in 30 day old larvae.

Most vitellogenin studies include the measurement of the protein (Vtg), but some studies focus on the mRNA (vtg). The method of vitellogenin detection depends on the nature and quantity of the biological material available, such as species used and life stages chosen (Wheeler et al., 2005). In the case of the early life stage animals from which blood withdrawal or liver dissection is impossible, the detection of mRNA can be the method of choice (Muncke et al., 2007). Moreover, vitellogenin 1 gene mRNA (vtg1) has been proposed by Muncke and Eggen (2006) as a molecular target and biomarker for estrogenicity in developing zebrafish. At least seven distinct vitellogenin genes (vtg1 to vtg7) have been identified in zebrafish with vtg1 being the most prominent species (57%) of vitellogenin mRNA (Tong et al., 2004).

Because vitellogenin induction can be measured not only in vivo but also in vitro, fish hepatocyte cultures have also been engaged in the studies of EDCs (Pelissero et al., 1993). However, since there are many factors influencing basal and induced vitellogenin production in cell culture, established standardized protocols will be necessary to minimize assay variability (Navas and Segner, 2006).
1.18 Aims

The overall aim of this study was to investigate the effects of BPA and genistein, individually and in combination, on the early development of zebrafish (*Danio rerio*).

The specific aims were as follows:

- To study the toxic and teratogenic effects of BPA and genistein, individually and in mixtures, on zebrafish larvae up to 120 hpf;
- To investigate the effects of BPA and genistein, individually and in mixtures, on the ultrastructure of the liver in 120 hpf larvae;
- To assess the estrogenicity of BPA and genistein, individually and in mixtures, based on the observation of vitellogenin 1 mRNA expression level in 120 hpf zebrafish larvae.
2 Materials and Methods

2.1 Materials

2.1.1 Animals

Zebrafish (Danio rerio) is a small tropical freshwater fish belonging to Cyprinidae Family and a popular aquarium fish. All the zebrafish embryos/larvae used in this and subsequent studies were obtained from the Zebrafish Facility in Dr. Lucy Byrnes’ Laboratory in the Biochemistry Department of the National University of Ireland, Galway. The fish in this facility were originally obtained from the International Resource Centre of Eugene, Oregon. Only the AB strain of zebrafish was used in all the studies presented in this work.

2.1.2 Consumables

ZM Ltd supplied ZM-500, Instant Ocean Sea Salts and Artemia. The following consumables were purchased from Sigma Aldrich: Bisphenol A (cat. no. 239658), Genistein (cat. no. G6649), DMSO (cat. no. 2650), 17β-oestradiol (cat. no. E8875), Chloroform Molecular Biology Tested (cat. no. C2432), 2-Propanol Molecular Biology Tested (cat. no. I9516), Chloroform: isoamyl Alcohol 24:1 (cat. no. C0549-1PT), Molecular Grade Ethanol 200 proof (cat. no. E7023), Phenol for Molecular Biology (cat. no. P4557), Paraformaldehyde (cat. no. P6148), Sodium Cacodylate Trihydrate (cat. no. C0250) and Propylene Oxide (cat. no. 110105). MicroAmp Fast Optical 96-well Reaction Plates (cat. no. 4346906), MicroAmp Optical Adhesive Film PCR compatible (cat. no. 4311971) and Fast Sybr GreenMaster Mix (cat. no. 4385612) were bought from Applied Biosystems. Invitrogen supplied Random Primers (cat. no. 48190-011), TRIzol® Reagent (cat. no. 15596-026) and Superscript II Reverse Transcriptase (cat. no. 100004925). Nuclease Free Water (cat. no. P1193) was bought from Promega. DNase Buffer (cat. no. AM8170G) and DNase Enzyme (cat. no. AM2222) were purchased from Ambion. 1 ml syringes (cat. no. 300015) and 21G needles (cat. no. 304432) were supplied by BD Biosciences. 6 well-plates (cat. no. 83.1839.300), 24 well-plates (cat. no. 83.1836.300) and 96 well-plates (cat. no. 83.1835.300) were purchased from Sarstedt and these plates were demonstrated to be BPA-free in the study by Biswanger et al. (2006). Agar Scientific supplied: Glutaraldehyde 25% EM Grade (cat. no. R1010), Osmium Tetroxide (cat. no. R1016), Agar Low Viscosity Resin kit (cat. no. R1078), Peel-A-Way Moulds 22 x 22 x 20 (cat. no. G3760), Flat Embedding Moulds (cat. no. G3531), 10 ml Glass Vials with Leak Proof Lids (cat. no. B793) and Athene 200 Mesh Thin Bar Grids Copper 3.05 mm (cat. no. G2002). Uranyl Acetate (cat. no. 705631095) and Lead Citrate (cat. no. 70553022) were purchased from Laurylab.
2.1.3 Equipment

Zebrafish embryos/larvae were incubated at 28°C in the LMS cooled incubator while exposed to the tested concentrations of BPA and/or genistein. A Nikon SMZ645 dissecting microscope was used regularly while changing solutions and to observe embryos/larvae, remove debris, dead embryos and/or floating chorions. Images of larvae were taken with a Nikon SMZ800 supplied with DXM 1200C camera and with the use of ACT-1C software. A Variable Speed Specimen Rotator (240 Volt) purchased form Agar Scientific was used through dehydration steps, propylene oxide and/or resin infiltration steps. Resin was polymerized in an oven purchased from Electron Microscopy Sciences (EMS). Ultracut Reicher Jung Microtome was used to cut semi- and ultra thin sections, the latter being cut with the use of a Diamond Knife from Diatome. A Heatblock (type 701201) was used to attach semi-thin sections to glass slides and to enhance toluidine blue staining intensity. Semi-thin sections were observed with a Leica DM500 light microscope to scout for the liver. Ultrathin sections were stained with uranyl acetate and lead citrate using Leica EM AC20 automatic contrasting instrument for ultrathin sections. The sections were viewed using a Hitachi H-7000 Transmission Electron Microscope fitted with a 1K Hamamatsu digital camera. Micrographs were taken with the use of AMTV542 Image Capture Engine™ software.

The following instruments were used to extract mRNA from the 120 hpf larvae and synthesize cDNA: Eppendorf microcentrifuges 5415R and 5415D, IKA Vortex Genius 3, Stuart and QBT1 Grant block heaters and LMS cooled incubator series 1. The concentration of the mRNA was measured with Nanodrop spectrophotometer ND-1000 using ND-1000 v3.5.2 Software. The PCR reaction plates were centrifuged with the use of refrigerated table top centrifuge Sorvall RT 6000D. Real time PCR amplification was performed with 7500 Fast Real Time PCR system (Applied Biosystems) using 7500 software v2.0.6.
2.2 Methods

2.2.1 Animal maintenance

Zebrafish were kept in filtered, aged, de-chlorinated tap water in normal glass aquaria. Aquaria were cleaned and water was changed at least once per week. Water in the aquaria was constantly filtered and its temperature was maintained at 28 ± 1°C. A cycle of 14:10 light: dark was maintained in the fish room. Temperature and humidity in the room were recorded daily. Zebrafish were fed twice a day with commercial dry flakes (ZM -500) and once a day with live brine shrimps (*Artemia salina*) on weekdays. During weekends fish were fed once daily with Artemia.

Artemia culture was conducted parallel to zebrafish culture. Each day 5 g encapsulated Artemia were added to 2 L of filtered tap water with 70 g of instant ocean sea salt. Water was aerated and temperature adjusted for around 24 hours before adding cysts. Artemia were incubated in the hatchery under constant aeration and 24 hours light cycle for at least 24 hours at 28°C. Hatched Artemia were separated from capsules and non-hatched brine shrimp and abundantly rinsed with tap water to get rid of salt before use as live food for zebrafish.

Spawning was performed as described in the Zebrafish book by Westerfield (2000). Only fish which did not show any macroscopically discernible symptoms of infection or disease prior to and while spawning were used to produce eggs. On the night before a planned experiment, the pairs of zebrafish (male and female) or groups of two females and one male were transferred to the spawning trays (20 x 9 x 9 cm) supplied with a lid and a stainless steel mesh. A mesh provided a physical barrier between parents and eggs to prevent the cannibalism of offspring. The eggs were collected the next morning shortly after spawning which normally takes place within 30 minutes after turning on the lights. Parental zebrafish were returned to the main tanks as soon as embryos were collected.

2.2.2 Exposure to endocrine disrupting compounds

All the collected embryos were transferred to Petri dishes filled with egg water and incubated at 28°C. Since fertilized eggs undergo the first cleavage after about 15 minutes, 2 cell-stage embryos were observed (~0.75 hpf) and distinguished from non-fertilized eggs. Only fertilized, 2-4 cell-stage embryos (0.75-1 hpf) which did not show obvious irregularities during cleavage (e.g., asymmetry) or injuries of the chorion were selected for the experiment.

Different rationales underlined the choice of the BPA/genistein concentrations tested in each of the studies. For the toxicity and teratogenicity study, high, environmentally irrelevant concentrations of BPA and genistein were tested in order to estimate lethal concentrations and
sublethal effects of these compounds. This study led to estimate the no-observed-effect-concentrations (NOEC) for BPA and genistein based on the three parameters assessed: the survival of zebrafish larvae up to 120 hpf, the heart rate at 48 and 72 hpf and for the hatching success of 72 hpf zebrafish larvae. For the remaining studies i.e. the study on the zebrafish liver ultrastructure and on the expression levels of vitellogenin 1 mRNA, the concentrations of BPA and genistein tested was lower than these NOECs. Additionally, higher concentrations of both compounds (2000 µg/L BPA and 1250 µg/L genistein) were introduced into the study on vtg1 to ensure the observation of a changed expression level of this gene. These concentrations were chosen based on previously published data. Muncke et al. (2007) reported that 2280 µg/L BPA significantly induced vtg1 in 120 hpf zebrafish larvae following exposure starting shortly after fertilization. The threshold level for genistein for the up-regulation of vtg1 was assessed as 5000 µg/L for male zebrafish following 11-day exposure (Kausch et al., 2008). Because this concentration caused mortality of the zebrafish larvae before 120 hpf (chapter 4, Figure 4.1), 1250 µg/L genistein was used as this was the highest concentration which did not affect the larvae survival up to 120 hpf. To facilitate the interpretation of the observed results, particularly those related to the oestrogenic potential of BPA or/and genistein, an additional treatment with the natural oestrogen (17β-oestradiol) was introduced in the ultrastructural and molecular studies. 20 µg/L 17β-oestradiol was used based on the significant induction of vtg1 in 120 hpf zebrafish observed for both 2.7 µg/L and 27 µg/L concentrations (Muncke et al., 2007).

Since both BPA and genistein are difficult to dissolve in water, dimethyl sulfoxide (DMSO) was used as it has been proved to be a useful solubilising agent for testing of difficult substances and mixtures (OECD, 2000). Stock solutions of BPA and genistein were prepared by dissolving each compound in DMSO as per protocol 1 in appendix A, aliquoted and stored at -20°C. Further dilutions were made in egg water, vortexing to ensure complete dissolution. DMSO concentrations equivalent to its highest concentrations in the treatment groups were used as control (0.1% for toxicity studies, 0.01% for liver fine structure and vtg1 expression studies).

Toxicity and teratogenicity of BPA and/or genistein

Single eggs (10) were randomly placed in the wells of the 96-well plates with 250 µl of chemical solution per well. The treatments were as follows:

- 2 mg/L BPA (8.76 µM)
- 4 mg/L BPA (17.52 µM)
- 8 mg/L BPA (35.04 µM)
- 16 mg/L BPA (70.09 µM)
- 32 mg/L BPA (140.18 µM)
- 1.25 mg/L genistein (4.63 µM)
- 2.5 mg/L genistein (9.25 µM)
• 5 mg/L genistein (18.5 μM)
• 10 mg/L genistein (37 μM)
• 4 mg/L BPA + 1.25 mg/L genistein
• 4 mg/L BPA + 2.5 mg/L genistein
• 8 mg/L BPA + 1.25 mg/L genistein
• 8 mg/L BPA + 2.5 mg/L genistein
• 16 mg/L BPA + 5 mg/L genistein

Solutions were changed daily. All exposures were performed in the incubator at 28°C. Each experiment was repeated three times with embryos drawn from different batches of fertilized eggs, i.e. collected on different days.

The mixture of BPA and genistein - both at NOECs (4 mg/L BPA + 1.25 mg/L genistein) was used to investigate their joint action with equitoxicity on the same endpoints. The mixtures composed of one compound at the concentration causing clear, medium range effect and the second substance at the concentration causing no effect (4 mg/L BPA + 2.5 mg/L genistein and 8 mg/L BPA + 1.25 mg/L genistein) were used to test whether the toxicity of the first compound is affected in the presence of the second one. The combination of BPA and genistein at concentrations causing individually medium effects (8 mg/L BPA + 2.5 mg/L genistein) could further reveal the mutual influence on their toxicity. An additional mixture of 16 mg/L BPA and 5 mg/L genistein was used to test the effects of combined exposure when at concentrations causing clear toxic effects.

➢ The effect of BPA on the liver structure of the 72 hpf zebrafish larvae – pilot study

Zebrafish embryos at 32-64 cells-stages (1.75-2 hpf) were randomly transferred into the wells of 24 well plates, each well containing 1 ml of a test solution. 10 embryos were used per well and the treatment was carried out in duplicates (20 embryos per treatment). The following concentrations of BPA were tested:

• 10 μg/L BPA (43.803 nM)
• 100 μg/L BPA (438.04 nM)
• 500 μg/L BPA (2.19 μM)
• 1000 μg/L BPA (4.38 μM)

Additionally 2 x 10 embryos were reared in both 0.01% DMSO and egg water for comparison and assessment of potential toxic effects of DMSO. Embryos were incubated until they reached 72 hpf. All solutions were changed daily. At 72 hpf all the embryos were placed on ice for about 20 minutes before fixation with the primary fixative for the observation with transmission electron microscopy.
The effects of BPA or/and genistein on the liver structure of the 120 hpf zebrafish larvae

Zebrafish embryos at 2-4 cells-stages (0.5-1 hpf) were randomly transferred into the wells of 24 well plates, each well containing 1 ml of the tested solution. Every exposure was done in duplicate. The following solutions of BPA, genistein and their combinations were tested:

- 10 μg/L BPA (43.80 nM)
- 100 μg/L BPA (438.04 nM)
- 312.5 μg/L genistein (1.16 µM)
- 625 μg/L genistein (2.31 µM)
- 10 μg/L BPA + 312.5 μg/L genistein
- 10 μg/L BPA + 625 μg/L genistein
- 100 μg/L BPA + 312.5 μg/L genistein
- 100 μg/L BPA + 625 μg/L genistein.

In addition to embryos exposed to BPA and/or genistein, 2 x 10 animals were reared in egg water, 0.01% DMSO or 20 μg/L 17β-oestradiol. All the solutions were changed daily. The animals were incubated in the tested solutions for five days. At 120 hpf, the plates were put on ice for ~ 20 minutes prior to fixation and processing for viewing with transmission electron microscope.

The effects of BPA or/and genistein on the expression of the vitellogenin 1 mRNA in the 120 hpf zebrafish larvae

Zebrafish embryos at 2-4 cells-stages (0.75-1 hpf) were randomly transferred into the wells of 6-well plates, each well containing 5 ml of the tested solution. Fifty embryos (n=50) were used per well. The experiment was performed four times using embryos drawn from different batches of fertilized eggs, i.e. collected on different days. All exposures were performed in an incubator at 28°C. Solutions were changed daily.

The following treatment groups were investigated in this study:

- 10 μg/L BPA (43.804 nM)
- 100 μg/L BPA (438.039 nM)
- 2000 μg/L (8.761 µM)
- 312.5 μg/L genistein (1.156 µM)
- 625 μg/L genistein (2.313 µM)
- 1250 μg/L genistein (4.626 µM)
- 10 μg/L BPA + 312.5 μg/L genistein
- 10 μg/L BPA + 625 μg/L genistein
- 100 μg/L BPA + 312.5 μg/L genistein
• 100 µg/L BPA + 625 µg/L genistein
• 2000 µg/L BPA + 1250 µg/L genistein
• 0.01% DMSO
• 20 µg/L 17β-oestradiol (73.4 nM)

All the treatments were finished when zebrafish larvae reached 120 hpf and at this time point the plates were put on ice for ~ 20 minutes before further processing for the polymerase chain reaction (PCR).

2.2.3 Processing zebrafish larvae for transmission electron microscopy

At the end of the exposure time (72 hpf or 120 hpf) the plates with the larvae were put on ice for about 20 minutes before transferring the larvae to 10 ml glass vials with lids. Then, the solutions were aspirated and replaced with primary fixative, i.e. 2.5% paraformaldehyde and 2.5% glutaraldehyde in 0.1M sodium cacodylate buffer (pH 7.6) (preparation of solution described in protocol 2, appendix A). This fixation and all the following steps were carried out on a rotator set at low speed, placed under a fume hood and at room temperature. After 3 hours fixation the larvae were washed 3 times with 0.1M sodium cacodylate buffer (5 minutes for each wash). Next, the larvae were fixed for two hours with the secondary fixative – 1% osmium tetroxide in 0.1M sodium cacodylate buffer. Larvae were dehydrated through a graded series of ethanol: 30%, 50%, 70% and 95%, 2 x 10 minutes each, and finally 3 x 10 minutes in 100% ethanol. After that the larvae were immersed for 2 x 10 minutes in propylene oxide and left overnight in the mixture of propylene oxide and low viscosity agar resin (50:50). The following morning this mixture was exchanged for the one composed of 25:75 propylene oxide and resin, in which the larvae were left for around 8 hours. Next, the mixture of propylene oxide and resin was replaced with the 100% resin in which the larvae were bathed overnight (stirring constantly). Next morning the larvae were immersed in freshly made 100% resin and left for several hours in the opened vials placed on a rotator to let any remaining traces of propylene oxide evaporate. Eventually, the larvae were transferred to the embedding moulds and resin was polymerized in an oven at 60°C for at least 48 hours.

Different types of embedding moulds were used depending on the age of larvae. In case of the 72 hpf zebrafish larvae, a larger surface area of the liver was obtained with saggital sections compared to transverse sections. Consequently, the 22 x 22 x 20 mm disposable moulds were used in which the larvae were laid flat on their left side to facilitate further sectioning of the resin blocks. Up to 6 larvae were embedded in a single mould. Polymerized resin blocks were cut using a fine toothed saw to get smaller blocks, each containing single animal.

An increase in the size of the liver in the 120 hpf versus 72 hpf zebrafish larvae, allowed for cross- instead of saggital sectioning of the larvae bodies. Consequently a different type of mould could be used and these were multiple-use, “coffin moulds”. Up to two larvae were
placed in each mould with heads facing the trapeze-shaped ends. The resin blocks used with
this method were ready to use for cutting.
All resin blocks were cut with the use of a microtome fitted with glass knife to obtain 1 μm
(semi-thin) sections. These sections were mounted on glass slides, stained with toluidine blue
and searched for the liver using a light microscope (20 x and 40 x magnification). At this level,
the liver was identified based on its localization. In the sagittal sections of the 72 hpf larvae, it
was localized ventrally, posterior to the heart, dorsal to the anterior part of the yolk sac and
roughly below the first 1-3 somites (Figure 2.1). The recognition of the liver was easier in the
cross-sections of the 120 hpf larvae as this organ was bigger and found ventrolaterally to the
intestine and in contact with the remaining yolk sac, as can be seen in Figure 2.2.

![Figure 2.1 The localization of the liver on semi-thin section of the 72 hpf zebrafish.](image1)

![Figure 2.2 The localization of the liver on semi-thin sections of 120 hpf zebrafish.](image2)
Following identification of the liver in the semi-thin sections, ultrathin sections of 90-100 nm thickness were cut with the use of a microtome fitted with a diamond knife. The ultrathin sections were transferred to 200 mesh, copper grids and stained with lead citrate and uranyl acetate to increase electron density of the tissue. This staining was carried out automatically in the Leica Instrument and using 36 minute programme (# 4). Finally, the sections were examined using a transmission electron microscope and the micrographs were taken with AMTV542 software.

2.2.4  Processing zebrafish larvae for the polymerase chain reaction (PCR)

In order to analyse vtg1 level in the exposed larvae, several steps were required and these included mRNA extraction, first strand cDNA synthesis, primer design and validation, PCR plate preparation and PCR amplification.

➢ RNA extraction

Following exposure to the tested solutions, zebrafish larvae at 120 hpf (48 ± 2 larvae/treatment) were transferred to 1.5 ml microcentrifuge tubes and put on ice. After a few minutes the solutions were carefully aspirated from the microcentrifuge tubes and replaced with 500 µl of TRIzol® and the larvae were homogenized using sterile syringes and needles. The homogenized material was either stored at -80°C until further processing or processed straight away.

On the day of processing, all the samples were removed from the -80°C freezer and incubated at room temperature for 5 minutes to thaw. An additional 500 µl TRIzol® was added into each microcentrifuge tube, as a pilot study showed that this effectively increased mRNA harvest. Consequently, each 1.5 ml tube contained tissue from 48 ± 2 zebrafish homogenized in 1 ml of TRIzol®. Next, 200 µl of chloroform was added into each microcentrifuge tube which was immediately vortexed for 15 seconds and then incubated at room temperature for 3 minutes. After that all the samples were centrifuged at 12,000g at 4°C for 15 minutes. Centrifugation of the tissue homogenized in TRIzol® led to the separation of the microcentrifuge tubes contents into upper (colourless phase containing RNA), middle (inter-phase with DNA) and lower phase (phenol-chloroform, red colour). Following centrifugation, the upper aqueous phase was transferred to new 1.5 ml microcentrifuge tubes. To precipitate RNA, 500 µl isopropanol was added into each tube which was then mixed, incubated at room temperature for 10 minutes and centrifuged at 13,200g at 4°C for 10 minutes. Following centrifugation, the supernatant was removed and discarded and the pellets of RNA remaining in the bottoms of the microcentrifuge tubes were washed through brief vortexing with 100 µl of 75% ethanol (pre-chilled on ice). Then the samples were again centrifuged at 4°C at 13,200g for 10 minutes. Then the supernatant (ethanol) was carefully removed leaving in the tubes RNA pellets which were then air dried for around 10 minutes. Each RNA pellet was then re-suspended with 88 µl DEPC
treated water and heated at 55°C for 5-10 minutes. To get rid of DNA remnants, 10 μl DNase buffer and 2 μl DNase enzyme were added into each tube which were then placed in 37°C incubator for 10 minutes. After the incubation, all the samples were placed back on ice and 20 μl 3M sodium acetate, 80 μl nuclease-free water (NFW) and 200 μl phenol was added. This was followed by quick vortexing and centrifugation at 13,200g at 4°C for 5 minutes. Next, the supernatant from each tube was removed and transferred to the new tubes - 200 μl chloroform:isoamyl alcohol (24:1) was added into each. Following centrifugation at 13,200g at 4°C for 5 minutes, the supernatants were once more transferred to the new tubes and 200 μl isopropanol was added to each tube which was then incubated at -80°C for a further 20 minutes. Next, the tubes were centrifuged at 13,200g at 4°C for 30 minutes, supernatants were removed and the RNA pellets were washed with 200 μl of pre-chilled 70% ethanol. Ethanol was removed after 10 minute-centrifugation at 4°C and the RNA pellets were air-dried for about 10 minutes. Finally, the RNA pellets were resuspended in 8 μl nuclease free water and stored at -80°C until further processing.

- **First strand cDNA synthesis**

Several steps were necessary in order to synthesise cDNA. First, all the RNA containing tubes were removed from the freezer (-80°C), centrifuged quickly to thaw and briefly vortexed. Next, the concentration of RNA was measured with the use of spectrophotometer and the purity of extracted RNA was determined by the ratio of 260/280. Next, the volume containing 1 μg of total RNA was calculated for each tube and this volume was then mixed with 1 μl random primers and nuclease-free water up to 12 μl. The whole mixture was spun down quickly and incubated at -70°C for 10 minutes. Following the incubation, the samples were centrifuged briefly and then 1 μl first strand buffer, 2 μl 0.1M DTT and 1 μl dNTPs were added into each tube followed by 2 minute-incubation at 42°C. Next, 1 μl superscript II enzyme was added into each microcentrifuge tube which was then incubated at 42°C for 1 hour. The reaction of cDNA synthesis was stopped by heating the samples to 70°C for 15 minutes. All the steps resulted in obtaining 17 μl of cDNA in all processed samples which were centrifuged and either directly used for PCR or frozen at -20°C until further use.

- **Primer design and validation**

Specific primers for the elongation factor 1 alpha - *ef1α* (NM_131263) and vitellogenin 1 mRNA – *vtg1* (NM_001044897) were designed with the use of NCBI primer design tools (http://www.ncbi.nlm.nih.gov/). Following primers were used in this experiment:

- *ef1α* forward primer (5’→3’): GTGGTATCACCATGGACATTCG (bp 280-301)
- *ef1α* reverse primer (5’→3’): TCAGCCTGAGAAGTACCAGTGA (bp 382-402)
- *vtg1* forward primer (5’→3’): CCTGGGTGAATTCCCTGCTGCAC (bp 3760-3782)
vgt1 reverse primer (5’→3’): GAATCCTGTGTCGTAAGCTGCTGT (bp 3857-3880)

Both primers sets were validated according to rules specified in the protocol by Bookout et al. (2006).

PCR plate preparation

Primer mixes containing 1.25 µM forward and 1.25 µM reverse primer in NFW were prepared for both ef1α and vgt1 primer sets. Next, each primer mix was added to SYBR Green and NFW in the amounts calculated according to the formula as per protocol by Bookout et al. (2006):

\[ [n \times 4.8] \mu l \text{ primer mix} + [n \times 20] \mu l \text{ SYBR Green} + [n \times 10.2] \mu l \text{ NFW} \]

\( n = \text{number of samples} + 1 \text{ non template control} + 1 \text{ extra} \)

Consequently the ef1α primer master mix and the vgt1 primer master mix were obtained. Next, 35 µl of ef1α primer master mix was added into each microcentrifuge tube containing 5 µl cDNA extracted from zebrafish exposed to the tested solutions. Similarly, 35 µl of vgt1 primer mix was added into the analogous set of 5 µl-samples. Non template controls, i.e. samples containing NFW instead of cDNA and either ef1α or vgt1 primer mix were also prepared. All the tubes were well stirred and kept on ice before the contents of each tube was distributed into the appropriate three wells of the optical reaction plate, each well receiving 10 µl solution. The arrangement of the tested samples was according to the diagram presented in Figure 2.3. As a final step, the plate was covered with the optical adhesive cover and briefly centrifuged (3 minutes) at 4°C before PCR reaction.

Figure 2.3 The schematic presentation of the arrangement of tested samples on the PCR plate.
Real-time PRC amplification

The plate was transferred to the real-time thermal cycler and real-time PCR was performed using 7500 v.2.0.6 software. Comparative Ct (ΔΔCt) method using SYBR Green qPCR Master Mix in 10 µl of reaction mixture was run. The following steps were performed:

- 1 cycle: 20 sec at 95°C (activation of the hot-start Taq DNA polymerase)
- 40 cycles: 3 sec at 95°C (collecting data throughout)

Following steps were added to the 40 cycles of the thermal cycling programme for melting curve analysis:

- 15 sec at 95°C
- 1 min at 60°C (collecting data)
- Increase from 60°C to 95°C at a 2% temperature ramping rate (collecting data)
- 15 sec at 95°C (collecting data)
- 15 sec at 60°C

Data analysis

Raw data was imported into a Microsoft Excel spreadsheet for analysis. The threshold cycle (CT) i.e. the cycle number at which enough amplified DNA accumulates to produce a detectable fluorescent signal, was recorded for every triplicate of each of the tested samples. All the data analysis steps were performed according to the method as per protocol by Bookout et al. (2006). For each of the three replicates of a single sample, the average threshold cycle (Ct) and standard deviation were calculated. Outlier wells from the averaged Ct values (standard deviation > 0.3) were removed (maximally one point per replicate).

A relative quantification approach was used to analyse the data. In this method the expression level of the gene of interest (GOI) is compared between control (a calibrator sample) and treated samples and fold differences are calculated. Expression levels were normalized against the reference gene elongation factor α (ef1α), which exhibits constant expression levels in all samples and is not affected by any investigated treatment.

Reaction efficiencies (E) of ef1α (a reference gene – ref) and vtg1 (gene of interest – GOI) were not similar, which means they were not within 5% of each other, consequently a Pfaffl method (Pfaffl, 2001) was used to calculate fold changes according to the formula:
\[
\text{Ratio (folds)} = E_{\text{GOI}}^{\Delta Ct \text{ GOI}} / E_{\text{ref}}^{\Delta Ct \text{ ref}}
\]

where:

\[
\Delta Ct \text{ GOI} = \text{Ct (GOI, calibrator)} - \text{Ct (GOI, test)}
\]

\[
\Delta Ct \text{ ref} = \text{Ct (ref, calibrator)} - \text{Ct (ref, test)}
\]

2.2.5 Observed parameters

Specific parameters were observed for each study and these have been specified below.

➢ Toxicity and teratogenicity of BPA and/or genistein

The survival of embryos was assessed every 24 h for all 5 days of the experiment. The following endpoints were used as indicators of acute lethality in zebrafish embryos:

- coagulation of fertilized eggs
- lack of detachment of the tail bud from the yolk
- lack of heart beat

To elaborate, coagulation is a clearly recognized sign of embryonic death and is identified by a milky white (dark under the microscope) egg appearance. Detachment of the tail from the yolk results from the posterior elongation of the embryonic body. Lack of this detachment can be easily seen and is indicative of embryonic death for both 24 and 48 hpf zebrafish. The lack of a beating heart was stated when there was no heart beat observed within at least 40 second periods. Zebrafish were considered dead if one of these endpoints was recorded as positive. Dead embryos were removed from the wells at each observation time.

Sublethal effects of tested solutions were investigated by the observation of heart rate and hatching success of the exposed embryos/larvae. The signs of a beating heart were observed at 24 hpf, however heart rate was assessed for 48 and 72 hpf zebrafish, when heart beat had fully developed. At 48 and 72 hpf the heart rate of 50 % of the embryos/larvae from each group was measured by counting number of heart beats per 30 seconds at room temperature of 25±1°C. Hatching success was estimated on the basis of the percentage of the larvae free of chorions at 72 hpf for each group under investigation.

The NOEC for BPA and genistein were estimated for each assessed endpoint and at every time point of the observation. In addition, the LC50 (lethal concentration 50%) was calculated for both BPA and genistein at each time point under investigation. Similarly, the effective concentration 50% (EC50) was estimated for both compounds for heart rate and hatching success of the treated larvae.
In addition to the lethal and sublethal endpoints, zebrafish embryos and larvae were evaluated for morphological defects. The morphology of the head, heart, tail, yolk sac and yolk extension as well as the skin pigmentation level was assessed using the following scoring system:

- score 5 – normal structure
- score 4 – mildly altered structures
- score 3 – moderately altered structure
- score 2 – severely altered structure
- score 1 – structure’s malformations equivalent to death of embryo/larva

Mean total morphological scores were obtained for embryos/larvae at 24, 48, 72, 96 and 120 hpf and for all the treatments tested. These values were acquired by summing the mean scores of all the observed parameters. The maximum possible score for 24 hpf embryos was 25 because skin pigmentation was not assessed at this stage. For 48, 72, 96 and 120 hpf embryos/larvae the maximum score was 30. Data were presented graphically with the use of Microsoft Excel programme.

The parameters of interest were observed with the use of a dissecting microscope with a minimum of 30-fold magnification. The study was finished with zebrafish reaching 120 hpf. This follows OECD guidelines on testing of chemicals using fish embryos which say that duration of the test may last no longer than 2 days post-hatch (OECD, 2006). In zebrafish this time coincides with the almost complete absorption of the yolk.

A test was classified as valid only if 90% of the larvae in the control group survived until the last day of experiment and showed no signs of abnormal development.

The effect of BPA on the liver structure of the 72 hpf zebrafish larvae – pilot study

In this study the cellular composition of 72 hpf zebrafish hepatocytes was observed and quantitatively described for control animals and larvae exposed to the tested concentrations of BPA. The following parameters were observed:

- volume of hepatocytes
- volume fraction and absolute volume of nuclei
- volume fraction and absolute volume of mitochondria
- volume fraction and absolute volume of rough endoplasmic reticulum
- volume fraction and absolute volume of bile canaliculi

Stereological tools were used to quantitatively describe observed parameters. First, ultrathin sections of the liver were obtained for five larvae (n=5) from each treatment group (water, 0.01 % DMSO, 10, 100, 500 and 1000 μg/L BPA). Next, the micrographs of the liver were taken using 8,000 x magnification and analyzed morphometrically with the use of ImagePro software.
A grid of 196 intersections was imposed on each micrograph and the number of intersections hitting elements other than hepatocytes (such as the bars of the copper grid, space of Disse, endothelial cells, sinusoids, etc.) were subtracted from the total number of intersections to calculate the hepatocyte-only fraction. In stereological terms this fraction is referred to as reference space. Next, the number of points falling on a structure of interest was counted and a volume fraction (Vv) of this structure was calculated according to the formula:

This procedure was performed for every structure of interest and led to obtaining the volume fractions of nuclei, mitochondria, rough endoplasmic reticulum (RER) and bile canaliculi. Following that, the absolute volumes of all examined elements were estimated as follows. First, the mean number weighted volume was determined for the nuclei of the hepatocytes from every examined larva. This was done by measuring the radii of the hepatocytes nuclei on the images of the semi-thin, toluidine blue- stained sections captured with Leica light microscope (100 x, oil objective). Four radii originating from a single point, such as the centre of a visible nucleolus, were drawn at 90 degrees to each other and measured using ImagePro Software. From a series of these measurements the mean volume of each observed nucleus in the number-weighted distribution was estimated from the formula:

In this formula \( r \) refers to the radii of each particle/nucleus measured (Howard and Reed, 1998). All the obtained volumes assessed per larvae were averaged out to obtain the mean nuclear volume. Next, knowing the mean number weighted volume of the nuclei and the nuclear volume fraction for each larva and assuming there was a single nucleus per cell, the volume of the hepatocytes, i.e. reference volume, was estimated. This was done by dividing the nuclear mean number weighted volume by its fraction. The volumes of all the remaining structures of interest were calculated by multiplying their volume fraction (Vv) by the reference space volume.

- The effects of BPA or/and genistein on the liver structure of the 120 hpf zebrafish larvae

In this study the liver ultrastructure of 120 hpf zebrafish larvae was observed and quantitatively described for control animals (0.01 % DMSO) and the larvae exposed to BPA or genistein individually and in combinations. As in the study on the liver ultrastructure in the 72 hpf zebrafish larvae exposed solely to BPA, here also the volumes of the hepatocytes and their nuclei, mitochondria, rough-endoplasmic reticulum and bile canaliculi were assessed. In
contrast to the previous study, glycogen volume was also estimated as at this stage this structure could be discerned much easier than at 72 hpf.

Stereological tools were used to quantitatively describe the fine structure of zebrafish liver. From each of 11 groups under investigation four random larvae were morphometrically analysed in the way analogous to this described for the study on 72 hpf zebrafish larvae. Minor changes in stereological procedures were introduced to facilitate analysis: the magnification of micrographs was increased up to 9000 and the density of stereological grid was increased up to 18 x 18 points. All the remaining procedures were the same and included the calculation of the reference space (nuclei only), volume densities (fractions) of all the hepatic structures of interest and the estimation of their absolute volume for each examined larva.

In addition to the stereological examination of the livers’ fine structure, an analysis of shape of the mitochondrial profiles observed in the hepatocytes of 72 hpf and 120 hpf zebrafish larvae was carried out. This analysis was performed with the use of ImageJ software, version 1.46r. Mitochondrial profiles were assessed for the same micrographs used in the stereological analysis. A single square-shaped grid was superimposed on each micrograph analysed. The grid’s boundaries were placed away from the edges of the micrographs and this distance was no smaller than the diameter of the largest mitochondrial profile observed for the zebrafish larvae’ hepatocytes. This procedure aimed to provide equal sampling probability for all size ranges of the mitochondrial profiles. In addition, a forbidden-edges rule was implemented in the sampling procedure. Consequently, all the mitochondrial profiles found within the grid and not touching the two forbidden lines were analysed. The analysis was done by drawing the outlines of the observed profiles with the use of a graphic, TRUST Mini Tablet. Surface, circularity, maximum and minimum feret diameters as well as major to minor axis ratio were estimated for each drawn profile with the use of functions available in ImageJ. These data were then analysed to search for the potential differences in any of the obtained shape descriptors between the treatment groups.

- The effects of BPA or/and genistein on the expression of the vitellogenin 1 mRNA in the 120 hpf zebrafish larvae

Gene expression level of the biomarker vitellogenin 1 mRNA (vtg1) was investigated in the 120 hpf zebrafish larvae exposed to the tested solutions based on the reverse-transcriptase, real-time polymerase chain reaction (rt PCR). The fold-changes in the expression level of vtg1 in the oestrogenic compound(s) exposed larvae were estimated based on the comparison to the control group (0.01% DMSO).
2.2.6 **Statistical analysis**

The data obtained from all studies were analysed with the use of Microsoft Office Excel 2007 and the statistical analysis was performed with the use of IBM SPSS Statistics Software, version 20. Most of the graphic presentations of data were performed with the use of GraphPad Prism software, version 5. Remaining graphs were created with Microsoft Excel programme.

Several steps of analysis were carried out to decide on the statistical test to be used. First, the presence of outliers was examined by inspection of a boxplot and the normality of data was assessed by a Shapiro-Wilk test. This procedure was carried out for each tested group of every study. Next, the homogeneity of variances was analysed with the use of Lavene’s Test of Homogeneity of Variance.

One way analysis of variance (ANOVA) was chosen if the assumptions of normal distribution (P>0.05, Shapiro-Wilk test) and lack of outliers for the data in each treatment group were met. If the variances were similar between all the treatment groups (P>0.05, Lavene’s Test of Homogeneity of Variance) ANOVA followed by post-hoc Tukey’s test were performed. However, if the assumption of homogeneity of variances was violated (P<0.05, Lavene’s Test of Homogeneity of Variance) the data were analysed with a robust, Welch’s ANOVA followed by Games-Howell post hoc tests.

An alternative statistical test was a non-parametric test for n-independent samples of Kruskal–Wallis. Pair-wise comparisons with the use of Mann-Whitney U test were performed to identify which treatment groups differed from each other in a significant way.

Because the data obtained from each part of study presented in this work was specific, different statistical tools were implemented to their analysis:

- **Toxicity and teratogenicity of BPA and/or genistein**

  The effects of BPA or and genistein on zebrafish survival, heart rate and hatching success were tested with a one way ANOVA followed by post-hoc multiple comparisons made with a Tukey’s test. The differences in the observed parameters between the embryos/larvae from different treatment groups were considered statistically significant for P≤0.05.

  The calculations of LC50s and EC50s were performed with the use of Probit analysis.

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1. Shapiro-Wilk H0: data’s distribution is equal to a normal distribution.
2. Lavene’s Test of Homogeneity of Variance H0: the variances between groups for the dependent variable are equal.
3. ANOVA H0: all group means are equal.
4. Kruskal-Wallis H0: the distribution of scores in each group is the same.
5. Mann-Whitney U H0: the distribution of scores for the two groups is equal.
The effect of BPA or/and genistein on the liver structure of the 72 hpf or 120 hpf zebrafish larvae

The absolute volumes and volume fractions of the observed parameters (hepatocytes, nuclei, mitochondria, RER, glycogen and bile canaliculi) were compared between the larvae from all the groups with the use of Kruskal-Wallis test. Specific comparison testing was carried with the use of Mann-Whitney U Test. Differences were considered to be significantly different if $P \leq 0.05$.

The effects of BPA or/and genistein on the expression of the vitellogenin 1 mRNA in the 120 hpf zebrafish larvae

Data were log10-transformed to meet the assumptions of normal distribution and lack of outliers for each of the treatment group. The differences in the expression level of vitellogenin 1 mRNA ($vtg1$) among all 13 treatments were investigated using one-way ANOVA or Welch’s ANOVA. Specific pair-wise comparisons were performed with the use of post hoc Tukey’s or Games-Howell tests. The differences in $vtg1$ expression were assessed as significant for $P \leq 0.05$. 
3 The Influence of Bisphenol A on the Early Development of Zebrafish

3.1 Aim of study

The aim of this study was to investigate the effects of BPA on the early development of zebrafish. Firstly, the toxic and teratogenic effects of BPA were studied. Both lethal and sublethal effects of the tested compound were assessed. Next, the potential changes in the liver ultrastructure caused by BPA at concentrations below the NOEC were studied for both 72 hpf and 120 hpf zebrafish larvae. The study with the use of 72 hpf zebrafish larvae was a pilot study which aimed to investigate whether zebrafish liver at this early stage of development can provide a suitable model to study the potential ultrastructural alterations in response to EDCs. Finally, the effect of BPA on the expression of the vitellogenin 1 mRNA – a widely accepted biomarker of oestrogenic exposure was investigated in 120 hpf zebrafish larvae.

3.2 Results

3.2.1 Embryotoxic and teratogenic effects of BPA on the early development of zebrafish

All embryos and larvae were exposed starting at 2-4 cell stage of development (0.75-1 hpf) until 120 hpf. Several aspects of the development were studied. Survival and morphology were assessed at 24, 48, 72, 96 and 120 hpf, heart rate was investigated at 48 and 72 hpf and hatching success at 72 hpf. No-observed effect concentrations (NOEC) and the concentrations causing 50% lethality (LC50) were estimated at every observed time point.

The effects of BPA exposure on zebrafish survival

The first stage of this study was the comparison of zebrafish survival between embryos and larvae reared in clean egg water and fish raised in the solvent control (0.1% DMSO). A statistical analysis demonstrated no significant difference in the observed parameter between these two groups of embryos/larvae for the whole course of the experiment, i.e. for first five days of zebrafish life. Consequently, zebrafish embryos/larvae raised in solvent control were used as a negative control for all the subsequent analysis of the influence of endocrine disruptors’ exposure on zebrafish survival.

BPA treatment had both concentration- and time-dependent effects on zebrafish embryo/larval survival under laboratory conditions, with increased concentration or increased
time in drug causing increased death rate as can be seen in Figure 3.1 (see also appendix B, Table 8.1). 32 mg/L BPA caused death of 100% of zebrafish population by 24 hpf, whereas 16 mg/L BPA had no significant effect on survival until 72 hpf, when it caused a significant increase in the mortality leading to death of all the exposed population at 120 hpf. Whereas there was no significant difference in the survival between the larvae exposed to 8 mg/L BPA and control group up to 96 hpf; this concentration led to a significantly higher mortality at 120 hpf ($P \leq 0.05$). Neither 2 mg/L nor 4 mg/L BPA affected the survival of zebrafish throughout the course of this experiment when compared to the control group (DMSO). Hence it is concluded that BPA at a concentration of 4 mg/L (17.52 µM) constitutes a NOEC for the survival of zebrafish up to 120 hpf.

Time-dependent toxicity of BPA can be deduced from the fact that the concentration of BPA causing mortality of 50% zebrafish population gradually decreased with the time of observation (see appendix B, Table 8.2). Whereas 16.77 mg/L BPA was estimated as LC50 for 24 hpf zebrafish, at 120 hpf this concentration had almost halved to 8.95 mg/L.

![Survival of zebrafish embryos exposed to BPA](image)

Figure 3.1 Survival of 24, 48, 72, 96 and 120 hpf zebrafish exposed to 2, 4, 8, 16 and 32 mg/L BPA. Graph plotted as means with standard deviation error bars. Asterisks indicate values significantly different from the controls (*$P \leq 0.05$, ** $P \leq 0.01$ and *** $P \leq 0.001$, Tukey’s test).
The effects of BPA exposure on zebrafish morphology

Embryo morphology was normal at 24 hpf following exposure to 2 or 4 mg/L BPA (Figure 3.2 B and C) when compared to control embryos (Figure 3.2 A). Embryos from both treatment groups had the highest possible total morphological score (Figure 3.3). 80% of embryos treated with 8 mg/L BPA looked normal (Figure 3.2 D) and 20% showed signs of mild oedema in the heart region. The mean total morphological score for these embryos was slightly reduced comparing to the controls (Figure 3.3). Zebrafish embryos treated with 16 mg/L had slightly reduced head size, mild heart oedema and somewhat bigger yolk extensions (Figure 3.2 E). In addition, their tails were slightly shorter and more tapered when compared to the controls. These observations are reflected in the reduced mean total morphological score for this treatment group (Figure 3.3). The whole population of 24 hpf embryos exposed to 32 mg/L BPA was coagulated (Figure 3.2 F) and consequently classified as dead. These embryos have received the lowest mean total morphological score as can be seen in Figure 3.3.

Figure 3.2 Morphology of the 24 hpf zebrafish embryos treated with BPA. A – control (DMSO); B – 2 mg/L BPA; C – 4 mg/L BPA; D – 8 mg/L BPA; E – 16 mg/L BPA; F – 32 mg/L BPA. Arrows indicate changed morphology of the heart region, yolk extension and tail.
When assessed at **48 hpf** embryos exposed to 2 or 4 mg/L BPA (Figure 3.4 B and C) were very similar to controls (Figure 3.4 A). A slightly reduced mean total morphological score for the 4 mg/L BPA treated embryos resulted from mildly reduced pigmentation and somewhat bigger yolk sac and yolk extension observed in 1 of 6 embryos scored (Figure 3.5). Concentration-dependent reduction in the body pigmentation was observed for the 8 mg/L BPA (Figure 3.4 D) and 16 mg/L BPA (Figure 3.4 E) treated embryos. In addition, embryos from these treatment groups had enlarged yolk extensions and yolk sacs when compared to the controls and consequently received lower mean total morphological scores (Figure 3.5). Furthermore, 10% of the embryos exposed to 8 mg/L and 15 % of the embryos treated with 16 mg/L BPA had heart oedema. 25% of the embryos treated with 16 mg/L BPA had also slightly reduced head size.
When assessed at 72 hpf zebrafish larvae treated with 2 mg/L BPA (Figure 3.6 B) were similar in their morphology to control larvae (Figure 3.6 A) and received a comparable mean total morphological score (Figure 3.7). The 4 mg/L BPA treated larvae received a slightly lower score of 28.50 because of somewhat increased size of the yolk sac and yolk extension and to some extent reduced skin pigmentation level in more than 20% of the scored larvae (Figure 3.7). The exposure to ≥ 8 mg/L BPA caused the inhibition of hatching (Figure 3.6 D), as will be described in a later section. 7% of the larvae treated with 8 mg/L BPA had heart oedema while in the 16 mg/L treated larvae this malformation was common. Skin pigmentation depletion increased with increasing concentration of BPA starting from 4 mg/L BPA (Figure 3.6 D and E).
At 96 hpf 2 mg/L or 4 mg/L of BPA had no effect on the morphology of zebrafish larvae (Figure 3.8 B) and these larvae received the mean total morphological scores similar to the control group (Figure 3.9). The only noticeable effect of 4 mg/L BPA on the larvae morphology was a little reduced skin pigmentation level. The larvae treated with 8 mg/L were still enclosed within their chorions and heart oedema was often observed in this treatment group (Figure 3.8 D). Moreover, the larvae exposed to 8 mg/L BPA (Figure 3.8 D) or 16 mg/L BPA (Figure 3.8 E) had reduced body sizes when compared to controls (Figure 3.8 A) indicating a general underdevelopment. 16 mg/L BPA caused 90% mortality at 96 hpf (previously shown in Figure 3.1) and the larvae which survived up to this time point were severely malformed and had reduced head, heart oedema, enlarged yolk and yolk extension and malformed tails as can be seen in Figure 3.8 E.
At **120 hpf** 2 mg/L BPA had no effect on zebrafish morphology (Figure 3.10 B) and only very mild changes in the pigmentation level were observed for the 4 mg/L treated larvae (Figure 3.10 C). Consequently, total morphological scores for the larvae treated with BPA at 2 mg/L or 4 mg/L concentrations were no different from the score given to the controls (Figure 3.11). Larvae from 8 mg/L BPA treatment group were still in their chorions and had reduced body size, decreased skin pigmentation and relatively larger yolk sacs comparing to the controls. Moreover, 60% of the larvae exposed to BPA at this concentration showed heart oedema (Figure 3.10 D). Treatment with 16 mg/L BPA resulted in 100% mortality at 120 hpf and these larvae were given the lowest mean total morphological score (Figure 3.10 E and 3.11).
The effects of BPA exposure on the heart rate of zebrafish embryos

There was a concentration-dependent decrease of the heart rate in the BPA treated zebrafish both at 48 hpf and 72 hpf (Figure 3.12). The heart rates of zebrafish embryos/larvae treated with 2 mg/L or 4 mg/L BPA were similar to controls on both days of examination. As a consequence, 4 mg/L BPA was interpreted to be a NOEC for the heart rate of zebrafish up to 72 hpf. Both 8 mg/L and 16 mg/L BPA caused a significant decrease in heart rate compared to controls at 48 and 72 hpf. 16 mg/L BPA, however, had a several times stronger effect on the
heart rate than 8 mg/L and these effects were significantly different when compared with each other (see also appendix B, Tables 8.3 and 8.4).

The concentrations of BPA causing 50% reduction in the heart rate of zebrafish (EC50) in reference to controls were calculated using Probit analysis and were equal to 8.41 mg/L (36.84 µM) for 48 hpf and 9.75 mg/L (42.72 µM) at 72 hpf.

The effects of BPA exposure on hatching success of zebrafish larvae

Starting at 8 mg/L increasing concentrations of BPA delayed hatching of larvae. At 72 hpf 100% of DMSO control zebrafish larvae were hatched, as was the case with the larvae raised in egg water. Neither 2 mg/L nor 4 mg/L BPA affected hatching success of the treated larvae (one way ANOVA, followed by Tukey’s post hoc test). Treatment with 8 mg/L or 16 mg/L BPA caused significant reduction in the hatching success of zebrafish larvae (Figure 3.13). Moreover, there was no significant difference in the effect of 8 and 16 mg/L BPA on this parameter when compared to each other (Figure 3.13, see also appendix B, Table 8.5). In addition, surviving zebrafish larvae exposed to either 8- or 16 mg/L BPA did not hatch on the following days and remained in their chorions up to 120 hpf.

The concentration of BPA causing a 50% reduction in the number of hatched larvae at 72 hpf was calculated as 5.65 mg/L (24.76 µM) with the use of Probit analysis.
Hatching of zebrafish embryos exposed to BPA

Figure 3.13 Hatching success of 72 hpf zebrafish larvae exposed to 2, 4, 8 and 16 mg/L BPA. % hatching is the percentage of live larvae that hatched and survival was: 100% for 0 mg/L, 96.7% for 2 mg/L, 93.3% for 4 mg/L, 90% for 8 mg/L and 66.7% for 16 mg/L. Graph plotted as means with standard deviation error bars. Asterisks indicate values significantly different from the controls (*** $P\leq0.001$, Tukey’s test).

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3.2.2 The effects of BPA on the 72 hpf zebrafish liver ultrastructure – pilot study

In the following section the effects of BPA on the ultrastructure of the liver in 72 hpf zebrafish larvae were investigated. This was a pilot study which aimed to resolve whether the zebrafish liver at this early stage of morphogenesis can serve as an organ to indicate structural alterations in response to treatment with endocrine disrupting compounds. In this study the zebrafish embryos at 32-64 cell stage (1.75-2 hpf) were exposed to 10, 100, 500 or 1000 μg/L BPA until they reached 72 hpf. The structure of the liver in control as well as in the BPA-treated larvae was assessed morphometrically with the use of the stereological tools. Other possible structural alterations were assessed based on the morphological analysis. Since the stock solution of BPA was prepared with the use of DMSO, the potential changes in the fine structure of the liver caused by this chemical were also studied.

Liver of normal 72 hpf zebrafish

To assess whether there are pathological changes in the fine structure of any organ or tissue in response to the tested treatment, the appearance and characteristic features of this anatomical structure in normal/healthy state must first be described. According to this reasoning, the ultrastructure of the liver of 72 hpf zebrafish larvae reared in normal conditions, i.e. in clean egg water, at appropriate temperature (28°C) and under the preferred light cycle (14:10 light : darkness) was studied and characterized in both quantitative and qualitative way.

To the best of our knowledge there has been no report on the ultrastructure of the liver in 72 hpf zebrafish larvae. Zebrafish liver at this stage of development showed a noticeable degree of inter-individual and inter-cellular variety and only partially resembled the liver of an adult fish which was described elsewhere (Braunbeck, 1998; Braunbeck et al., 1990; Burkhardt-Holm et al., 1999; Oulmi and Braunbeck, 1996; Strmac and Braunbeck, 1999). Hepatocytes - undoubtedly the main component of the liver parenchyma – were assembled in rather compact way with relatively few sinusoids in between (Figure 3.14). A characteristic arrangement of the hepatocytes into cords or tubules could not be identified. The hepatocytes were of oval or polygonal shapes with the volume estimated as 217.79 ± 32.93 μm³ and possessed single nuclei. The nuclei were primarily oval or round and occupied around 24% of the hepatocytes’ volume (52.73 ± 14.02 μm³). They were located in the centre of most of the hepatocytes observed. Most of the nuclei were euchromatic and had a conspicuous, single nucleolus in their centres. Nuclei with two nucleoli, however, were also encountered. Nuclear surface was mainly relatively smooth but somewhat irregular or wavy nuclear outlines could also be found (Figure 3.14). Mitochondria in the hepatocytes showed a wide range of shapes and sizes. Small, roundish mitochondria were the most common, but longitudinal or irregular, sometimes very big forms could also be observed (Figure 3.14 and 3.15). Mitochondria took up about 11% of the volume of hepatocytes (24.01 ± 5.22 μm³) and were predominantly located around the nuclei and very often associated with the rough endoplasmic reticulum (RER). The membranes of RER could be found both around the nuclei and associated with mitochondria as
well as interspersed in the cytoplasm (Figure 3.15). No stacks of RER membranes surrounding the nucleus – a feature typical for the older fish, were observed. The volume of RER was estimated to be 13.83 ± 5.04 μm³. Golgi fields and smooth endoplasmic reticulum (SER) were very rare. Bile canaliculi (Figure 3.16) together with the bile preductules were estimated to have volume of 3.77 ± 1.11 μm³ and occupied about 1.7% of the hepatocytes’ volume. Preductules were identified based on the presence of preductule epithelial cells which were much smaller than hepatocytes and often had longitudinal nuclei of irregular shapes (Figure 3.17). The lumen of most observed bile canaliculi and preductules was filled with microvilli projecting from the hepatocytes. Neither the amount nor the arrangement of glycogen was well defined for zebrafish of this stage of development. The amount of glycogen in the hepatocytes was observed to be subjected to appreciable variation due to inter-individual variability. Moreover, the differences in the amount and pattern of glycogen fields were observed between hepatocytes of the same organ. No lipid droplets were observed in the hepatocytes of the 72 hpf zebrafish liver.

Figure 3.14 Liver of the normal, 72 hpf zebrafish larva, overview. N – nuclei of hepatocytes, M – mitochondria, G – glycogen, E – nucleated erythrocyte, S – sinusoid, En – endothelial cell, Y – yolk sac material. Scale bar – 2 μm.
Figure 3.15 Liver of the normal, 72 hpf zebrafish. N – nucleus of a hepatocyte, M – mitochondria, both small and round as well as large and longitudinal forms; → – RER; G – glycogen, S – sinusoid, E – a fragment of a nucleated erythrocyte. Scale bar – 1 μm.

Figure 3.16 Liver of the normal, 72 hpf zebrafish. N – nucleus of a hepatocyte, M – mitochondria, → – RER; G – glycogen, B – bile canaliculus occluded by microvilli projecting from the hepatocytes. Scale bar – 1 μm.
The effects of DMSO on the liver ultrastructure of 72 hpf zebrafish

The first stage of this study was to investigate whether there is any effect of DMSO on the ultrastructure of zebrafish larvae. In order to answer this question the absolute volumes of all hepatic structures of interest in 72 hpf zebrafish reared in 0.01 % DMSO solution were compared with their equivalents in larvae raised in clean egg water (Figure 3.18). Statistically significant differences were searched with the use of Mann-Whitney U test and regarded as such for $p \leq 0.05$.
The absolute volume of the hepatocytes was similar ($P=0.046$, Mann-Whitney U test) when compared between the DMSO treated ($244.13 \pm 52.97 \, \mu m^3$) larvae and the egg water control group ($217.79 \pm 32.93 \, \mu m^3$).

Similarly, there was no statistically significant difference ($P=0.060$, Mann-Whitney U test) in the Nuclear volume of the hepatocytes between zebrafish from DMSO ($48.83 \pm 8.53 \, \mu m^3$) and water ($52.73 \pm 14.02 \, \mu m^3$) treatment groups.

The volume of mitochondria in the hepatocytes of the DMSO treated larvae was estimated as $28.77 \pm 6.42 \, \mu m^3$ and was similar to the volume of these organelles in the larvae reared in egg water ($24.01 \pm 5.22 \, \mu m^3$; $P=0.12$, Mann-Whitney U test).

Rough endoplasmic reticulum in the egg water-control group occupied $13.83 \pm 5.04 \, \mu m^3$of the volume of hepatocytes whereas in the larvae treated with DMSO – $14.33 \pm 8.85 \, \mu m^3$. This difference was of no statistical significance ($P=0.75$, Mann-Whitney U test).

Likewise, there was no statistically significant difference when the volume of bile canaliculi in the liver of the zebrafish larvae reared in egg water ($3.77 \pm 1.11 \, \mu m^3$)was compared with the analogous volume in the larvae exposed to 0.01% DMSO ($3.82 \pm 1.42 \, \mu m^3$; $P=0.092$, Mann-Whitney U test).

To summarize, this study showed that DMSO at a concentration of 0.01% has no effect on the hepatic ultrastructure of 72 hpf zebrafish and can be used in the subsequent study to investigate the effects of oestrogenic compounds dissolved in it. As a consequence, zebrafish larvae reared in 0.01% DMSO were used as a control group to which all the larvae exposed to BPA were compared.
The effects of bisphenol A on the ultrastructure of the liver of 72 hpf zebrafish – morphometric analysis

In the following section 72 hpf larvae were treated with 10 μg/L, 100 μg/L, 500 μg/L or 1000 μg/L bisphenol A (BPA). The exposure commenced at a 32-64 cell-stage (1.5-2 hpf) and finished at 72 hpf. The fine structure of the liver was analyzed for each treatment group and compared between the BPA-treated larvae and the controls (0.01% DMSO). The differences in the absolute volumes of the hepatocytes, the hepatocytes’ nuclei, mitochondria and rough endoplasmic reticulum, as well as the bile canaliculi were analyzed with the use of statistical tools.

- Volume of hepatocytes

The volume of the hepatocytes was similar when compared across all the treatment groups, i.e. 0.01% DMSO, 10 μg/L BPA, 100 μg/L BPA, 500 μg/L BPA and 1000 μg/L BPA ($P=0.057$, Kruskal-Wallis test). A subsequent pair-wise comparison testing revealed, however, that the hepatocytes of the larvae treated with 10 μg/L BPA ($159.14 \pm 37.37 \, \mu m^3$) were significantly smaller ($P=0.028$, Mann-Whitney U test; Figure 3.19) than the hepatocytes of the control larvae ($244.13 \pm 52.97 \, \mu m^3$). Although there was no statistical difference there was a trend towards smaller volume in all the remaining treatment groups in respect to controls ($P=0.076$ for all the comparisons, Mann-Whitney U test).

![Volume of hepatocytes in 72 hpf zebrafish exposed to BPA](image)

**Figure 3.19** Absolute volume of hepatocytes in the 72 hpf zebrafish exposed to BPA. Data presented as boxplots (median and quartiles) with whiskers from minimum to maximum. Asterisks indicate values significantly different from the controls ($*P<0.05$, Mann-Whitney U test).
• Nuclear volume of hepatocytes

The nuclear volume fraction was similar when compared across the treatment groups ($P=0.111$, Kruskal-Wallis test). The specific comparison testing revealed, however, that the nuclear fraction in the 10 µg/L BPA treated larvae was significantly higher than in the control group ($P=0.047$, Mann Whitney test). A trend towards the increased nuclear volume fraction when compared to the control group ($P=0.094$, Mann-Whitney U test) was noticeable for the 500 µg/L BPA treatment group (Figure 3.20).

When the mean absolute volumes of the hepatocytes’ nuclei were compared across all the treatment groups no significant difference was revealed ($P=0.756$, Kruskal-Wallis test). Post hoc analysis confirmed that the volumes of the nuclei from the larvae exposed to all the tested concentrations of BPA were similar to that of the control group (Figure 3.21). The biggest disparity in the nuclear volumes was between the controls (52.73 ± 14.02 µm$^3$) and the zebrafish exposed to 10 µg/L BPA (42.63 ± 4.14 µm$^3$).

![Volume fraction of nuclei in hepatocytes of 72 hpf zebrafish exposed to BPA](image)

Figure 3.20 Volume fraction of nuclei in the hepatocytes of the 72 hpf zebrafish exposed to BPA. Data presented as boxplots (median and quartiles) with whiskers from minimum to maximum. Asterisks indicate values significantly different from the controls (*$P \leq 0.05$, Mann-Whitney U test).
• Mitochondrial volume of hepatocytes

The volume fractions of mitochondria in the hepatocytes of the larvae treated with BPA at tested concentrations (10, 100, 500 and 1000 µg/L) were similar to each other ($P=0.981$, Kruskal-Wallis test) and the fraction of mitochondria in the BPA treated larvae was similar to the control group (Mann-Whitney U test, Figure 3.22).

Similarly, the comparison of the absolute volumes of the mitochondria across the treatment groups revealed no statistically significant differences ($P=0.073$, Kruskal-Wallis test). A specific comparison testing uncovered, however, that the mitochondrial absolute volume was significantly lower ($P=0.016$, Mann-Whitney U test) in the 10 µg/L BPA exposed larvae (18.04 ± 4.51 µm$^3$) than in the controls (28.77 ± 6.42 µm$^3$; Figure 3.23). A noticeable trend towards reduced mitochondrial volume was observed for the 1000 µg/L BPA treated larvae ($P=0.076$).
Volume fraction of mitochondria in hepatocytes of 72 hpf zebrafish exposed to BPA

Figure 3.22 Volume fraction of pooled mitochondria in the hepatocytes of the 72 hpf zebrafish exposed to BPA. Data presented as boxplots (median and quartiles) with whiskers from minimum to maximum.

Absolute volume of mitochondria in hepatocytes of 72 hpf zebrafish exposed to BPA

Figure 3.23 Absolute volume of mitochondria in the hepatocytes of the 72 hpf zebrafish exposed to BPA. Data presented as boxplots (median and quartiles) with whiskers from minimum to maximum. Asterisks indicate values significantly different from the controls (*P≤0.05, Mann-Whitney U test).

- Volume of rough endoplasmic reticulum in hepatocytes

The volume fractions of rough endoplasmic reticulum (RER) in the hepatocytes of the larvae exposed to 10, 100, 500 or 1000 µg/L BPA were similar across the treatment groups (P=0.762, Kruskal-Wallis test) and no differences were found comparing the RER volume fraction in the BPA treatment groups to the controls (Mann-Whitney U test, Figure 3.24)
The comparison of the mean absolute volumes of RER across all the treatment groups under investigation showed no significant difference ($P=0.193$, Kruskal-Wallis test). A specific comparison testing (Mann-Whitney U test) revealed, however, that the RER mean absolute volume in the hepatocytes of the 10 µg/L BPA larvae (6.42 ± 2.58 µm$^3$) was significantly lower ($P=0.028$) than that in the controls (14.33 ± 8.85 µm$^3$) as seen in Figure 3.25.

Figure 3.24 Volume fraction of rough endoplasmic reticulum in the hepatocytes of 72 hpf zebrafish exposed to BPA. Data presented as boxplots (median and quartiles) with whiskers from minimum to maximum.

Figure 3.25 Absolute volume of rough endoplasmic reticulum in the hepatocytes of 72 hpf zebrafish exposed to BPA. Data presented as boxplots (median and quartiles) with whiskers from minimum to maximum. Asterisks indicate values significantly different from the controls (*$P\leq0.05$, Mann-Whitney U test).
• Volume of bile canaliculi

The volume fraction of bile canaliculi as well as the mean absolute volume of this structure was similar across the following treatment groups: 0.01% DMSO, 10, 100, 500 or 1000 µg/L BPA ($P=0.231$ and 0.302, respectively; Kruskal-Wallis test). Moreover, both the volume fraction and the mean absolute volume of bile canaliculi in the BPA treated larvae were similar to the control group (Mann-Whitney U test) as presented in Figures 3.26 and 3.27.

Figure 3.26 Volume fraction of bile canaliculi in the hepatocytes of 72 hpf zebrafish exposed to BPA. Data presented as boxplots (median and quartiles) with whiskers from minimum to maximum.

Figure 3.27 Absolute volume of bile canaliculi in the hepatocytes of 72 hpf zebrafish exposed to BPA. Data presented as boxplots (median and quartiles) with whiskers from minimum to maximum.
A noticeable degree of morphological heterogeneity was observed for the hepatocytes of 72 hpf larvae raised in the solvent control (0.01% DMSO). The hepatocytes were mostly of polygonal shapes and easily discernible borders were observed between most of them (Figure 3.28). The majority of the nuclei were euchromatic with little heterochromatin and single or two nucleoli, however, nuclei with increased amount of heterochromatin were also encountered. The outline of nuclei was generally smooth and regular but irregularly delineated forms were also observed. The variation in nuclear size of the hepatocytes was visible. The cytoplasm of most of the hepatocytes was disordered, and showed no clear organization of intracellular organelles into clear compartments. The granularity of the cytoplasm seriously impeded the recognition of most of the organelles. Mitochondria, which could be easily identified, were numerous and relatively small. Most of these organelles were roundish or oval and some were longitudinal (Figure 3.28 and 3.29). Generally, the whole mitochondrial lumen was filled with cristae (Figure 3.29). Another structure which was relatively easily recognized in the hepatocytes was bile canaliculi. The initial, intracellular portions of the bile canaliculi were found towards the cells’ centres and were mostly seen as small profiles composed of round, electron-lucent vesicles. Larger bile canaliculi with microvilli projecting into their lumen were also observed. RER was hardly discerned in the granular cytoplasm. The majority of endoplasmic membranes were associated with mitochondria or/and nuclei. No clear organization of RER into the stacks of more than three membranous tubules was observed (Figure 3.29). The organization of the glycogen showed a high degree of diversity compared to the other observed intracellular structures. Its amount and organization not only varied between the individual larvae but also between the cells of the same organ. This storage product was observed sometimes as fields with clear boundaries which were localized throughout the cytoplasm but mostly towards the hepatocytes’ periphery. In most cells, however, glycogen was dispersed and hardly distinguished from other granular components of the cytoplasm. No SER and very rare Golgi fields could be found in the hepatocytes of the DMSO treated larvae. Macrophages were rarely observed amidst hepatic parenchymal cells.
Figure 3.28 Liver of 72 zebrafish exposed to DMSO. N – nuclei of hepatocytes, M – mitochondria, BC – bile canaliculi, → – rough endoplasmic reticulum. The borders of the hepatocytes can be easily recognized. Scale bar = 2 μm.

Figure 3.29 Liver of the 72 zebrafish exposed to DMSO. N – nucleus of a hepatocyte, M – mitochondria, → RER; G – glycogen. Scale bar = 500 nm.
No prominent alterations in the morphology of the hepatocytes were observed as the result of the treatment with 10 µg/L BPA. The nuclei of the hepatocytes in the BPA exposed larvae resembled those observed in the control group, there was, however, a slight increase in the number of nuclei with irregular outline (Figure 3.30). Most mitochondria were very small and were mainly of round or oval shapes. Mitochondria of bigger sizes and of different than roundish shapes were encountered much less often. The amount of RER varied between cells – some had amounts of RER similar to that observed in controls, but other cells showed a clear reduction in the amount of this organelle. In most observed hepatocytes glycogen was dispersed or absent, however some hepatocytes had clear compartments of this storage material. The hepatocytes were clearly delineated by cellular membranes.

Increased morphological heterogeneity of the hepatocytes was observed as the result of the treatment with 100, 500 and 1000 µg/L BPA. For these treatment groups some of the observed hepatocytes were similar in their appearance to the hepatocytes in the controls and had easily recognizable borders between the hepatocytes (Figure 3.31 and 3.33). In other cases, however, the clarity of the intercellular borders was observably reduced, and a kind of intracellular disarray was observed with the reduced clarity of the organelles’ outlines, including the nuclei (Figure 3.32 and 3.34). The amount of heterochromatin in the nuclei was variable but there were no apparent differences between the BPA treated larvae and controls. A degree of concentration-dependent increase in the number of nuclei of shapes deviating from regular ovals has been observed (Figure 3.30 and 3.36). Most of the observed mitochondria were of small sizes and of round shapes but longitudinal forms as well as mitochondria with irregular outlines could also be observed (Figures 3.31-3.36). The amount and arrangement of RER varied from cell to cell and no obvious influence of BPA treatment could be determined based on morphological assessment of this organelle. No difference was observed in the appearance of bile canaliculi when the liver architecture was compared between the larvae treated with xenoestrogen and the controls. Furtermore, no apparent changes in the amount of macrophages in response to the exposure to BPA has been observed in this study.
Figure 3.30 Liver of 72 zebrafish exposed to 10 µg/L BPA. N – nuclei of hepatocytes, some with irregular outline; M – mitochondria, → RER. Notice clear borders between the hepatocytes. Scale bar ~2 µm.

Figure 3.31 of 72 zebrafish exposed to 100 µg/L BPA. N – nuclei of hepatocytes, notice a slight expansion of heterochromatin in one of the nuclei; M – mitochondria, B – bile canaliculi, → rough endoplasmic reticulum. Notice clear borders between the hepatocytes. Scale bar ~2 µm.
Figure 3.32 Liver of 72 zebrafish exposed to 100 µg/L BPA. N – nuclei of hepatocytes; M – mitochondria; → RER; G - glycogen. Notice lack of clear nuclear border and small mitochondria. Scale bar – 2 µm.

Figure 3.33 Liver of the 72 zebrafish exposed to 500 µg/L BPA. N – nuclei of hepatocytes, notice a slight expansion of heterochromatin in one of the nuclei; M – mitochondria, B – bile canaliculi, → RER. Scale bar – 2 µm.
Figure 3.34 Liver of 72 zebrafish exposed to 500 µg/L BPA. N – nuclei of the hepatocytes; M – mitochondria, rough endoplasmic reticulum. Scale bar – 1 µm.

Figure 3.35 Liver of 72 zebrafish exposed to 1000 µg/L BPA, overview. Notice expansion of heterochromatin in some nuclei. Scale bar – 2 µm.
Shape analysis of the mitochondrial profiles in the hepatocytes of the 72 hpf zebrafish larvae exposed to BPA.

Morphometrical analysis and morphological assessment of the fine liver structure revealed that the volume and morphology of mitochondria can be affected by BPA. To further investigate this phenomenon the shape of individual mitochondrial profiles was studied. The area, maximum and minimum feret diameters, major to minor axis ratio as well as circularity of each analyzed mitochondrial profiles were estimated. For the circularity description a value of 1.0 indicates a perfect circle while the value approaching 0.0 indicates an increasingly elongated form.

- Area of the mitochondrial profiles

There was no difference in the area of the mitochondrial profiles in the hepatocytes of 72 hpf zebrafish larvae when compared between all the treatment groups, i.e. DMSO, 10, 100, 500 and 1000 µg/L BPA (P=0.189, Kruskal-Wallis test). A specific comparison of the profiles’ areas between the larvae treated with 10, 100, 500 or 1000 µg/L BPA and the control groups (DMSO) showed no significant differences (a Mann-Whitney U test, Figure 3.37).
The analysis of the distribution (Chi-squared test) revealed no significant difference between the BPA-exposed larvae and the larvae treated with DMSO, however there was a clear trend (0.05 > p < 0.1) towards smaller areas of the mitochondrial profiles in the 10 µg/L BPA treated larvae. Comparably larger profiles (0.05 > p < 0.1) were in turn observed in the 1000 µg/L BPA treatment group (Figure 3.38).

**Figure 3.37** Area of the mitochondrial profiles in the hepatocytes of the 72 hpf zebrafish exposed to BPA. Each symbol represents the median of 119 ± 24 mitochondria measured per larva (n=5).

**Figure 3.38** Distribution of the area of the mitochondrial profiles in the hepatocytes of the 72 hpf zebrafish larvae exposed to BPA. Data presented as the mean (n=5) of the percentages of the mitochondrial profiles of a given area range.
- **Circularity of the mitochondrial profiles**

When the circularity of the mitochondrial profiles observed in the hepatocytes of the larvae was compared among the treatment groups no significant difference was observed ($P=0.473$, Kruskal-Wallis test). Moreover, a specific comparison of the profiles between larvae treated with BPA and the control larvae (Mann Whitney U test) revealed no statistically significant differences (Figure 3.39). There was, however, a noticeable trend ($P=0.058$) towards increased circularity of the mitochondrial profiles observed for the 10 µg/L BPA treatment groups when compared to the control.

The comparison of the circularity distributions (*Chi-squared* test) between the BPA-treated and the control larvae showed that the mitochondrial profiles were similar (Figure 3.40).

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**Figure 3.39** Circularity of the mitochondrial profiles in the hepatocytes of the 72 hpf zebrafish larvae exposed to BPA. Each symbol represents the median of 119 ± 24 mitochondria measured per larva (n=5).
Figure 3.40 Distribution of the circularity of the mitochondrial profiles in the hepatocytes of the 72 hpf zebrafish larvae exposed to 10, 100, 500 or 1000 µg/L BPA. Data presented as the mean (n=5) of the percentages of the mitochondrial profiles of a given circularity range.

- Maximum feret diameter of the mitochondrial profiles

Maximum feret diameters of the mitochondrial profiles observed in the hepatocytes of the larvae from all the treatment groups (DMSO, 10, 100, 500 and 1000 µg/L BPA) were similar (P=0.156, Kruskal-Wallis test) and a subsequent specific comparison of the profiles (a Mann-Whitney U test) between the BPA-treated larvae and the controls showed no significant differences (Figure 3.41).

Similarly, no differences in the maximum feret diameters of the observed mitochondrial profiles were found when the distributions of the values were compared between the BPA- and DMSO-treated larvae (Chi-squared test) as presented in Figure 3.42.
Max feret diameters of mitochondrial profiles in the hepatocytes of the 72 hpf zebrafish exposed to BPA

![Graph showing feret diameters of mitochondrial profiles in zebrafish](image)

Figure 3.41 Maximum feret diameters of the mitochondrial profiles in the hepatocytes of the 72 hpf zebrafish larvae exposed to BPA. Each symbol represents the median of 119 ± 24 mitochondria measured per larva (n=5).

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Maximum feret diameters of mitochondrial profiles

![Bar chart showing percentage of mitochondrial profile lengths](image)

Figure 3.42 Distribution of the maximum feret diameters of the mitochondrial profiles in the hepatocytes of the 72 hpf zebrafish larvae exposed to BPA. Data presented as the mean (n=5) of the percentages of the mitochondrial profiles of a given length range.
• Minimum feret diameter of the mitochondrial profiles

When the minimum feret diameter of the mitochondrial profiles was compared among all the treatment groups no difference was revealed ($P=0.245$, Kruskal-Wallis test). In addition, a specific comparison of this parameter (Mann-Whitney U test) between the BPA-treated larvae and the controls revealed no differences (Figure 3.43). The comparison of the minimum feret diameter distribution (Chi-squared test) also showed lack of significant differences. There was, however, a noticeable trend (0.05 > p < 0.1) towards increased length of the minimum feret diameter in the 1000 µg/L BPA treated larvae comparing to the controls (Figure 3.44).

![Minimum feret diameter of mitochondrial profiles in the hepatocytes of the 72 hpf zebrafish exposed to BPA](image)

Figure 3.43 Minimum feret diameters of the mitochondrial profiles in the hepatocytes of the 72 hpf zebrafish larvae exposed to BPA. Each symbol represents the median of 119 ± 24 mitochondria measured per larva (n=5).
Figure 3.44 Distribution of the minimum feret diameters of the mitochondrial profiles in the hepatocytes of the 72 hpf zebrafish larvae exposed to BPA. Data presented as the mean (n=5) of the percentages of the mitochondrial profiles of a given length range.

- Major to minor axis ratio of the mitochondrial profiles.

When the major to minor axis ratio of the mitochondrial profiles observed in the hepatocytes was compared between all the treatment groups no significant difference was found ($P=0.399$, Kruskal-Wallis test). A specific comparison of this parameter (Mann-Whitney U test) revealed, however that for both the 10 µg/L BPA and 1000 µg/L BPA treatment groups the major to minor aspect ratio was significantly lower than in controls ($P=0.035$ and $P=0.046$, respectively; Figure 3.45).

However, the distributions of the mitochondrial profiles aspect ratios in the larvae treated with 100, 500 or 1000 µg/L BPA were significantly different than that observed in the control group (Figure 3.46).
Major to minor axis ratio of mitochondrial profiles in the hepatocytes of the 72 hpf zebrafish exposed to BPA

Figure 3.45 Major to minor axis ratios of the mitochondrial profiles in the hepatocytes of the 72 hpf zebrafish larvae exposed to BPA. Each symbol represents the median of 119 ± 24 mitochondria measured per larva (n=5). Asterisks indicate values significantly different from the controls (* P≤0.05, Mann-Whitney U test).

Major to minor axis ratio of mitochondrial profiles

Figure 3.46 Distribution of the major to minor axis ratios of the mitochondrial profiles in the hepatocytes of the 72 hpf zebrafish larvae exposed to BPA. Data presented as the mean (n=5) of the percentages of the mitochondrial profiles of a given aspect ratio range. Asterisks indicate values significantly different from the controls (* P≤0.05, ** P≤0.01 and *** P≤0.001, Chi-squared test).
3.2.3 The effects of BPA on the 120 hpf zebrafish liver ultrastructure

Liver ultrastructure of control 120 hpf zebrafish

To study the effect of the tested endocrine disruptors on the liver of 120 hpf zebrafish, the ultrastructure of this organ was characterized for the zebrafish at this specific age.

Liver of 120 hpf zebrafish was mainly composed of hepatocytes which formed a rather compact structure interspersed with occasional sinusoids (Figure 3.47). Sinusoids were lined by the endothelial cells which were characterized by the presence of oval to elongated nuclei with abundant, clumped heterochromatin (Figure 3.48). Spaces of Disse occupied relatively small areas and were filled with microvilli projecting from the neighbouring hepatocytes. No clear organization of hepatocytes into cords or tubules could be observed.

Hepatocytes were of oval or polygonal shapes and were assessed to have the volume of 452.58 ± 70.30 µm$^3$. Around 11% of the hepatocytes’ volume was occupied by their nuclei whose volume was assessed to be 51.54 ± 1.17 µm$^3$. Nuclei were predominantly of oval to roundish shape and were located in the centre of the hepatocytes. Most of the nuclei were euchromatic with one or two conspicuous nucleoli in their centre. Nuclear surface was relatively smooth (Figure 3.47).

Mitochondria were very conspicuous organelles in the hepatocytes of 120 hpf zebrafish. Their pooled volume reached 89.26 ± 21.33 µm$^3$ which constituted around 20% of hepatocytes’ volume. The shape of mitochondria was very diverse. Small and roundish as well as very big, round to longitudinal mitochondria could be observed (Figure 3.47 – 3.50). The average volume of a single mitochondrial was calculated to be 7.07 ± 3.11 µm$^3$.

Rough endoplasmic reticulum was mainly observed in the association with the nuclei and/or mitochondria but could also be found as an independent structure in the cytoplasm between the other organelles (Figure 3.48 and 3.49). The membranes of RER were estimated to occupy 24.07 ± 3.59 µm$^3$ which was around 5% of the volume of observed hepatocytes.

Both cross and longitudinal profiles of bile canaliculi and bile preductules were found in the liver of 120 hpf zebrafish. Small profiles were observed as spaces in large part occupied by microvilli projecting from the hepatocytes (Figure 3.50) while sections of larger diameter had a relatively bigger lumen occupied by finger-like projections to a lesser degree. The volume of these biliary passages occupied 4.30 ± 1.57 µm$^3$. Bile structures of higher organizational order such as bile ductules or bile ducts were observed very rarely. Both bile preductule (Figure 3.50) and bile ductular cells were identified based on their appearance as cells smaller than hepatocytes and dominated by irregular and relatively more heterochromatic nuclei.

There was no specific pattern in the localization of glycogen in the hepatocytes of zebrafish at this stage of development. Moreover, the amount of this storage product showed inter-
individual variation which was reflected by a high standard deviation in its amount between individual larvae (12.31 ± 12.79 µm³). If present, glycogen was mainly located at the periphery of hepatocytes, although small glycogen fields could also be observed towards the cell centre and in between the other organelles.

Macrophages which can be recognized based on their varied shapes and nuclei with moderate marginated and clumped heterochromatin, were rarely identified amidst observed hepatic cells. Moreover, neither smooth endoplasmic reticulum (SER) nor lipid droplets were seen in the hepatocytes of 120 hpf zebrafish and Golgi apparatus was very rare.

Figure 3.47 Liver of normal 120 hpf zebrafish reared in egg water. N – nuclei of hepatocytes; M – mitochondria of various sizes and shapes; G – glycogen; S – sinusoid with two nucleated erythrocytes (E); B – bile canaliculi. Scale bar ~2 µm.
Figure 3.48 Liver of normal 120 hpf zebrafish reared in egg water. N – nucleus of a hepatocyte; M – mitochondria; S – sinusoid lined with endothelial cell (En), fragment of nucleated blood cell can be seen (Er); R→ rough endoplasmic reticulum. Scale bar ~2 μm.

Figure 3.49 Liver of normal 120 hpf zebrafish reared in egg water. N – nucleus of hepatocytes with single nucleolus (Nu); M – mitochondria – notice variable sizes and shapes of this organelle; R→ rough endoplasmic reticulum. Scale bar ~2 μm.
Figure 3.50 Liver of normal 120 hpf zebrafish reared in egg water. N – Fragment of a hepatocyte’s nucleus; M – mitochondria; bile canaliculi (B) and bile preductule with a preductule epithelial cell (PE). Scale bar ~2 μm.
The effects of DMSO on the ultrastructure of the liver of 120 hpf zebrafish

The absolute volumes of all the structures under investigation were compared between the 120 hpf DMSO treated and egg water reared zebrafish larvae (Figure 3.51).

![Figure 3.51 Volume of observed structures in the 120hpf zebrafish reared in DMSO (solvent control) vs. normal zebrafish raised in egg water. Graph plotted as means with standard deviation error bars.](image)

The volume of the hepatocytes was similar ($P=0.248$) when compared (Mann-Whitney U test) between the larvae treated with the solvent control (503.92 ± 40.18 $\mu m^3$) and the larvae reared in egg water (452.58 ± 70.30 $\mu m^3$).

DMSO had no influence on the volume of hepatocytes' nuclei (55.37 ± 7.20 $\mu m^3$) which was similar ($P=0.248$, Mann-Whitney U test) to the analogous volume in the zebrafish larvae reared in egg water (51.54 ± 1.17 $\mu m^3$).

In addition, the pooled volume of mitochondria in the hepatocytes (89.26 ± 21.33 $\mu m^3$ vs. 91.39 ± 3.59 $\mu m^3$) as well as the volume of a single mitochondrion (7.07 ± 3.11 $\mu m^3$ vs. 7.76 ± 0.68 $\mu m^3$) were highly similar between egg water and DMSO treated larvae (Mann-Whitney U test).

Similarly, no significant difference was revealed ($P=0.564$) when the volume of rough endoplasmic reticulum in the hepatocytes of DMSO treated larvae (25.23 ± 4.26 $\mu m^3$) was compared to the analogous volume in the larvae reared in egg water (24.07 ± 3.59 $\mu m^3$).

The volume of glycogen in the hepatocytes of the larvae reared in egg water was 12.31 ± 12.79 $\mu m^3$ while in the DMSO treated larvae this storage material occupied 29.76 ± 19.58 $\mu m^3$ of the
hepatocytes’ volume. These values, although clearly diverse, were not significantly different ($P=0.149$, Mann-Whitney U test).

Similarly, even though the mean volume of bile canaliculi in the zebrafish larvae treated with DMSO (11.12 ± 7.34 µm$^3$) was higher than in the larvae reared in egg water (4.30 ± 1.57 µm$^3$), the volumes were highly variable and statistical analysis revealed no significant difference between these two values ($P=0.149$, Mann-Whitney U test).

Summarizing, 0.01% DMSO had no effect on the volume of any of the observed parameters and could be used for the subsequent analysis of the effects of BPA and/or genistein on the fine structure of 120 hpf zebrafish larvae. Consequently, any potential, significant alteration in the volume of the observed structure upon treatment with BPA or/and genistein could be attributed to these compounds and not to DMSO used as the solvent for preparation of their solutions.

- The effects of BPA on the liver ultrastructure of 120 hpf zebrafish – morphometric analysis
  
  - Volume of the hepatocytes

There was a significant difference in the hepatocyte volumes ($P=0.018$, Kruskal-Wallis test) when compared across all the treatment groups (DMSO, 10 µg/L BPA, 100 µg/L BPA, 20 µg/L β-oestradiol). Pair-wise comparisons (Mann-Whitney U test) between the larvae treated with BPA and the control group (503.92 ± 40.18 µm$^3$) revealed that 10 µg/L BPA did not affect the volume of the hepatocytes ($P=0.386$) but there was a noticeable trend ($P=0.083$) towards increased cell volume in the 100 µg/L BPA treatment group (Figure 3.52).

A significant decrease in the volume of the hepatocytes was observed in the larvae from the positive control-oestradiol-treated group (367.17 ± 101.50 µm$^3$) when compared to the controls (503.92 ± 40.18 µm$^3$).

The volumes of the hepatocytes in the BPA treated larvae were noticeably ($P=0.083$, for 10 µg/L) or significantly ($P=0.021$, for 100 µg/L) increased when compared to the volume of the cells in the β-oestradiol treated larvae (Figure 3.52, see also appendix B, Tables 8.14 and 8.15).
Nuclear volume of hepatocytes

There was a significant difference in the volume fraction of nuclei when compared among all the treatment groups ($P=0.036$, Kruskal-Wallis test). Further analysis revealed that none of the compounds (10 µg/L BPA, 100 µg/L BPA or β-oestradiol) affected the volume fraction in a significant way in comparison to the control group (Mann-Whitney U test). A statistically significant difference was however revealed in the nuclear volume fraction between the 100 µg/L BPA and the β-oestradiol treated larvae ($P=0.021$, Mann-Whitney U test; Figure 3.53).

The absolute volume of the nuclei in the hepatocytes of zebrafish in all the treatment groups was similar ($P=0.277$, Kruskal-Wallis test). Moreover, none of the treatments caused alterations in the nuclear absolute volume when compared to the controls ($p>0.05$, Mann-Whitney U tests). In addition, the volumes of the nuclei in the hepatocytes of the larvae treated with BPA (either 10 or 100 µg/L) were similar when compared to the analogous volume in the positive control group (Figure 3.54, see also appendix B, Tables 8.14 and 8.15).
Figure 3.53 Volume fractions of the nuclei in the hepatocytes in 120 hpf zebrafish exposed to BPA. Data presented as boxplots (median and quartiles) with whiskers from minimum to maximum. The capital letters indicate a significant difference from: E – positive controls (β-oestradiol). Asterisks specify statistical significance of the differences (*P≤0.05, Mann-Whitney U test).

Figure 3.54 Absolute volumes of the nuclei in the hepatocytes in 120 hpf zebrafish exposed to BPA. Data presented as boxplots (median and quartiles) with whiskers from minimum to maximum.
Pooled mitochondrial volume of hepatocytes

Volume fraction of mitochondria in the hepatocytes of the zebrafish larvae was similar when compared across all the treatment groups ($P=0.304$, Kruskal-Wallis test). A specific comparison revealed however that the treatment with 10 µg/L BPA caused a significant decrease in the volume fraction of the mitochondria when compared to the controls ($P=0.043$, Mann-Whitney U test). A noticeable difference in this parameter was observed also between the β-oestradiol and DMSO treatment groups, this difference however was not significant statistically ($P=0.059$; Figure 3.55).

There was a statistically significant difference in the absolute volume of pooled mitochondria across the treatment groups ($P=0.007$, Kruskal-Wallis test). Both 10 µg/L BPA and 20 µg/L β-oestradiol caused a significant decrease in the volume of mitochondria when compared to the DMSO treated larvae ($P=0.021$ for both comparisons, Mann-Whitney U test). In addition, a significant difference ($P=0.021$) was revealed when this volume was compared between the 100 µg/L BPA and 20 µg/L β-oestradiol treated larvae (Figure 3.56, see also appendix B, Tables 8.14 and 8.15).

![Figure 3.55 Volume fraction of the pooled volume of mitochondria in the hepatocytes in 120 hpf zebrafish exposed to BPA. Data presented as boxplots (median and quartiles) with whiskers from minimum to maximum. The capital letters indicate a significant difference from: C - controls (DMSO). Asterisks specify statistical significance of the differences (*$P<0.05$, Mann-Whitney U test).](image-url)
Absolute volume of pooled mitochondria in hepatocytes of 120 hpf zebrafish exposed to BPA

Figure 3.56 Absolute volume of pooled mitochondria in the hepatocytes in 120 hpf zebrafish exposed to BPA. Data presented as boxplots (median and quartiles) with whiskers from minimum to maximum. The capital letters indicate a significant difference from: C - controls (DMSO), E – positive controls (β-oestradiol). Asterisks specify statistical significance of the differences (*P≤0.05, Mann-Whitney U test).

- Individual mitochondrial volume of hepatocytes

Volume fraction and absolute volume of individual mitochondria was analysed in order to investigate whether their alterations were linked with the observed changes in the pooled mitochondrial volume. Volume fraction of individual mitochondria was similar across the treatment groups (P=0.135, Kruskal-Wallis test). A specific comparison testing revealed that the volume fraction of individual mitochondria was significantly lower in the 10 µg/L BPA treated larvae compared to the controls (P=0.019). Noticeably smaller fraction comparing to the control group was observed in the β-oestradiol treatment group (Figure 3.57) but this difference was of no statistical significance (P=0.059).

A significant difference was found when the mean absolute volume of individual mitochondria in the zebrafish hepatocytes was compared across all the treatment groups (P=0.042, Kruskal-Wallis test). Pair-wise comparisons showed that both 10 µg/L BPA and 20 µg/L β-oestradiol caused a significant reduction in the absolute volume of individual mitochondria compared to controls (P=0.21, Mann-Whitney U test; Figure 3.58).
Volume fraction of individual mitochondria in hepatocytes of 120 hpf zebrafish exposed to BPA

![Graph showing volume fraction (Vv) of mitochondria with different treatments.]

Figure 3.57 Volume fraction of single mitochondrion in the hepatocytes in 120 hpf zebrafish exposed to BPA. Data presented as boxplots (median and quartiles) with whiskers from minimum to maximum.

Absolute volume of individual mitochondria in hepatocytes of 120 hpf zebrafish exposed to BPA

![Graph showing absolute volume of mitochondria with different treatments.]

Figure 3.58 Absolute volume of single mitochondrion in the hepatocytes in 120 hpf zebrafish exposed to BPA. Data presented as boxplots (median and quartiles) with whiskers from minimum to maximum. The capital letters indicate a significant difference from: C - controls (DMSO). Asterisks specify statistical significance of the differences (*P≤0.05, Mann-Whitney U test).
Volume of rough endoplasmic reticulum in hepatocytes

Both the volume fraction \( (P=0.415) \) and the mean absolute volume \( (P=0.068) \) of rough endoplasmic reticulum (RER) were similar across the treatment groups (Kruskal-Wallis test). Moreover no differences were found \( (p>0.05) \) when the RER volume fraction was compared between individual treatment groups and the controls or between the BPA-treated larvae and the β-oestradiol treatment group (Mann-Whitney U test) as presented in Figure 3.59.

Similarly, pair-wise comparisons of the RER absolute mean volume between the larvae treated with oestrogenic substances and the controls revealed no statistically significant differences, although there was a noticeably increased amount of RER in the 100 µg/L BPA treated larvae \( (P=0.083, \) Mann-Whitney U test). Moreover, the volume of RER in the 100 µg/L treatment group was significantly smaller \( (P=0.043) \) the analogous volume in the β-oestradiol treated larvae (Figure 3.60, see also appendix B, Tables 8.14 and 8.15).

![Volume fraction of RER in hepatocytes of 120 hpf zebrafish exposed to BPA](image)

Figure 3.59 Volume fraction of rough endoplasmic reticulum in the hepatocytes in 120 hpf zebrafish exposed to BPA. Data presented as boxplots (median and quartiles) with whiskers from minimum to maximum.
Absolute volume of RER in hepatocytes of 120 hpf zebrafish exposed to BPA

![Graph showing absolute volume of RER in hepatocytes]

Figure 3.60 Absolute volume of rough endoplasmic reticulum in the hepatocytes in 120 hpf zebrafish exposed to BPA. Data presented as boxplots (median and quartiles) with whiskers from minimum to maximum. The capital letters indicate a significant difference from: E – positive controls (β-oestradiol). Asterisks specify statistical significance of the differences (*P<0.05, Mann-Whitney U test).

- **Volume of glycogen**

There were significant differences found when the glycogen volume fraction and the absolute mean volume of glycogen were compared among the treatment groups (P=0.039 and 0.023 respectively, Kruskal-Wallis test). Pair-wise comparison testing revealed that both the 10 µg/L BPA (P=0.043) and 100 µg/L BPA (P=0.043) but not β-oestradiol treatment increased the volume fraction of glycogen (Mann-Whitney U test). Moreover, the glycogen volume fraction for the larvae treated with 10 µg/L BPA was significantly higher (P=0.043) than in the positive control group, i.e. the larvae exposed to 20 µg/L β-oestradiol (Figure 3.61).

The mean absolute volume of glycogen was significantly smaller in the hepatocytes of the larvae treated with 10 µg/L BPA (P=0.043) or 100 µg/L BPA (0.043) comparing to the controls (Mann-Whitney U test). Moreover, the volume of glycogen in the 10 µg/L BPA and the 100 µg/L BPA treated larvae was significantly smaller than in the positive control group (P=0.043 and P=0.021, respectively; Figure 3.62; see also appendix B, Tables 8.14 and 8.15).
Volume fraction of glycogen in hepatocytes of 120 hpf zebrafish exposed to BPA

![Graph showing volume fraction of glycogen](image)

Figure 3.61 Volume fraction of glycogen in the hepatocytes in 120 hpf zebrafish exposed to BPA. Data presented as boxplots (median and quartiles) with whiskers from minimum to maximum. The capital letters indicate a significant difference from: C - controls (DMSO), E - positive controls (β-oestradiol). Asterisks specify statistical significance of the differences (*P≤0.05, Mann-Whitney U test).

Absolute volume of glycogen in hepatocytes of 120 hpf zebrafish exposed to BPA

![Graph showing absolute volume of glycogen](image)

Figure 3.62 Absolute volume of glycogen in the hepatocytes in 120 hpf zebrafish exposed to BPA. Data presented as boxplots (median and quartiles) with whiskers from minimum to maximum. The capital letters indicate a significant difference from: C - controls (DMSO), E - positive controls (β-oestradiol). Asterisks specify statistical significance of the differences (*P≤0.05, Mann-Whitney U test).
- **Volume of bile canaliculi**

Both the volume fraction and the absolute mean volume of bile canaliculi were similar when compared across the treatment groups ($P=0.135$ and $P=0.146$, respectively, Kruskal-Wallis test). Specific comparison testing revealed that the volume fraction of bile canaliculi in the 10 µg/L BPA treated larvae was significantly lower than in the controls ($P=0.042$, Mann-Whitney U test; Figure 3.63).

No differences were found when the individual treatment groups were compared to the controls or when the BPA-treated larvae were compared to the larvae reared in 20 µg/L β-oestradiol (Mann-Whitney U test, Figure 3.64. See also appendix B, Tables 8.14 and 8.15). A noticeable difference, however, was observed between the controls and the 10 µg/L BPA treated larvae ($P=0.083$, Mann-Whitney U test).

![Volume fraction of bile canaliculi in hepatocytes of 120 hpf zebrafish exposed to BPA](image)

Figure 3.63 Volume fractions of bile canaliculi in the hepatocytes in 120 hpf zebrafish exposed to BPA. Data presented as boxplots (median and quartiles) with whiskers from minimum to maximum. The capital letters indicate a significant difference from: C - controls (DMSO). Asterisks specify statistical significance of the differences (*$P<0.05$, Mann-Whitney U test).
The effects of BPA on the liver ultrastructure of 120 hpf zebrafish – morphological assessment

The hepatocytes of the larvae reared in the solvent control (0.01% DMSO) were mostly oval or round and contained single nuclei containing one or two prominent nucleoli. The vast majority of the nuclei were oval with a relatively regular outline (Figure 3.65). Mitochondria in the hepatocytes presented remarkable diversity of shapes and sizes, small, round or oval as well as very big longitudinal, twisted or branched forms could be observed (Figure 3.65 and 3.66). RER was found mainly in the vicinity of the nuclei or the mitochondria (Figure 3.66 and 3.67) and in most cases formed stacks of three or more membrane-bound cisterns. The size of the bile canaliculi also varied and both small, filled with microvilli profiles (Figure 3.66) as well as large lumens only partially occupied by the hepatocytes’ projections (Figure 3.67) were observed. Associated with the biliary tracts were electron dense vesicles (Figure 3.67), which on account of lack of crystalline core were identified as lysosomes, as opposed to peroxisomes. The amount of glycogen could be described as small to moderate, and if present – the storage material was mainly localized towards the periphery of the cells. Myelin whorls were observed in the DMSO treated zebrafish (Figure 3.66) however their amount was moderate. Macrophages were occasionally encountered among hepatic parenchymal cells.
Figure 3.65 Overview of the liver in the 120 hpf zebrafish reared in solvent control (DMSO). N – nuclei of hepatocytes with single or two nucleoli; M – mitochondria; S – sinusoid, B – bile canaliculus, G – glycogen. Scale bar – 2 μm.

Figure 3.66 Liver of the 120 hpf zebrafish reared in solvent control (DMSO). N – nuclei of hepatocytes; M – mitochondria; → rough endoplasmic reticulum, B – bile canaliculus, mw – myelin whorls. Scale bar – 2 μm.
The liver of 120 hpf zebrafish exposed to 10 µg/L BPA in many aspects was similar to that observed in the larvae reared in DMSO. The shape of the hepatocytes was highly similar and the borders between neighbouring cells were clear. Nuclei were mostly euchromatic with one or two nucleoli and of oval shapes with rather smooth surface (Figure 3.68). There were no differences in the appearance of RER when compared to the controls. Most of RER membranes were arranged in stacks of more than two and were associated with the nuclei and mitochondria (Figure 3.68). Comparing to the liver of the DMSO treated larvae, there was clearly more glycogen in the hepatocytes of the larvae from this treatment group (Figure 3.68). In this case, the storage material was generally accumulated towards the periphery and in a few hepatocytes it was clearly the most dominant component. Another clear difference between control and 10 µg/L BPA treated larvae was the size of mitochondria. These organelles were visibly smaller in the hepatocytes of the larvae exposed to BPA (Figure 3.68 vs. 3.65). Moreover, the diversity of mitochondrial shapes typical for control larvae was reduced in the BPA treated larvae and the vast majority were oval or round. The sizes of the bile canaliculi in the larvae exposed to BPA were somewhat reduced and with higher density of microvilli in the observed lumens (Figure 3.69) when compared to the some analogous structures in the controls (Figure 3.67). Several macrophages were identified amidst parenchymal cells. These were recognized by their irregularly shaped nuclei of the sizes approximate to the hepatocytes’ nuclei and containing considerable amount of heterochromatin. Macrophages were often

![Figure 3.67 Liver of the 120 hpf zebrafish reared in solvent control (DMSO). N – nucleus of one of the hepatocytes; M – mitochondria; B - bile canaliculus and electron dense transporting vesicles in its vicinity (*). Scale bar –2 µm.](image-url)
localized in the areas close to the elements of the biliary tract (Figure 3.69) but their amount was comparable to that observed in the control group. Clear indicators of BPA toxicity based on the morphology of the hepatocytes were very rare and included presence of the mitochondria with partially lysed cristae and increased amount of unidentified vesicular profiles (Figure 3.70).

Figure 3.68 Liver of the 120 hpf zebrafish exposed to 10 µg/L BPA. N – nuclei of the hepatocytes, M – mitochondria; G – glycogen, → RER. Scale bar –2 µm.
Figure 3.69 Liver of the 120 hpf zebrafish exposed to 10 µg/L BPA. NM - nucleus of a macrophage in the vicinity of the biliary passages; M – mitochondria; B – bile canaliculi; G – glycogen; → RER. Scale bar –2 µm

Figure 3.70 Liver of the 120 hpf zebrafish exposed to 10 µg/L BPA. M – mitochondria with disturbed morphology and numerous vesicular profiles may be observed. G – glycogen; → RER. Scale bar –2 µm
The ultrastructure of the liver in the 100 µg/L BPA treated larvae showed conspicuous inter-individual variation. Although most of the hepatocytes’ nuclei seemed to have a typical appearance with prominent nucleoli and relatively little heterochromatin, the nuclei of one of the BPA treated larvae had noticeably increased amount of heterochromatin organized in patches (Figure 3.71). Moreover, there was a conspicuous inter-individual heterogeneity of the mitochondrial appearance in this treatment group. Both small and round, often clustered together mitochondria (Figure 3.72), as well as huge mitochondria of diameters larger than that of the nuclei, could be found (Figure 3.74). These giant forms demonstrated an evident reduction in the amount of intra-mitochondrial cristae which were limited to the mitochondrial periphery and did not reach to the centre of the organelles. Moreover, some mitochondria were almost entirely devoid of cristae (Figure 3.74). RER also showed intercellular variation regarding its amount and organization as some hepatocytes had clearly higher content of the RER membranes than others (Figure 3.72). In addition, in some liver cells RER was observed to have dilated and fractionated cisternae which more resembled smooth endoplasmic reticulum (Figure 3.73). Glycogen organized into clearly delineated fields (Figure 3.71 and 3.74) was often observed in the hepatocytes of the larvae from this treatment group. Bile canaliculi did not show any obvious differences in their appearance and the amount of intra-hepatocytic macrophages did not vary when the liver was compared between the 100 µg/L BPA treated larvae and the controls.

![Figure 3.71](image.png)
Figure 3.71 Liver of the 120 hpf zebrafish exposed to 100 µg/L BPA. N – hepatocytes’ nuclei, notice increased amount of heterochromatin in some of them; M – mitochondria; NM- nuclei of macrophages in the vicinity of a biliary passage; G – glycogen; S – sinusoid. Scale bar – 2 µm.
Figure 3.72 Liver of the 120 hpf zebrafish exposed to 100 µg/L BPA. N – hepatocytes’ nuclei; M – mitochondria; →RER. Notice increased amount of RER and less clear borders between the cells. Scale bar – 2 µm.

Figure 3.73 Liver of the 120 hpf zebrafish exposed to 100 µg/L BPA. M – mitochondria, some with disturbed morphology; →RER, notice dilated and fragmented cisterns; S – sinusoid. Scale bar – 2 µm.
Figure 3.74 Liver of the 120 hpf zebrafish exposed to 100 µg/L BPA. N – nuclei of hepatocytes; Nu – nucleolus; M – mitochondria, notice huge mitochondrion with cristae present only at its edges; → rough endoplasmic reticulum, G – glycogen. Scale bar –2 µm.
Shape analysis of the mitochondrial profiles in the hepatocytes of the 120 hpf zebrafish larvae exposed to BPA.

Because changes were observed in the volume of mitochondria in the BPA-treated group, it was decided to further investigate the mitochondrial morphology, by examining mitochondrial profile area, circularity, maximum feret diameter, minimum feret diameters and major to minor axis ratio.

- Area of the mitochondrial profiles

A statistically significant difference was revealed ($P=0.027$, Kruskal-Wallis test) when the areas of the mitochondrial profiles in the hepatocytes of zebrafish larvae were compared across the treatment groups (DMSO, 10 µg/L BPA, 100 µg/L BPA, 17β-oestradiol). Pair-wise comparison testing (Mann-Whitney U test) demonstrated that the areas of the mitochondrial profiles in the hepatocytes of the larvae treated with 10 µg/L BPA, 100 µg/L BPA or 20 µg/L β-oestradiol were significantly smaller than in the control larvae ($P=0.019, 0.042$ and $0.042$, respectively). In addition, a significant difference in the measured parameter was revealed between the 10 µg/L BPA and the 20 µg/L β-oestradiol treated larvae (Figure 3.75).

The comparison of the mitochondrial profiles’ areas distribution between the larvae treated with oestrogenic compounds and the controls (Chi-squared test) revealed significant differences for the 10 µg/L BPA and 100 µg/L BPA treatment groups. BPA-treated larvae had more mitochondrial profiles of smaller area and fewer larger profiles comparing to the controls (Figure 3.76).
Figure 3.75 Area of the mitochondrial profiles in the hepatocytes of the 120 hpf zebrafish larvae exposed to BPA. Each symbol represents the median of 86 ± 16 mitochondria measured per larva (n=4). The capital letters indicate a significant difference from: C - controls (DMSO), E – positive controls (β-oestradiol). Asterisks specify statistical significance of the differences (*P≤0.05, Mann-Whitney U test).

Figure 3.76 Distribution of the areas of the mitochondrial profiles in the hepatocytes of the 120 hpf zebrafish larvae exposed to BPA. Data presented as the mean (n=4) of the percentages of the mitochondrial profiles of a given area range. Asterisks indicate values significantly different from the controls (*P≤0.05, Chi-squared test).
• Circularity of the mitochondrial profiles

The circularity of the mitochondrial profiles in the hepatocytes of the 120 hpf zebrafish larvae was similar amidst all the treatment groups \((P=0.368, \text{Kruskal-Wallis test; Figure 3.77})\). Moreover, none of the treatments with oestrogenic compounds (BPA and \(\beta\)-oestradiol) altered the circularity of the mitochondrial profiles in a significant way when compared to the controls \((P>0.05, \text{Mann-Whitney U test})\). There was also no difference when mitochondrial profiles were compared between the larvae treated with BPA (both 10 \(\mu\)g/L BPA and 100 \(\mu\)g/LBPA) and the positive control group.

The distribution of the mitochondrial profiles’ circularity was significantly different for both the 100 \(\mu\)g/L BPA and the \(\beta\)-oestradiol treated larvae when compared to the controls \((P<0.05 \text{ and } P<0.005, \text{respectively; Chi-squared test})\). There was a higher percentage of the more circular mitochondrial profiles (the value approaching 1) in the hepatocytes of the larvae from the 100 \(\mu\)g/L BPA treatment group. In turn, there were more mitochondrial profiles deviating from the circular shape in the \(17\beta\)-oestradiol group comparing to the control larvae (Figure 3.78).
Figure 3.78 Distribution of the circularity of the mitochondrial profiles in the hepatocytes of the 120 hpf zebrafish larvae exposed to BPA. Data presented as the mean (n=4) of the percentages of the mitochondrial profiles of a given circularity range. Asterisks indicate values significantly different from the controls (*$P \leq 0.05$ and ***$P \leq 0.001$, Chi-squared test).

- **Maximum feret diameter of the mitochondrial profiles**

When the maximum feret diameters of the mitochondrial profiles in the hepatocytes of the zebrafish larvae were compared across the treatment groups no statistically significant difference was found ($P=0.131$, Kruskal-Wallis test). The subsequent specific comparison testing exhibited, however, that the maximum feret diameter was significantly smaller in the 10 µg/L BPA treated larvae when compared to the controls ($P=0.021$, Mann-Whitney U test, Figure 3.79). The observed parameter in both the 10 µg/L BPA and 100 µg/L BPA treatment groups was similar when compared to the larvae treated with β-oestradiol.

Both 10 µg/L BPA and 100 µg/L BPA affected the distribution of the mitochondrial profiles’ maximum feret diameters comparing to the controls ($P<0.005$ for both comparisons, Chi-squared test). In both cases there were significantly more mitochondrial profiles with smaller maximum feret diameters then in the control group (Figure 3.80).
Max caliper of mitochondrial profiles in the hepatocytes of the 120 hpf zebrafish exposed to BPA

Figure 3.79 Maximum feret diameters of the mitochondrial profiles in the hepatocytes of the 120 hpf zebrafish larvae exposed to BPA. Each symbol represents the median of 86 ± 16 mitochondria measured per larva (n=4). The capital letters indicate a significant difference from: C - controls (DMSO). Asterisks specify statistical significance of the differences (*P≤0.05, Mann-Whitney U test).

Figure 3.80 Distribution of the maximum feret diameters of the mitochondrial profiles in the hepatocytes of the 120 hpf zebrafish larvae exposed to BPA. Data presented as the mean percentage (n=4) of the mitochondrial profiles of a given length range. Asterisks indicate values significantly different from the controls (** P≤0.01, Chi-squared test).
Minimum feret diameter of the mitochondrial profiles

A statistically significant difference was exhibited when the minimum feret diameters of the mitochondrial profiles in the hepatocytes of the larvae were compared across the treatment groups ($P=0.028$, Kruskal-Wallis test). The specific comparison of the individual treatments with the control group (Mann-Whitney U test) revealed that the treatments 10 µg/L BPA, 100 µg/L BPA and 20 µg/L β-oestradiol affected influenced the shape of the mitochondrial profiles in a significant fashion ($P=0.020$, 0.021 and 0.020, respectively; Figure 3.81). Moreover, the minimum feret diameters of the mitochondrial profiles in the BPA treated larvae was similar comparing to the larvae exposed to 20 µg/L β-oestradiol.

The distribution of the minimum feret diameters in the larvae exposed to the oestrogenic compound (10 µg/L BPA, 100 µg/L BPA or 20 µg/L β-oestradiol) was significantly different then in the control larvae (Chi-squared test). In all cases there were more mitochondrial profiles with smaller minimum feret diameters than in the controls (Figure 3.82).

![Figure 3.81 Minimum feret diameters of the mitochondrial profiles in the hepatocytes of the 120 hpf zebrafish exposed to BPA. Each symbol represents the median of $86 \pm 16$ mitochondria measured per larva (n=4). The capital letters indicate a significant difference from: C - controls (DMSO). Asterisks specify statistical significance of the differences (*$P \leq 0.05$, Mann-Whitney U test).](image)
Major to minor axis ratio of the mitochondrial profiles

The major to minor axis ratio of the mitochondrial profiles observed in the zebrafish larvae’ hepatocytes was similar when compared across all the treatment groups \((P=0.136, \text{Kruskal-Wallis test})\). Moreover, a pair-wise comparison of the mitochondrial profiles (Mann-Whitney U test) between the larvae treated with BPA or β-oestradiol and the controls showed no significant differences (Figure 3.83). A significant difference was, however, revealed when the profiles of the mitochondria in the 100 µg/L BPA treated larvae were compared to the 20 µg/L β-oestradiol treatment group \((P=0.043, \text{Mann-Whitney U test})\).

When the distribution of the aspect ratio was analyzed a statistically significant difference \((P<0.005)\) was exposed between the larvae treated with 20 µg/L 17β-oestradiol and the controls (Figure 3.84).
Figure 3.83 Major to minor axis ratio of the mitochondrial profiles in the hepatocytes of the 120 hpf zebrafish larvae exposed to BPA. Each symbol represents the median of 86 ± 16 mitochondria measured per larva (n=4). The capital letters indicate a significant difference from: E = positive controls (β-oestradiol), Asterisks specify statistical significance of the differences (*P≤0.05, Mann-Whitney U test).

Figure 3.84 Distribution of the major to minor axis ratios of the mitochondrial profiles in the hepatocytes of the 120 hpf zebrafish larvae exposed to BPA. Data presented as the mean percentage (n=4) of the mitochondrial profiles of a given aspect ratio range. Asterisks indicate values significantly different from the controls (** P≤0.01, Chi-squared test).
The effects of BPA on the expression of vitellogenin 1 mRNA in 120 hpf zebrafish

In this study the estrogenicity of BPA was assessed based on its effect on the expression of vitellogenin 1 mRNA (vtg1) in the 120 hpf zebrafish larvae exposed to 10, 100 or 2000 μg/L BPA. The embryos were immersed in the tested solutions starting from 0.75-1 hpf (2-4 cell stage) and the exposure was terminated at 120 hpf when the processing of the larvae for PCR commenced.

There was a significant difference in the level of vtg1 when compared across the treatment groups, i.e. 0.01% DMSO, 10 μg/L BPA, 100 μg/L BPA, 2000 μg/L BPA and 20 μg/L β-oestradiol (P<0.001, ANOVA). A specific comparison testing revealed that neither 10 μg/L BPA nor 100 μg/L BPA significantly altered the vtg1 expression when compared to the controls (P>0.85 for both comparisons, Tukey’s test) as one can see in Figure 3.85. Only BPA at 2000 μg/L concentration affected the expression of vtg1 in a statistically significant fashion comparing to the controls (P=0.001, Tukey’s test). A significantly higher level of vtg1 than in controls was also observed for the 20 μg/L β-oestradiol treated larvae (P<0.001, Tukey’s test).

The comparison of the treatment groups with the positive control group showed that the level of vtg1 in the BPA treated larvae at all tested concentrations was significantly lower than in the β-oestradiol treated larvae (P <0.001 for all comparisons, Tukey’s test).

![Vitellogenin expression in 120 hpf zebrafish exposed to BPA](image)

Figure 3.85 Vitellogenin 1 mRNA (vtg1) expression fold changes in the 120 hpf zebrafish larvae exposed to BPA. Data presented as boxplots (median and quartiles) with whiskers from minimum to maximum. The capital letters indicate a significant difference from: C - controls (DMSO), E – positive controls (β-oestradiol). Asterisks specify statistical significance of the differences (*P≤0.05, Tukey’s test).
3.3 Discussion

This study aimed to investigate the influence of BPA on the early development of zebrafish. BPA is one of the most widely found endocrine disruptors in the environment (Maffini et al., 2006) and is known for its oestrogenic properties resulting from the ability to bind to ERs (vom Saal et al., 2007).

This study found that BPA caused concentration and time dependent toxicity to zebrafish embryos and larvae including morphologic defects, reduction in heart rate, delay in hatching and lethality. The alterations in the liver ultrastructure included changes in the volume of the hepatocytes, pooled mitochondria, individual mitochondria, RER and glycogen. The morphology of mitochondrial profiles was also significantly affected by BPA treatments. High concentration of BPA caused increased expression of vitellogenin 1 mRNA in 120 hpf zebrafish.

On the lethal and sublethal effects of BPA on the zebrafish development

The concentration- and time dependent toxicity of BPA observed in this study corroborates the previous reports by Duan and co-workers (2006; 2008). The LC50 for 24 hpf in the present study (14.78 mg/L BPA) is similar to the studies of Duan et al. (16.36 mg/L and 16.75 mg/L). In the study of Lam et al. (2011) the LC50 for 168 hpf zebrafish treated with BPA from 3 hpf was assessed as 8.6 mg/L which is similar to the LC50 for 120 hpf larvae in the current study. In addition, while in the present study all the larvae exposed to 16 mg/L BPA were dead on the day 4, in the study of Lam et al. above 10% of the larvae treated with 15 mg/L were still alive on the 7th day. Together these studies indicate that earlier exposure to BPA (0.75 – 1 hpf vs. 3 hpf) can increase the lethal effects.

The EC50 for hatching in the current study was similar to that found by Chow et al (2012) for 96 hpf larvae, but lower than that found by Duan and Zhu (2006) for 72 hpf larvae (5.65 mg/L, 5.25 and 13.00 mg/L respectively). These differences may be due to differences in fish strain (not stated by Duan and Zhu) or incubation temperature (26 vs. 28°C). The temperature can affect the toxicity of chemical compounds dissolved in water (degradation rate) and/or influence their metabolism by ectothermal fish (Scheil and Kohler, 2009).

The hatching of 72 hpf larvae was significantly reduced by BPA at concentrations ≥ 8 mg/L. This effect could be the result of the overall underdevelopment of the larvae. Other pathways, however, could have been affected by this EDC. The hatching process is the combination of biochemical and behavioural phenomena which involve the enzymes digesting the chorion as well as the active movements of the developing embryo which help to tear the chorion up (Fraysse et al., 2006). Since active movements of the embryo are dependent on the proper development of neuromuscular systems, BPA could affect hatching success by its potentially neurotoxic properties. In fact, lethargy of the embryos/larvae treated with BPA was noticed during the course of the present study and a study to scrutinize this phenomenon is planned.
for the near future. It is also possible that BPA may affect the hatching process by influencing the hatching enzyme, i.e. chorionase. Such a hypothesis has been proposed for cadmium – also an EDC, which was observed to affect the hatching rate in rainbow trout embryos (Lizardo-Daudt and Kennedy, 2008). Further studies are necessary to investigate whether there is any relationship between BPA and zebrafish chorionase activity.

The phenotypic analysis revealed that up to 120 hpf, neither 2 mg/L nor 4 mg/L BPA had obvious effect on the heart morphology but 8 mg/L BPA caused heart malformation in 60% of 120 hpf larvae. This contrasts with the study of Lam et al. (2011) who observed that 168 hpf larvae treated with 1.5 and 4.5 mg/L BPA had significantly higher frequency of heart oedema than control animals. Since Lam et al. did not describe younger larvae, it is possible that at 120 hpf heart oedema was not observed, particularly that the frequency of this malformation was ≤ 10% for the 1.5 mg/L BPA and 25 ± 10 % (mean ± SD) for the 4.5 mg/L BPA treatment groups. Together these studies indicate that the toxicity of BPA increases with time.

The frequency of cardiac oedema was both concentration and time dependent and it was observed at 24 hpf for the 16 mg/L BPA treated embryos and at 72 hpf in the larvae from the 8 mg/L BPA treatment group. Since both 8 mg/L BPA and 16 mg/L BPA caused decrease in the heart rate at 48 and 72 hpf, it may suggest that there was a correlation between the heart rate and heart oedema. Particularly that the 4 mg/L BPA treated larvae, which had heart rate similar to the control group, did not show the signs of the cardiac malformation. One of the possible explanations could be that heart oedema leads to the reduction of the heart rate. The alternative would be that reduced heart rate causes pooling of the blood in this organ leading to oedema. It has been previously reported that BPA can induce oxidative stress in zebrafish embryos following exposure from 4 to 168 hpf (Wu et al., 2011a). Since one of the effects of oxidative stress may be the depletion of ATP (Lee and Shacter, 1999) one might assume that BPA-related oxidative stress could affect the pathways responsible for providing energy to the heart and through this reduce its rate.

The morphological analysis of the embryos/larvae treated with BPA revealed that the skin pigmentation level of the developing zebrafish was also affected. It cannot be ruled out that BPA could affect the skin morphology by interfering with the pigmentation developmental pathways. It is possible, however, that this effect of BPA is related to its influence on heart function. Since reduced heart rate and oedema affect the rate of blood flow, the disturbances in the distribution of the necessary nutrients may occur. This, in turn, would lead to the malnutrition of the tissues, including the skin. This study showed also that BPA affects the size of the yolk sac and/or yolk extension in the exposed zebrafish embryos and larvae. Enlarged reservoirs of the yolk may indicate that, in fact, the catabolic processes are disturbed leading to the decrease in the amount of nutrients released and supplied to the tissues of the developing organism.

The observations of a reduced head size in the ≥ 8 mg/L BPA treated embryos/larvae may further confirm the underdevelopment resulting from the disturbances in the metabolism and
malnutrition of the developing tissues. It is also possible that BPA affected directly the brain tissue leading to changes in the development of this organ. The study by Lam et al. (2011) demonstrated that the exposure of zebrafish larvae to BPA led to suppressed neuron branching from the spinal cord and significantly affected the expression of sp4 – a transcription factor related to disorders in the CNS development and function in rodents (Zhou et al., 2007) and humans (Zhou et al., 2009).

On the liver development of zebrafish larvae between 72 hpf and 120 hpf

There have been several reports on the arrangement of hepatocytes in the zebrafish liver. Some authors claim that hepatocytes in zebrafish liver are arranged in tubules with sinusoids (Paris-Palacios et al., 2000) or biliary passages (Lorent et al., 2004) in their centres. Others report that the hepatic parenchyma is composed of cords consisting of bilayered hepatocytes between which preductule biliary cells are located (Yao et al., 2012) or describe zebrafish liver architecture simply as cord-like structures limited by sinusoids (Rodrigues and Fanta, 1998). These reports, however, are focused on older animals and not on embryonic or early larval stages of zebrafish. Several reports describe the liver of zebrafish at early development (Burkhardt-Holm et al., 1999; Oulmi and Braunbeck, 1996; Strmac and Braunbeck, 1999) but are devoted to the parenchymal ultrastructure of the liver and do not give any information on the histological arrangement of this organ. Consequently, the present study has contributed to filling this gap in the information.

No clear architectural pattern was observed in the liver of either 3 or 5 dpf zebrafish. Hepatocytes were seen to form a compact mass of cells interspersed with sinusoids without any clear organization and no arrangement of hepatocytes into tubules or cords could be observed. This seems to indicate that the organization of hepatocytes into tubules/cords occurs at later stages of liver development, i.e. after 120 hpf. This reasoning can be supported by the study on carp – another member of Cyprinidae family, which revealed that the formation of the hepatic tubules was first seen in the larvae at 10 dpf (Fishelson and Becker, 2001). Moreover, since different organizations of hepatocytes (cords, plates, tubules) were reported for various fish species, Fishelson and Becker (2001) reason that it may, in fact, represent different stages in the liver development. These scientists suggest also that a concomitant growth of blood vessels is the factor which establishes the blueprint for the evolving architecture of the liver. The paucity of blood vessels observed in the liver of 72 and 120 hpf larvae in this study could be therefore another marker of not fully completed development of this organ and a reason for the lack of clear pattern in the organization of the parenchyma. Even though the liver buds out of the gut wall as early as 24-28 hpf, is a well defined structure by 50 hpf (Field et al., 2003a; 2003b) and some authors describe it as “mature” for 96 hpf zebrafish (Passeri et al., 2009), our observations indicate that not all aspects of the liver maturity are present in zebrafish up to 120 hpf.
The analysis of the liver ultrastructure for 72 and 120 hpf zebrafish larvae revealed several important cytological alterations occurring within a 48 hour period. To the best of our knowledge, 4 and 6 day old larvae are the earliest stages of zebrafish development for which the fine liver structure has been reported (Oulmi and Braunbeck, 1996; Strmac and Braunbeck, 1999). These studies have constituted a vital point of reference for the current study.

The hepatocytes of 72 and 120 hpf zebrafish larvae were mostly polygonal in shape and contained single centrally located nuclei which is similar to reports of Braunbeck’s group for the 96 and 144 hpf fish. Mean hepatocyte volume more than doubled between 72 hpf and 120 hpf larvae from 217.79 ± 32.93 µm$^3$ at 72 hpf to 452.58 ± 70.30 µm$^3$ at 120 hpf. Since the mean volume of hepatocytes nuclei remained without a major change (52.73 ± 5.22 µm$^3$ at 72 hpf and 51.54 ± 1.17 µm$^3$ at 120 hpf) it can be concluded that the enlargement of the hepatocytes was due to the increase in the cytoplasm and intracellular organelles other than the nucleus. This reasoning is further confirmed by the fact that the volume fraction of the nuclei decreased from 24% to 11% between 72 and 120 hpf. These observations agree with results reported for the hepatocytes in the developing liver of the domestic carp (Fishelson and Becker, 2001). While this statement is true for the zebrafish liver between 72 and 120 hpf, it is only partially accurate when the observations from the liver structure in older animals are taken under consideration. Whereas there was no significant difference in nuclear volume between 3 and 5 day old larvae, a substantial increase in this volume was revealed between 10 and 31 day-old fish (Burkhardt-Holm et al., 1999). Moreover, the comparison of the nuclear volume between 5 dpf zebrafish in the current study (51.54 ± 1.17 µm$^3$) with that reported for 10 day old fish by Burkhardt-Holm et al. (2233 µm$^3$) demonstrates clear enlargement of this organelle with time.

While the enlargement of the hepatocytes reported for the liver of carp was assigned mainly to the increase in the volume of glycogen (Fishelson and Becker, 2001) this was not the reason for the change in the volume of hepatocytes observed in the current study. Firstly, no quantification of glycogen was carried out for the hepatocytes of 72 hpf larvae due to the difficulties related to correct identification of its granules in the cytoplasm. Secondly, glycogen was the most inconsistent component, as to its amount and localization in the cell, of the hepatocytes and no clear pattern could be observed for the control animals in either 72 or 120 hpf animals. As a consequence, no distinguishable difference was detected in the amount of this storage material between 3 and 5 dpf zebrafish.

The difficulties related to the identification of glycogen granules in the cytoplasm of 5 and, even more, 3 dpf zebrafish were mainly caused by the high cytoplasmic density, which obscured clarity of the intracellular structures, including glycogen. Dense cytoplasm was mentioned by Fishelson and Becker in the already mentioned report on the cytological changes in the carp liver occurring with time of development and aging. These authors reported an age dependent and clear reduction in the density of the hepatocytes cytoplasm based on the comparison between 16 dpf fish to younger animals. Furthermore, Spornitz (1978) who studied the ultrastructure of the developing liver in *Xenopus laevis*, found it to be extremely difficult to distinguish between free ribosomes and smaller glycogen particles when analysing the organ.
before hatching of the embryos. Spornitz (1978) used a differential glycogen/RNA stain to differentiate between glycogen and ribosomes and revealed that more than half of the cytoplasmic particles contained RNA. Thus, it cannot be ruled out that the density of the cytoplasm in the hepatocytes of developing carp (Fishelson and Becker, 2001) and of zebrafish (the present study) may be related to the content of free ribosomes.

In addition to an increased electron density in the cytoplasm, disruption of normal organelle organisation was evident in both embryonic and larval zebrafish; this disorder affected the recognition of intracellular structures, including glycogen. Moreover, neither 3 nor 5 dpf animals demonstrated separation of the intracellular content into characteristic peri-nuclear, organelle containing regions of cytoplasm and enlarged areas of peripheral glycogen deposits. Such organization has been repeatedly reported as characteristic for the hepatocytes in older zebrafish (Braunbeck, 1998; Burkhardt-Holm et al., 1999). Lack of intracellular compartmentation in 3 and 5 dpf zebrafish larvae is in accordance with the report of Strmac and Braunbeck (1999) who first observed it in the hepatocytes of 8 and 10 day old but not younger larvae. This further confirms that the liver of 120 hpf zebrafish is not yet a fully developed organ.

In spite of reduced intracellular order, Braunbeck and co-workers (1996; 1999) regularly observed small glycogen fields in the periphery of hepatocytes of 4 and 6 dpf zebrafish. This clearly contrasts with the results in the present study for 3 dpf zebrafish where the amount of glycogen, its localization and appearance varied considerably between the cells. Both clear glycogen fields as well as granules hardly distinguishable from other cytoplasmic content were often observed in neighbouring cells. The situation clearly progressed over the next two days since it was much easier to recognize glycogen in the hepatocytes of 120 hpf zebrafish. However, even at this stage the cells lacking glycogen were seen amidst those clearly showing its presence in the form of smaller or larger fields found rather randomly throughout the cells, including their peripheries. Therefore, the present results do not reflect this reported regularity in the pattern of intracellular glycogen deposits.

The fact that glycogen was more easily recognized at 120 hpf than at 72 hpf may result from the increase of this storage material over time. Time-dependent increase of glycogen in the cell periphery was observed for zebrafish from 8- and 10- day old larvae (Strmac and Braunbeck, 1999). Several factors, in turn, might have caused the differences in the glycogen amount between the larvae used for this work and those described in the referred studies. The development of any organ is orchestrated by the synergic action of time, physiology and ambient environmental conditions (Fishelson and Becker, 2001), that is why differences in any of these factors could have resulted in the observed variation in the amount of glycogen. The observed differences may be simply related to the early age of the observed animals. The demarcation of the border between normal and abnormal phenotype is made particularly difficult in the case of early development when all the intracellular structures are constantly changing to adapt to altering physiology. Moreover, even small differences in the pace of organogenesis may result in considerable diversity of the observed phenotypes. There are
many factors which could potentially affect the rate of zebrafish development. Temperature, for example, is of great importance since it has been shown that the differences of 2-3°C may produce up to a 25-35% difference in the rate of organogenesis in exothermic species (Fishelson, 1996). Consequently, to estimate what is “typical” or “normal” when dealing with the structures in their early development is particularly difficult, especially when dealing with structures influenced by a multitude of internal and external parameters, to which hepatic storage products, including glycogen, belong (Braunbeck, 1998).

One of the intracellular organelles which clearly increased its volume during the observed period is mitochondria. The stereological analysis revealed that the volume occupied by mitochondria increased more than three times starting from 24.01 ± 5.22 µm³ at 72 hpf and taking up as much as 89.26 ± 21.33 µm³ at 120 hpf. This increase not only kept up with the enlargement of the hepatocytes, which would be true if the mitochondria: cell fraction did not change, but it exceeded the growth of the cells since mitochondrial volume increased from 11% of cell volume in 72 hpf zebrafish to 20% at 120 hpf. This could be interpreted as an indicator of the increased energetic needs of the hepatocytes in 120 hpf zebrafish when compared to younger, 72 hpf larvae. Mitochondria in the hepatocytes of 3 dpf zebrafish were mostly spherical or ovoid and this agrees with the description of shape of mitochondria in 4 dpf and 6 dpf zebrafish by Strmac and Braunbeck (1999) and Oulmi and Braunbeck (1996). Much larger mitochondria and a greater variability of profiles were observed in the hepatocytes of 120 hpf larvae. These profiles varied from spherical to elongated but twisted and branched forms were also commonly seen. In addition, the mitochondria encircling small profiles of cytoplasm were frequently encountered which seems to indicate active movement of these organelles throughout the cytoplasm or undergoing processes of fission and fusion. Although mitochondria of similar morphology have not been reported for zebrafish larvae, Braunbeck et al. (1990) stated that “a typical feature of zebrafish liver is the occurrence of round “giant” mitochondria reaching 8 µm in length and 1.5 µm in width”. This observation, although somehow contradictive, indicates that it was not unusual for the authors to observe really large and probably longitudinal mitochondria in the zebrafish liver. Increased irregularity in liver mitochondrial shape was also reported with increasing age of zebrafish from 10, 31 and 45-day old larvae (Burkhardt-Holm et al., 1999). In addition, the study on ultrastructural changes of the developing liver in Xenopus laevis revealed that the morphology of the mitochondria changes with the time of development (Spornitz, 1978). While small and spheroid mitochondria were observed in the Xenopus hepatocytes before hatching, considerably larger forms and of irregular outlines were viewed after hatching. All these reported observations together with the results of the current study indicate that the changes in liver ultrastructure between 72 hpf and 120 hpf is an age-related increase in the size and morphological diversity of these organelles.

The volume of rough endoplasmic reticulum (RER) also increased with time of development in the hepatocytes of zebrafish larvae. While in the 72 hpf larvae this organelle occupied 13.83 ± 5.04 µm³, two days later this volume was equal to 24.07 ± 3.59 µm³. This enlargement in the
absolute volume was, however, not accompanied by the increase in the proportional volume of this structure which at 72 hpf amounted to 6.4% while at 120 hpf it was equal to 5%. This decrease in the volume fraction of RER is consistent with previous observations by Strmac and Braunbeck (1999) who reported a slight reduction in volume of the RER in 6-day-old-larvae were compared to that of 4-day-old larvae. This is likely due to the cell growth rate exceeding the growth rate of RER. The cisternae of RER were mostly associated with mitochondria and nucleus and could also be found aggregated to small stacks in the periphery of hepatocytes which is in agreement with the previous reports.

Similarly to RER, the absolute volume of the bile canaliculi increased while the proportional volume decreased between 3rd and 5th day of zebrafish development. These differences, however, were much less significant since the absolute volume of the bile canaliculi increased from $3.77 \pm 1.11$ to $4.30 \pm 1.57 \mu m^3$ while its volume fraction decreased from 1.7% to 0.95% from 3 dfp to 5 dpf. As in the case of RER, decreasing volume fractions result probably from the significantly greater growth rate of hepatic cells when compared to the growth/development rate of bile canaliculi.

On the effects of BPA on the liver ultrastructure of zebrafish larvae

Because of its oestrogenic properties, BPA is believed to particularly affect those organs which express ERs (Asahi et al., 2010). The majority of studies on this compound have, however, been dedicated to reproductive organs, the mammary gland and the nervous system. Nevertheless, other organs are known to express ER, such as the liver, kidney, thyroid, skin, bladder, lungs, cartilage, bone and gastrointestinal tract (Pelletier, 2000) and these have received considerably less attention. Little has been done, for example, regarding histopathological effects of BPA in the liver and this study is the first to investigate the possible ultrastructural alterations of this organ with the use of an in vivo model. In addition, the present study focused on the possible adverse effects of BPA on the liver at early developmental stages since it has been reported that it is during early life that living organisms are the most vulnerable to risks posed by EDCs. Moreover, there is evidence that disturbances in the development of a given organ can increase its susceptibility of disease later in life. Taking into consideration that the liver is a vital organ responsible for multiple indispensable functions, its early developmental disturbances may lead to consequences affecting the quality of life. The present study aimed to investigate whether early exposure to BPA may affect the development of this vital organ based on the observations of its fine structure. The use of stereological tools allowed the objective assessment of the observed phenotypes.

Treatment with BPA tended to decrease the volume of hepatocytes at 72 hpf, with the reduction being statistically significant for the 10 µg/L concentration of BPA. As already discussed, the size of the hepatocytes was increasing with the time of development for normal larvae when compared between 72- and 120- hpf. The reduction in cell size could therefore
reflect the underdevelopment of the liver following BPA treatment. A link between the nutrition state and the rate of development is well understood. In addition, a clear correlation between the volume of hepatocytes and food intake (two-fold reduction following fasting and a return to 83% of the control values when re-fed) was observed in rats by Uhal and Roehrig (1982). Based on the observations of enlarged yolk sacs or/and yolk extensions in the BPA treated larvae one might surmise that there was a decrease in the release of nutrients which could potentially lead to their reduced supply to the developing organs. That release of the yolk materials could perhaps be reduced due to disturbances in the blood vessel development. A significantly suppressed vascularisation in the abdominal region was reported by Lam et al. (2011) for transgenic zebrafish larvae treated with 500 and 5000 μg/L BPA.

The possibility that reduction in the volume of hepatocyte was a reflection of the delay caused by BPA in the rate of zebrafish development is further validated by the nucleus to cytoplasm ratio analysis. Comparing to mature hepatocytes, hepatoblasts have a large nuclear to cytoplasmic ratio which decreases with time of development due to an increase in the number of intracellular organelles (Luzzatto, 1981). The results of the present study correspond to a decreasing nuclear: cytoplasmic ratio in the hepatocytes of control larvae occurring between 72 and 120 hpf. An increase in the nuclear volume fraction was observed for the 10 μg/L BPA and, to a lesser degree, 500 μg/L BPA treated larvae at 72 hpf. These alterations perhaps suggest a delayed/slower development of hepatocytes in the BPA treated larvae.

It cannot be ruled out that other, more direct effects of BPA underlie reduction in the volume of hepatocytes as observed at 72 hpf. Decrease in cell size was observed in the hepatocytes of zebrafish exposed to low doses of DDT, another EDC, by Weis (1974). Rapid glycogenolysis observed in the cells of the DDT treated fish could perhaps be the reason behind the cell size reduction reported by Weis. Particularly that the reduction in the glycogen content was one of the main reasons (besides lipid depletion) of the decline in the hepatocytes volume observed in the carp exposed to endosulfan – an endocrine disrupting pesticide (Braunbeck and Appelbaum, 1999). Since, as will be discussed later, depletion in glycogen was not observed in the present study for BPA treated larvae, glycogenolysis was not the reason of the observed decrease in the hepatocytes volume.

BPA was also observed to affect mitochondria in the hepatocytes of both 72 and 120 hpf zebrafish larvae. These effects are particularly interesting because the assessment of mitochondrial morphology in fish liver has been recognized as having a diagnostic value with respect to attempts to identify the effects of specific compounds (Braunbeck, 1998). The most common alteration observed in this study was reduction in the pooled mitochondrial volume. Interestingly, 10 μg/L, which is an environmentally relevant concentration, caused a significant reduction in the mitochondrial absolute volume both at 72 and 120 hpf. The mitochondria in the hepatocytes of the 100 μg/L BPA treated 120 hpf larvae showed the highest variability of sizes including giant forms with diameters exceeding those of the nuclei. Significant effects of BPA on mitochondria were further exhibited when their shape was analysed. The predominating round and oval mitochondrial profiles indicated a spheroid shape of these
organelles, which was further confirmed by reduction in the minimum and maximum feret diameters and/or decreased major to minor axis ratios. In addition, some treatments were observed to cause significant reduction in the area of mitochondrial profiles indicating their smaller sizes. Based on these observations we can conclude that BPA caused proliferation of small and more spherical mitochondria when compared to controls. These mitochondrial alterations are of crucial importance taking into consideration how important these organelles are for the cells. Such changes indicate that BPA may affect the energy metabolism of the hepatocytes at these early developmental stages in zebrafish. Moreover, mitochondrial depletion may indicate their damage which in turn might play a role in the induction of apoptosis (Asahi et al., 2010). It is well established that mitochondria play crucial role in the production of reactive oxygen species (ROS). In aerobic organisms there is a state of equilibrium between production of ROS and their destruction by antioxidant defence mechanisms (Wu et al., 2011a). In pathological processes, however, excess ROS can overcome antioxidant defences leading to oxidative stress. It has been recently demonstrated by Hassan et al. (2012) that BPA induces hepatotoxicity through oxidative stress. In their study, significantly decreased levels of ROS scavengers as well as increased level of by-products of lipid peroxidation (TBARS) and increased nitric oxide production were revealed in the livers of rats exposed to BPA at 50 and, to a lesser extent, at 10 mg/kg body weight/day. In addition, the capability of BPA to generate oxidative stress in developing zebrafish has also been revealed. Wu et al. (2011a) identified the oxidative stress indices and decreased measurements of antioxidant parameters in 168 hpf larvae exposed to BPA at concentrations comparable to those used in the present study. Since Wu et al. performed their study on whole-body homogenates we can only assume that one of the organs affected by the oxidative stress could be the liver. If that is the case, then the change in the morphology of mitochondria in the hepatocytes may reflect the oxidative stress related processes. This reasoning may be further confirmed by the study of Yu et al. (2008) who revealed that mitochondrial fragmentation was an upstream factor for ROS overproduction for the cardiovascular system cells incubated in high glucose. This fragmentation was revealed based on the analysis of the mitochondrial morphology which showed increase in smaller and shorter forms.

It is well established that the mitochondrial architecture oscillates between longitudinal tubules and small round vesicles resulting in a dynamic organizational equilibrium (Benard et al., 2009). This equilibrium is accomplished through the processes of opposing nature, namely fusion and fission. Moreover, these processes are tightly regulated and under physiological conditions mitochondrial fission is evenly counterbalanced by their fusion (Benard et al., 2009). It is known, for example, that mitochondrial fission, i.e. breaking into smaller pieces, is observed early in programmed cell death (Hales, 2010; Youle and Karbowski, 2005) . The presence of small, round mitochondrial profiles in the hepatocytes of the BPA treated larvae as opposed to larger, more longitudinal and irregular forms observed in the controls, may, therefore, indicate that the fusion – fission equilibrium has been disturbed. In addition, it has been reported that cells lacking mitofusins, i.e. GTPases essential for mitochondrial fusion, grow very slowly (Chen et al., 2005). A slower development of the BPA treated larvae could
also be proposed as a reason for the observed mitochondrial profiles, especially that it was already suggested for other ultrastructural alterations. Based on the observations of Chen et al., one might imply that this potential delay in the liver development is linked to the disturbances in mitochondrial fusion and fission caused by BPA.

Distinctive effects were observed in the 120 hpf larvae treated with 100 µg/L BPA. In this treatment group a trend towards increased, as opposed to decreased volume of hepatocytes was observed. Furthermore, this treatment resulted in a substantial increase in the absolute volume of RER. Lack of change in the proportional volume of RER suggests that the increase in the cell volume was directly related and proportional to the increase in the volume of this organelle. Increase in the amount of RER was observed in the hepatocytes of carp exposed to endosulfan (Braunbeck and Appelbaum, 1999). Both smooth endoplasmic reticulum (SER) and RER are involved in fish hepatic biotransformation processes (Lester et al., 1993) and consequently the increase in their amounts may be interpreted as an adaptive response to toxicity. An increase in the amount of RER and SER was, for example, seen in the hepatocytes of carp in response to the treatment with endosulfan (Braunbeck and Appelbaum, 1999). In addition, the amount of RER is probably an indicator of higher metabolic activity and stimulation of hepatic protein synthesis and could provide the basis for the proliferation of the SER (Braunbeck and Appelbaum, 1999). Furthermore, RER proliferation has been suggested by Braunbeck and Appelbaum (1999) to be an unspecific, adaptive response of the fish liver to toxicity. One might therefore surmise that the alterations observed in the hepatocytes of the larvae treated with 100 µg/L BPA indicate that defence mechanisms have been triggered. In the current study SER was not observed in the hepatocytes of control zebrafish larvae or as a result of BPA exposure. The former is in accordance with previous reports on zebrafish larvae (Burkhardt-Holm et al., 1999). No proliferation of SER was, for example, observed in the hepatocytes of mature zebrafish treated with DDT, although it was expected based on the observations from mammalian studies (Weis, 1974).

Another effect of the 100 µg/L BPA was dilation and, what appeared to be, a fragmentation of RER membranes in some hepatocytes from this treatment group. Similar alterations were observed by Braunbeck et al. (1990) in the liver of mature zebrafish exposed to 4-chloroaniline and were interpreted as a general morphological evidence of the induction of biotransformation processes.

A significant reduction of the RER volume was, in turn, observed in the 10 µg/L BPA treated larvae at 72 hpf. An overall reduction of RER accompanied by fenestration and fragmentation of cisternae was observed in the hepatocytes of 4 and 6 dpf (Strmac and Braunbeck, 1999) and 10 and 31 dpf (Burkhardt-Holm et al., 1999) zebrafish larvae exposed to the fungicide triphenyltin acetate. Taking into consideration that RER is the site of the synthesis, folding and modifications of proteins, such changes might indicate disturbances in these pathways.

BPA treatment affected also the amount of glycogen in the hepatocytes of 120 hpf zebrafish larvae. Both the volume fraction and the absolute volume of this storage product were
increased comparing to the control group. In the light of great diversity in the amount and localization of this storage product, even among the control larvae, it is difficult to assess whether this observation was a BPA related phenomenon. Particularly that glycogen depletion, and not its increase, was reported as a non-specific reaction to xenobiotics (Braunbeck, 1998).

The contribution of BPA in the observed enlargement of the hepatocytes glycogen stores cannot, however, be ruled out in the light of the recent studies which show that this compound may affect the glucose homeostasis. The study of Alonso-Magdalena et al. (2008) has revealed that the content of insulin, the main hormone involved in the regulation of blood glucose levels, was up-regulated by environmentally relevant doses of this EDC. Moreover, BPA, similarly to 17β-oestradiol, increased the concentration of insulin in a non-monotonic manner, with significant effect observed at 1 and 10 nM and no effect for 100 and 1000 nM. Non-monotonic behaviour of BPA has been confirmed in vivo: daily exposure of mice to 100 μg/kg but not to 100 mg/kg caused a statistically significant increase in the content of insulin. One of the important effects of insulin on intracellular metabolism is its ability to stimulate the synthesis of glycogen in liver (Cohen et al., 1978). Moreover, it was reported that excess glycogen accumulation in the liver is seen in the 80% of diabetic persons (Stone and Van Thiel, 1985). Such observations led us to surmise that the glycogen accumulation observed in the zebrafish hepatocytes is related to the insulin level alterations in the BPA exposed larvae. Furthermore, it has been demonstrated that BPA affects glucagon-releasing α-cells mimicking the silencing effect produced by 17β-oestradiol and through this influences their ability to properly respond to hypoglycemia (Ropero et al., 2008). Observations of this type have led to the assumption that BPA may contribute to metabolic disorders relevant to glucose homeostasis such as diabetes mellitus type II (Batista et al., 2012).

The interference of BPA with the enzymes responsible for gluconeogenesis or glycogenesis also seems possible. Accumulation of glycogen in the hepatocytes of adult zebrafish was observed following a treatment with mercury and correlated with the up-regulation of gluconeogenesis as assessed based on transcriptase analysis (Ung et al., 2010). Real time PCR further revealed a concentration-dependent up-regulation of glycogen phosphorylase and glycogen synthase 3 in the mercury-treated fish.

BPA treatment has also caused a reduction in the volume of bile canaliculi in 120 hpf zebrafish larvae when compared to the controls. This observation may again indicate slower or “delayed” development of the liver in the BPA treated larvae. Such reasoning is supported by the observation of the time-dependent increase in the absolute volume of bile canaliculi as assessed based on the comparison between normal larvae at 72 and 120 hpf.
On the effects of BPA on the vitellogenin 1 mRNA expression in 120 hpf zebrafish larvae

The oestrogenic effects of BPA were studied based on the measurement of the vitellogenin 1 mRNA \( (vtg1) \). Vitellogenin has been extensively used as a biomarker of exposure to oestrogenic compounds in aquatic vertebrates, including fish (Sumpter and Jobling, 1995).

The larvae treated with 20 μg/L 17β-oestradiol constituted the positive control for the oestrogenic exposure in the present study. Taking into account that vitellogenesis is an ER-dependent process, the potential of this natural oestrogen to significantly affect the level of vitellogenin is understandable and has been repeatedly confirmed. The study of Ortiz-Zarragoitia and Cajaraville (2005) revealed that adult male zebrafish exposed to 10 μg/L of 17β-oestradiol had 609- and 1350-fold higher concentration of the vitellogenin protein (Vtg) in comparison to the control fish when exposed for 7 and 15 days, respectively. Concentration-dependent up-regulation of the vitellogenin 1 mRNA \( (vtg1) \) following treatment with β-oestradiol was observed by Muncke and Eggen (2006). In their study a statistically significant up-regulation of \( vtg1 \) was observed in 120 hpf larvae treated with 2.7 μg/L and 27 μg/L β-oestradiol. Based on this report, the concentration of 20 μg/L has been chosen for a positive control group in the present study. While 27 μg/L β-oestradiol caused 2772.62 ± 375.26 fold induction of \( vtg1 \) (mean ± SEM) in the study of Muncke and Eggen, the present study showed that β-oestradiol at 20 μg/L increased the level of \( vtg1 \) 1037.55 ± 301.77 fold (mean ± SEM) in respect to controls. The use of different concentrations of the natural hormone, possibly different time at which the exposure to the compound commenced (0.75-1 hpf vs. “freshly fertilized”) as well as biological variation of the tested larvae could lead to the observed discrepancies in the expression levels.

In the present study BPA at 10 or 100 μg/L (43.9 and 439 nM, respectively) did not affect \( vtg1 \) expression in a significant way when compared to the controls. These observations are in accordance with previous studies in which zebrafish embryos exposed to 1140 μg/L BPA showed no statistically significant change in the \( vtg1 \) at 120 hpf (Muncke and Eggen, 2006). In addition, no induction of vitellogenin was observed by the authors for the 0.5-2 μM concentrations of BPA. The present study demonstrates that BPA at concentrations below those reported previously does not alter the expression level of \( vtg1 \) in a statistically significant manner. These observations are important because BPA is a hormonally active compound and thus may cause non-monotonic dose responses. Since linear extrapolation is not possible for the compounds able to cause non-monotonic responses (Welshons et al., 2003), data obtained with higher concentrations of such compounds should not be used to predict their effects at low doses. Consequently, the studies of EDCs, including BPA, ought not to be limited only to the high concentrations and should include those below no observed effect concentrations (NOEC).

Only the highest concentration of BPA tested in the present study (2000 μg/L) up-regulated \( vtg1 \) in a significant way in respect to controls. This concentration far exceeds environmentally relevant doses but it was included because of the previously reported, clear oestrogenic effect
of BPA at a similar concentration, i.e. 2280 μg/L (Muncke and Eggen, 2006). While in the present study BPA at 2000 μg/L caused a 24.33 ± 14.27 fold increase (mean ± SEM) in the expression level of vtg1, the larvae treated by Muncke and Eggen showed 50.87 ± 16.55 fold up-regulation. A considerably higher expression level observed in their study is probably related to the higher concentration of BPA. It is also possible that the physiological differences between zebrafish strains have contributed to the difference in vtg1 expression levels (AB vs. WIK, pet shop fish and WT mix). In addition, the difference in the exposure time may also have played its role (0.75-1 hpf vs. “freshly fertilized eggs”) since one might surmise that a shorter exposure would lead to the lower expression of the measured biomarker. This reasoning is based on the previous reports. Van den Belt et al. (2003), for example, observed a significant increase in plasma vitellogenin (Vtg) level in adult male zebrafish exposed for three weeks to 1000 μg/L BPA (4.4 μM). Furthermore, 375 μg/L BPA was observed to significantly up-regulate Vtg in both male and female zebrafish when the exposure lasted from fertilization to adult stage (Segner et al., 2003a). Because these results concern the protein levels further studies are necessary to investigate the relationship between the length of exposure and vtg1 expression in zebrafish larvae.
4 The Influence of Genistein on the Early Development of Zebrafish

4.1 Aim of study

The aim of this study was to investigate the effects of genistein on the early development of zebrafish. Lethal and sublethal effects of genistein were studied for zebrafish at 24, 48, 72, 96 and 120 hpf. In addition the effects of genistein on the liver ultrastructure of 120 hpf zebrafish larvae were investigated. To assess the oestrogenic potential of genistein at concentrations below NOEC the study of the vitellogenin 1 mRNA expression in 120 hpf zebrafish larvae was carried out.

4.2 Results

4.2.1 Embryotoxic and teratogenic effects of genistein on the early development of zebrafish

Zebrafish embryos/larvae were exposed to genistein at 1.25, 2.5, 5 and 10 mg/L, starting from 0.75-1 hpf and until 120 hpf. Embryos and larvae were examined for survival and morphological defects at 24, 48, 72, 96 and 120 hpf. Moreover, the heart rate of the genistein exposed embryos (48 hpf) and larva (72 hpf) as well as the hatching successes of 72 hpf larvae were investigated. LC50 and EC50 for heart rate and hatching success were assessed at the time points of observation.

The effects of genistein exposure on zebrafish survival

Genistein exposure caused a concentration and time-dependent increase in mortality of zebrafish embryos and larvae (one-way ANOVA followed by Tukey’s post-hoc test). 10 mg/L constituted a lethal concentration for 100% embryos at 24 hpf. Genistein at 5 mg/L caused a significant increase in zebrafish embryos mortality both at 24 and 48 hpf when compared to the control group. At 72 hpf all the embryos treated with this concentration of genistein were dead. No significant difference was observed in the survival of zebrafish embryos treated with 2.5 mg/L genistein up to 72 hpf when compared to the controls. At 96 hpf, however, this concentration of genistein caused a major decrease in the survival of the exposed larvae and at 120 hpf over 90% of the exposed population was dead (Figure 4.1, see also appendix B, Table 8.6).
The highest concentration of genistein that did not affect zebrafish survival throughout the length of the experiment was 1.25 mg/L. This concentration of genistein was therefore estimated to be the NOEC for the survival of zebrafish up to 120 hpf. The LC50s decreased with each subsequent day of exposure indicating a time-dependent toxicity of genistein (appendix B, Table 8.7). Whereas LC50 calculated for 24 hpf was 4.99 mg/L genistein, at 120 hpf it was almost 3 times lower (1.76 mg/L).

Survival of zebrafish embryos exposed to genistein

![Graph showing survival of zebrafish embryos exposed to genistein](image)

Figure 4.1 Survival of the 24, 48, 72, 96 and 120 hpf zebrafish exposed to 1.25, 2.5, 5 and 10 mg/L genistein. Graph plotted as means with standard deviation error bars. Asterisks indicate values significantly different from the controls (*** P≤0.001, Tukey’s test).

The effects of genistein exposure on zebrafish morphology

There was a concentration- and time-dependent increase in severity of morphological defects following genistein treatment. There were no apparent changes in the morphology of 24 hpf embryos treated with 1.25 mg/L genistein (Figure 4.2 B) and this group received the mean total morphological score equal to the controls (Figure 4.3). Embryos treated with 2.5 mg/L (Figure 4.2 C) had mildly reduced heads and somewhat larger yolk sacs. In addition, 6.6% embryos from this treatment group had heart oedema. Treatment with 5 mg/L genistein caused severe malformations in the exposed embryos which involved heart oedema, yolk oedema and altered morphology of tail (Figure 4.2 D). Moreover, head size was clearly reduced and the brain tissue appeared dark in all embryos from this treatment group indicating neurotoxic effect of genistein at this concentration. As the heart was seen to contract these embryos were not classified as dead. Treatment with 10 mg/L genistein resulted in even more advanced malformations of exposed embryos (brain/head necrosis, heart and yolk oedema, tail...
malformations) as seen in Figure 4.2 E. Since these embryos did not have beating hearts they were classified as dead and obtained the lowest mean total morphological score (Figure 4.3).

![Figure 4.2 Morphology of the 24 hpf zebrafish exposed to genistein. A – control (DMSO); B – 1.25 mg/L genistein; C – 2.5 mg/L genistein; D – 5 mg/L genistein; E – 10 mg/L genistein. Arrows indicate malformations in the head, heart and tail regions.]

![Figure 4.3 Mean total morphological scores of the genistein treated embryos at 24 hpf. The percentage of the embryos scored for the 1.25 and 2.5 mg/L was ≥10% and for the remaining treatment groups ≥20%.]

Genistein caused concentration dependent toxicity in zebrafish embryos when assessed at 48 hpf. The effects (Figure 4.5) were more severe than at 24 hpf (Figure 4.3) indicating a time dependent toxicity of genistein. While embryos treated with 1.25 mg/L genistein did not show any obvious changes in their morphology (Figure 4.4 B) when compared to controls (Figure 4.4...
A), 36.7% of the embryos treated with 2.5 mg/L genistein showed heart oedema (Figure 4.4 C). Moreover, embryos from this treatment group had reduced head size and necrotic brain tissue as well as visibly malformed tails (kinked tails). The 5 mg/L genistein treated embryos had global malformations of the body (Figure 4.4 D) which included severe head size reduction, enlarged yolk sacs, heart and yolk oedema and major tail malformations. However, although acutely deformed, they had beating hearts, and therefore were classified as alive. Reduction in the body pigmentation was observed both for the 2.5 mg/L and 5 mg/L genistein treated embryos.

Figure 4.4 Morphology of the 48 hpf zebrafish embryos exposed to genistein. A – control (DMSO); B – 1.25 mg/L genistein; C – 2.5 mg/L genistein; D – 5 mg/L genistein. Arrows indicate reduced head, heart and yolk sac oedema, and malformed tail.

Morphology of 48 hpf zebrafish

There were no alterations in the morphology of 72 hpf zebrafish larvae treated with 1.25 mg/L genistein (Figure 4.6 B) when compared to controls (Figure 4.6 A) as reflected in the mean total morphological score (Figure 4.7). 96.3% of the zebrafish larvae exposed to 2.5 mg/L genistein were still in their chorions and severe body malformations were characteristic for all individuals from this treatment group (Figure 4.6 C). These malformations included heart
oedema, reduced head and eye size, enlarged yolk sac, malformed tail and decreased level of skin pigmentation. None of the 72 hpf larvae treated with 5 mg/L genistein showed signs of a beating heart and consequently were classified as dead as indicated by the lowest mean total morphological score (Figure 4.7).

Figure 4.6 Morphology of the 72 hpf zebrafish larvae exposed to genistein. A – control (DMSO); B – 1.25 mg/L genistein; C – 2.5 mg/L genistein. Arrows indicate reduced head, heart and yolk sac oedema and malformed tail.

Figure 4.7 Mean total morphological scores of the genistein treated larvae at 72 hpf. The percentage of the larvae scored for all the treatment groups was ≥20%.

At 96 hpf genistein caused concentration-dependent toxicity when assessed based on the morphology of the treated larvae (Figure 4.9). Around 80% of the larvae treated with 1.25 mg/L genistein showed no difference in the morphology when compared to control zebrafish (figure 4.8 A). However, 20.7% of the larvae from this treatment group demonstrated heart oedema (Figure 4.8 B) and had weaker body pigmentation compared to the control group.
Very high mortality (76.66%) and severe malformations in the larvae which survived up to this observation point were observed in the 2.5 mg/L genistein treatment group (Figure 4.8 C). These malformations affected all the anatomical body parts observed in the study (Head, heart, yolk sac, yolk extension and tail) as well as body’s pigmentation level.

![Figure 4.8 Morphology of the 96 hpf zebrafish larvae exposed to genistein. A – control (DMSO); B – 1.25 mg/L genistein; C – 2.5 mg/L genistein. Arrows indicate heart oedema and malformed tail.]

Further increase in the toxicity of the genistein was seen at 120 hpf based on the assessment of the treated larval morphology (Figure 4.11). 61% of the larvae treated with 1.25 mg/L genistein showed heart oedema (Figure 4.10 B). In addition, the size of yolk sacs and yolk extensions was increased in the larvae from this treatment group in comparison to the controls. The size of head was also affected by genistein at this concentration but severity of the effect varied between individuals (Figure 4.10 B and C). In addition, 1.25 mg/L genistein
observably reduced larval skin pigmentation when compared with controls (Figure 4.10 C). The treatment with 2.5 mg/L genistein resulted in 97% mortality of 120 hpf larvae and the bodies of larvae which survived up to this point were globally malformed (Figure 4.10 D).

Figure 4.10 Morphology of the 120 hpf zebrafish exposed to genistein. A – control (DMSO); B, C - 1.25 mg/L genistein, D – 2.5 mg/L genistein. Arrows indicate enlarged yolk sac and yolk extension, heart oedema and reduced head.

Figure 4.11 Mean total morphological scores of the genistein treated larvae at 120 hpf. The percentage of the larvae scored for all the treatment groups was ≥20%.
The effects of genistein exposure on the heart rate of zebrafish at 48 and 72 hpf

Genistein caused a concentration- and time- dependent decrease in the heart rates of 48 and 72 hpf zebrafish (Figure 4.12). A significant difference in the heart rate of embryos/larvae across all studied groups was revealed with one-way ANOVA (P≤0.05). A specific comparison testing (Tukey’s test) demonstrated that 5 mg/L genistein caused a significant reduction in the number of heart beats per minute at 48 hpf when compared to the control group (see also appendix B, Tables 8.8 and 8.9). No heart rate could be recorded for this group at 72 hpf since all the treated larvae were dead. Although there was no influence of 2.5 mg/L genistein on the survival of zebrafish at both 48 and 72 hpf as previously shown (Figure 4.1) this concentration caused a significant decrease in the heart rate at both time points of observation. Moreover, the difference between heart rate of the 2.5 mg/L genistein treated larvae and controls was clearly much bigger at 72 hpf than at 48 hpf. There was no significant difference in the heart rate of embryos/larvae exposed to 1.25 mg/L genistein and control group at any of the investigated time points and this concentration was classified as a NOEC for the zebrafish’ heart rate up to 72 hpf.

The concentrations of genistein causing 50% decline in the heart rate of zebrafish (EC50) were estimated with the use of Probit analysis as 3.61 mg/L (13.36 µM) for 48 hpf embryos and 2.25 mg/L (8.32 µM) for 72 hpf zebrafish larvae.

Figure 4.12 Heart rate of the 48 and 72 hpf zebrafish exposed to 1.25, 2.5 and 5 mg/L genistein. Graph plotted as means with standard deviation error bars. Asterisks indicate values significantly different from the controls (** P≤0.01 and *** P≤0.001, Tukey’s test)
The effects of genistein exposure on hatching success of zebrafish larvae at 72 hpf.

There was no effect of 1.25 mg/L genistein on the 72 hpf zebrafish larvae hatching success compared to the controls (one-way ANOVA followed by a Tukey’s test). Consequently, 1.25 mg/L genistein was characterized as a NOEC for the zebrafish hatching at 72 hpf.

Although survival of the 72 hpf larvae exposed to 2.5 mg/L was similar to the controls as demonstrated in previous section (Figure 4.1), this concentration of genistein severely disturbed the hatching of zebrafish larvae (Figure 4.13; see also appendix B, Table 8.1). Furthermore, the larvae exposed to 2.5 mg/L genistein did not hatch on the following days of the observation and, if alive, remained in chorions until the end of the experiment (120 hpf).

Genistein at a concentration of 1.93 mg/L (7.15µM) was calculated using Probit Analysis to constitute the EC50 for the hatching of 72 hpf larvae.

![Hatching success of the 72 hpf zebrafish larvae exposed to 1.25 and 2.5 mg/L genistein. % hatching is the percentage of live larvae that hatched and survival was: 100% for 0 mg/L, 96.7% for 1.25 mg/L, 93.3% for 2.5 mg/L genistein. Graph plotted as means with standard deviation error bars. Asterisks indicate values significantly different from the controls (**P≤0.001, Tukey’s test).](image-url)
4.2.2 The effects of genistein on the 120 hpf zebrafish liver ultrastructure

- The effects of genistein on the liver ultrastructure of 120 hpf zebrafish – morphometric analysis

In this section the effect of genistein on the fine structure of the liver in 120 hpf zebrafish larvae was investigated. The morphometric analysis was carried out to assess the volume fractions and the absolute volumes of the hepatocytes, the nuclei, mitochondria, RER and glycogen in the hepatocytes and the bile canaliculi. Next, the data were compared between the genistein exposed larvae and the controls (0.01% DMSO). The morphometrical and morphological description of the liver ultrastructure in the 120 hpf zebrafish larvae reared in DMSO has been presented in Section 3.2.3.

- Volume of hepatocytes

The volume of hepatocytes was similar across the DMSO, 312.5 µg/L genistein, 625 µg/L genistein and 20 µg/L β-oestradiol treatment groups \((P=0.292, \text{Kruskal-Wallis})\). The subsequent pair-wise comparisons revealed that neither 312.5 µg/L nor 625 µg/L genistein altered the volume of the hepatocytes compared to the controls \((P=0.773 \text{ and } P=0.248, \text{respectively; Mann-Whitney } U \text{ test})\). A significant reduction in the volume of hepatocytes comparing to the controls was revealed in the β-oestradiol treatment group \((503.92 \pm 40.18 \mu m^3 \text{ vs. } 367.17 \pm 101.51 \mu m^3, P=0.043, \text{Mann-Whitney } U \text{ test})\) as presented in Figure 4.14 (see also appendix B, Tables 8.14 and 8.15). There was no statistically significant difference when either 312.5 µg/L genistein or 625 µg/L genistein treatment group was compared to the β-oestradiol treated larvae \((P=0.248 \text{ for both comparisons, Mann-Whitney } U \text{ test})\).
Nuclear volume of hepatocytes

Both the nuclear volume fraction and the absolute volume of the nuclei was similar when compared across the treatment groups ($P=0.484$ and $P=0.375$, respectively; Kruskal-Wallis test). Moreover, there were no statistically significant differences in the nuclear volume fraction or the absolute volume of nuclei when the 312.5 µg/L genistein, 625 µg/L genistein or 20 µg/L β-oestradiol treated larvae were compared to the controls (Mann-Whitney U test). In addition, both the volume fraction and the absolute volume of the nuclei in the larvae treated with genistein at either concentration were similar to the volumes observed for the positive controls (Figure 4.15 and 4.16, see also appendix B, Tables 8.14 and 8.15).
Figure 4.15 Volume fractions of the nuclei in the hepatocytes in 120 hpf zebrafish exposed to genistein. Data presented as boxplots (median and quartiles) with whiskers from minimum to maximum.

Figure 4.16 Absolute volume of the nuclei in the hepatocytes in 120 hpf zebrafish exposed to genistein. Data presented as boxplots (median and quartiles) with whiskers from minimum to maximum.
Pooled mitochondrial volume of hepatocytes

There was a statistically significant difference in the volume fraction ($P=0.021$) but no difference in the absolute volume of pooled mitochondria ($P=0.067$) when compared across the treatment groups (Kruskal-Wallis test). Subsequent pair-wise comparisons revealed that 312.5 µg/L genistein did not alter the volume fraction or the absolute volume of mitochondria in comparison to the controls ($P=0.386$ and $P=0.564$, respectively; Mann-Whitney U test). The treatment with 625 µg/L genistein caused significant reduction ($P=0.021$) in the volume fraction of mitochondria and considerably affected ($P=0.083$) their absolute volume when compared to the control larvae (Mann-Whitney U test, Figure 4.17 and 4.18. See also appendix B, Tables 8.14 and 8.15). The natural oestrogen, in turn, caused a significant decrease in the mitochondrial absolute volume ($P=0.021$) and noticeably reduced their volume fraction ($P=0.059$) in comparison to the DMSO group (Mann-Whitney U test). No differences were found in the volume fraction or the absolute volume of mitochondria when the genistein treated larvae (both 312.5 µg/L genistein and 625 µg/L genistein) were compared to the positive control group (20 µg/L β-oestradiol).

![Volume fraction of pooled mitochondria in hepatocytes of 120 hpf zebrafish exposed to genistein](image)

Figure 4.17 Volume fraction of the pooled mitochondria in the hepatocytes in 120 hpf zebrafish exposed genistein. Data presented as boxplots (median and quartiles) with whiskers from minimum to maximum. The capital letters indicate a significant difference from: C - controls (DMSO). Asterisks specify statistical significance of the differences (*$P<0.05$, Mann-Whitney U test).
Absolute volume of pooled mitochondria in hepatocytes of 120 hpf zebrafish exposed to genistein

**Figure 4.18** Absolute volume of pooled mitochondria in the hepatocytes in 120 hpf zebrafish exposed to genistein. Data presented as boxplots (median and quartiles) with whiskers from minimum to maximum. The capital letters indicate a significant difference from: C - controls (DMSO). Asterisks specify statistical significance of the differences (*P≤0.05, Mann-Whitney U test).

- Individual mitochondrial volume of hepatocytes

Statistically significant differences were exhibited when the volume fraction (*P=0.036) and the absolute volume of individual mitochondrial profiles (*P=0.028) were compared across the treatment groups (Kruskal-Wallis test). Specific comparison testing demonstrated that 312.5 µg/L genistein caused significant reductions in the volume fraction and the absolute volume of mitochondrial profiles in comparison to the controls (*P=0.021 for both comparisons, Mann-Whitney U test) as presented in Figure 4.19 and 4.20 (see also appendix B, Tables 8.14 and 8.15). Similarly, both the volume fraction and the absolute volume of individual mitochondria in the 625 µg/L genistein treated larvae were significantly lower than in the control group (*P=0.020 and *P=0.021, respectively). A significant reduction in the absolute volume of individual mitochondria (*P=0.021) and a noticeable trend towards reduction in their volume fraction (*P=0.059) were exhibited comparing the β-oestradiol treated larvae and the controls (Mann-Whitney U test). No differences were seen when the volume fraction or the absolute volumes of the individual mitochondria were compared between the genistein treated larvae (both 312.5 µg/L and 625 µg/L) and the larvae from the positive control treatment group (Mann-Whitney U test).
Volume fraction of individual mitochondria in hepatocytes of 120 hpf zebrafish exposed to genistein

![Boxplot showing volume fraction of individual mitochondria](image)

Figure 4.19 Volume fraction of individual mitochondria in the hepatocytes in 120 hpf zebrafish exposed to genistein. Data presented as boxplots (median and quartiles) with whiskers from minimum to maximum. The capital letters indicate a significant difference from: C - controls (DMSO). Asterisks specify statistical significance of the differences (*P≤0.05, Mann-Whitney U test).

Absolute volume of individual mitochondria in hepatocytes of 120 hpf zebrafish exposed to genistein

![Boxplot showing absolute volume](image)

Figure 4.20 Absolute volume of individual mitochondria in the hepatocytes in 120 hpf zebrafish exposed to genistein. Data presented as boxplots (median and quartiles) with whiskers from minimum to maximum. The capital letters indicate a significant difference from: C - controls (DMSO). Asterisks specify statistical significance of the differences (*P≤0.05, Mann-Whitney U test).
- **Volume of rough endoplasmic reticulum in hepatocytes**

The volume fraction and the absolute volume of rough endoplasmic reticulum (RER) in the hepatocytes of 120 hpf zebrafish larvae was similar when compared across all the treatment groups (\(P=0.201\) and \(P=0.113\), respectively; Kruskal-Wallis test). Subsequent pair-wise comparisons exhibited that 625 µg/L genistein was the only treatment which significantly increased the volume fraction of RER comparing to the controls (\(P=0.043\), Mann-Whitney U test; Figure 4.21. See also appendix B, Tables 8.14 and 8.15). None of the genistein treatments affected the absolute volume of RER in comparison to the DMSO treatment group (Figure 4.22). Moreover, no differences were found by the comparison of the genistein (both 312.5 µg/L and 625 µg/L) treated larvae with the 20 µg/L \(\beta\)-oestradiol treatment group.

![Volume fraction of RER in hepatocytes of 120 hpf zebrafish exposed to genistein](image)

**Figure 4.21** Volume fraction of rough endoplasmic reticulum in the hepatocytes in 120 hpf zebrafish exposed to genistein. Data presented as boxplots (median and quartiles) with whiskers from minimum to maximum. The capital letters indicate a significant difference from: C - controls (DMSO). Asterisks specify statistical significance of the differences (*\(P\leq0.05\), Mann-Whitney U test).
Volume of glycogen

The volume fraction as well as the absolute volume of glycogen were different when compared across the treatment groups ($P=0.042$ and $P=0.046$, respectively; Kruskal-Wallis test). Specific comparisons demonstrated that the larvae treated with 312.5 µg/L genistein had a significantly higher volume fraction and the absolute volume of glycogen than the control larvae ($P=0.043$ for both comparisons, Mann-Whitney U test) as presented in Figures 4.23 and 4.24 (see also appendix B, Tables 8.14 and 8.15). The treatment with 625 µg/L genistein noticeably increased ($P=0.083$) the volume fraction of glycogen comparing to the controls. The same treatment had no effect on the absolute volume of the storage material ($P=0.149$, Mann-Whitney U test). Both the volume fraction and the absolute volume of glycogen were similar between the 20 µg/L β-oestradiol and the DMSO treatment groups. Further comparisons revealed that the volume fraction and the absolute volume of glycogen in the liver of the larvae treated with 20 µg/L β-oestradiol were significantly lower than in the larvae from both genistein treatment groups ($P=0.043$ for all the comparisons, Mann-Whitney U test).
Volume fraction of glycogen in hepatocytes of 120 hpf zebrafish exposed to genistein

Figure 4.23 Volume fraction of glycogen in the hepatocytes in 120 hpf zebrafish exposed to genistein. Data presented as boxplots (median and quartiles) with whiskers from minimum to maximum. The capital letters indicate a significant difference from: C - controls (DMSO), E - positive controls (β-oestradiol). Asterisks specify statistical significance of the differences (*P≤0.05, Mann-Whitney U test).

Absolute volume of glycogen in hepatocytes of 120 hpf zebrafish exposed to genistein

Figure 4.24 Absolute volume of glycogen in the hepatocytes in 120 hpf zebrafish exposed to genistein. Data presented as boxplots (median and quartiles) with whiskers from minimum to maximum. The capital letters indicate a significant difference from: C - controls (DMSO), E - positive controls (β-oestradiol). Asterisks specify statistical significance of the differences (*P≤0.05, Mann-Whitney U test).
• Volume of bile canaliculi

Both the volume fraction and the absolute volume of bile canaliculi were similar when compared across the treatment groups (P=0.224 and P=0.353, respectively; Kruskal-Wallis test). Subsequent specific comparison testing showed that none of the treatments (312.5 µg/L genistein, 625 µg/L genistein or 20 µg/L β-oestradiol) caused a significant alteration either in volume fraction or the absolute volume of bile canaliculi when compared to the controls (Mann-Whitney U test). Moreover, both the volume fraction and the absolute volume of bile canaliculi was similar when compared between the genistein treated larvae (312.5 µg/L or 625 µg/L genistein) and the positive control treatment group (Figure 4.25 and 4.26, see also appendix B, Tables 8.14 and 8.15).

![Volume fraction of bile canaliculi in hepatocytes of 120 hpf zebrafish exposed to genistein](image)

Figure 4.25 Volume fractions of bile canaliculi in the hepatocytes in 120 hpf zebrafish exposed to genistein. Data presented as boxplots (median and quartiles) with whiskers from minimum to maximum.
The effects of genistein on the liver ultrastructure of 120 hpf zebrafish – morphological assessment

The exposure to genistein at either tested concentration, i.e. 312.5 or 625 µg/L (Figure 4.27 and 4.28) did not cause any apparent alterations in the shape of hepatocytes. Similarly, the size and shape of the hepatocytes nuclei was mostly similar to what was observed in the DMSO treated larvae which were previously described (Section 3.2.3, Figures 3.65-3.67) although there was somewhat higher variation of nuclear sizes in the 312.5 µg/L genistein treated larvae. The amount of the nuclear heterochromatin and the number and appearance of the nucleoli did not show any obvious alterations due to the genistein treatment. In contrast, mitochondria in the hepatocytes of the larvae exposed to both tested concentrations of genistein were visibly much smaller than in the controls (Figure 4.27 and 4.29). In addition, although there were some mitochondria of longitudinal or even twisted or branched forms, the amount of these forms was noticeably reduced compared to the controls. Furthermore, the number of these small and roundish mitochondria was to some degree increased. This observation is justified by the fact that despite the smaller sizes of mitochondria their pooled volume was not different from the control larvae, as demonstrated in the previous section. Although morphometric comparison in the amount of the RER showed no differences between the genistein exposed larvae and the controls, some hepatocytes had a noticeably increased amount of membranes. In addition, dilated and fractionated cisternae of the RER could be observed (Figure 4.29) and their amount appeared to increase in a concentration-dependent manner. The liver of the 312.5 µg/L
genistein treated larvae was rich in glycogen and in some hepatocytes this storage product occupied nearly all the cellular space. Much higher inter- and intra-individual variation in the amount of glycogen was observed in the larvae reared in 625 µg/L genistein. No apparent differences were observed in the morphology of the bile canaliculi and the preductules or in the amount and distribution of the macrophages.

Figure 4.27 Liver of the 120 hpf zebrafish exposed to 312.5 µg/L genistein. N – nuclei of the hepatocytes; M – mitochondria; → rough endoplasmic reticulum, G – glycogen. Scale bar ~2 µm.
Figure 4.28 Liver of the 120 hpf zebrafish exposed to 312.5 µg/L genistein. N – nuclei of the hepatocytes; M – mitochondria; → rough endoplasmic reticulum, G – glycogen; mw – myelin whorls in the glycogen. Scale bar ≈2 µm.

Figure 4.29 Liver of the 120 hpf zebrafish exposed to 625 µg/L genistein. N – nuclei of the hepatocytes; M – mitochondria; → rough endoplasmic reticulum, notice distended membranes; B – bile canaliculi. Scale bar ≈2 µm.
Shape analysis of the mitochondrial profiles in the hepatocytes of the 120 hpf zebrafish larvae exposed to genistein.

- Area of the mitochondrial profiles

The comparison of the mitochondrial profiles’ areas across the treatment groups (DMSO, 312.5 μg/L genistein, 625 μg/L genistein and 20 μg/L 17β-oestradiol) revealed a statistically significant difference ($P=0.025$, Kruskal-Wallis test). Subsequent pair-wise comparison revealed that the treatments with either 312.5 μg/L genistein or 625 μg/L genistein caused a significant reduction in the areas of the mitochondrial profiles when compared to the controls ($P=0.020$ for both comparisons, Mann-Whitney U test). The areas of the mitochondrial profiles in the β-oestradiol treated larvae were also significantly lower than in the control group ($P=0.042$, Mann-Whitney U test) and were similar to those observed in the genistein treated larvae (Figure 4.30).

The comparison of the mitochondrial profiles areas distribution between the larvae treated with genistein or β-oestradiol and the controls (Chi-squared test) revealed significant differences in the 312.5 μg/L genistein and 625 μg/L genistein treatment groups. In both cases there were more mitochondrial profiles of smaller area range and fewer profiles of larger area comparing to the control group (Figure 4.31).

Figure 4.30 Area of the mitochondrial profiles in the hepatocytes of the 120 hpf zebrafish exposed to genistein. Each symbol represents the median of 86 ± 16 mitochondria measured per larva (n=4). The capital letters indicate a significant difference from: C - controls (DMSO), E - positive controls (β-oestradiol). Asterisks specify statistical significance of the differences (*$P<0.05$, Mann-Whitney U test).
Figure 4.31 Distribution of the areas of the mitochondrial profiles in the hepatocytes of the 120 hpf zebrafish larvae exposed to genistein. Data presented as the mean (n=4) of the percentages of the mitochondrial profiles of a given area range. Asterisks indicate values significantly different from the controls (*P≤0.05, **P≤0.01, Chi-squared test).

- **Circularity of the mitochondrial profiles**

The circularity of the mitochondrial profiles in the hepatocytes of the 120 hpf zebrafish larvae was similar across the treatment groups (P=0.077, Kruskal-Wallis test). Moreover, none of the treatments with genistein altered the circularity of the mitochondrial profiles in a significant way when compared to the controls (P=0.243 for 312.5 µg/L, P=0.081 for 625 µg/L, Mann-Whitney U test). Similarly, the treatment with 20 µg/L β-oestradiol did not change this parameter comparing to the DMSO treatment group (P=0.191, Mann-Whitney). Further comparisons revealed that the circularity of the mitochondrial profiles in the larvae treated with β-oestradiol was significantly lower than in the 625 µg/L genistein treatment group (P=0.042) but similar to the profiles observed for the 312.5 µg/L genistein treated larvae (P=0.080, Figure 4.32).

The comparison of the distributions of the mitochondrial profiles circularity (Chi-squared test) revealed statistically significant differences between the β-oestradiol treatment group and the control larvae. There was a significantly higher frequency of the mitochondrial profiles with shapes deviating from circular in the β-oestradiol treated larvae (Figure 4.33).
Circularity of mitochondrial profiles in the hepatocytes of the 120 hpf zebrafish exposed to genistein

Figure 4.32 Circularity of the mitochondrial profiles in the hepatocytes of the 120 hpf zebrafish larvae exposed to genistein. Each symbol represents the median of 86 ± 16 mitochondria measured per larva (n=4). The capital letters indicate a significant difference from: E – positive controls (β-oestradiol). Asterisks specify statistical significance of the differences (*P≤0.05, Mann-Whitney U test).

Figure 4.33 Distribution of the circularity of the mitochondrial profiles in the hepatocytes of the 120 hpf zebrafish larvae exposed to genistein. Data presented as the mean (n=4) of the percentages of the mitochondrial profiles of a given circularity range Asterisks indicate values significantly different from the controls (*P≤0.05, Chi-squared test).
• Maximum feret diameter of the mitochondrial profiles

There was a statistically significant difference revealed when the maximum feret diameter of the mitochondrial profiles was compared across the treatment groups ($P=0.033$, Kruskal-Wallis test). Genistein at both 312.5 µg/L and 625 µg/L concentrations caused a significant reduction in the length of the maximum feret diameter comparing to the controls ($P=0.021$, for both comparisons; Mann-Whitney U test). In turn, the maximum feret diameter in the β-oestradiol treatment group was similar to the control group (Figure 4.34). No differences were found when comparing this parameter between the genistein treated larvae (both 312.5 µg/L and 625 µg/L) and the positive control treatment group.

The distribution analysis also revealed significant differences in the maximum feret diameters of the mitochondrial profiles between the genistein treated larvae and the control group (Chi-squared test). For both the 312.5 µg/L genistein and 625 µg/L genistein treatment groups there were more profiles with smaller maximum feret diameters and fewer with larger maximum feret diameters comparing to the control group (Figure 4.35).

![Max feret diameter of mitochondrial profiles in the hepatocytes of the 120 hpf zebrafish exposed to genistein](image)

Figure 4.34 Maximum feret diameters of the mitochondrial profiles in the hepatocytes of the 120 hpf zebrafish larva exposed to genistein. Each symbol represents the median of 86 ± 16 mitochondria measured per larva (n=4). The capital letters indicate a significant difference from: C - controls (DMSO), E – positive controls (β-oestradiol). Asterisks specify statistical significance of the differences (*$P<0.05$, Mann-Whitney U test).
Figure 4. Distribution of the maximum feret diameters of the mitochondrial profiles in the hepatocytes of the 120 hpf zebrafish larvae exposed to genistein. Data presented as the mean percentage (n=4) of the mitochondrial profiles of a given length range. Asterisks indicate values significantly different from the controls (**P≤0.001, Chi-squared test).

- Minimum feret diameter of the mitochondrial profiles

The comparison of the minimum feret diameter of the mitochondrial profiles across the treatment groups revealed a statistically significant difference (P=0.032, Kruskal-Wallis test). Pair-wise comparisons revealed that both treatments with genistein as well as the treatment with β-oestradiol caused a decrease in the length of the minimum feret diameter compared to the control group (P=0.021, 0.020 and 0.021 for 312.5 µg/L genistein, 625 µg/L genistein and 20 µg/L β-oestradiol, respectively; Mann-Whitney U test). Moreover, the minimum feret diameters of the mitochondrial profiles observed for the larvae treated with either 312.5 µg/L or 625 µg/L genistein were similar to the positive control treatment group (Figure 4.36).

All the tested treatments, i.e. 312.5 µg/L genistein, 625 µg/L genistein and 20 µg/L β-oestradiol had a significant effect on the distribution of the mitochondrial profiles minimum feret diameters based on the comparison to the control group (Chi-squared test; Figure 4.37). More profiles of smaller- and fewer of larger minimum feret diameters were observed in all these treatment groups.
Min feret diameter of mitochondrial profiles in the hepatocytes of the 120 hpf zebrafish exposed to genistein

Figure 4.36 Minimum feret diameter of the mitochondrial profiles in the hepatocytes of the 120 hpf zebrafish larvae exposed to genistein. Each symbol represents the median of 86 ± 16 mitochondria measured per larva (n=4). The capital letters indicate a significant difference from: C - controls (DMSO), E – positive controls (β-oestradiol). Asterisks specify statistical significance of the differences (*P≤0.05, Mann-Whitney U test).

Figure 4.37 Distribution of the minimum feret diameters of the mitochondrial profiles in the hepatocytes of the 120 hpf zebrafish larvae exposed to genistein. Data presented as the mean percentage (n=4) of the mitochondrial profiles of a given length range. Asterisks indicate values significantly different from the controls (*P≤0.05 and ***P≤0.001, Chi-squared test).
- Major to minor axis ratio of the mitochondrial profiles

The major to minor axis ratio of the mitochondrial profiles observed in the zebrafish larvae hepatocytes was similar among all the treatment groups (0.116, Kruskal-Wallis test). Pair-wise comparisons of the mitochondrial profiles in the hepatocytes of the larvae treated with genistein or β-oestradiol with the control group did not reveal any statistically significant differences (p≥0.149 for all comparisons, Mann-Whitney U test). Further analysis revealed that mitochondrial profiles in the positive control group had significantly larger aspect ratios than in the 625 µg/L genistein treatment group (P=0.043, Mann-Whitney U test; Figure 4.38).

When the distribution of the mitochondrial profiles aspect ratios was analyzed, a statistically significant difference was exposed between the larvae treated with 20 µg/L 17β-oestradiol and the controls (P<0.005, Chi-squared test). There were more mitochondrial profiles with larger aspect ratio in the hepatocytes of the larvae treated with β-oestradiol than in the control group (Figure 4.39).

![Figure 4.38](image)

Figure 4.38 Major to minor axis ratio of the mitochondrial profiles in the hepatocytes of the 120 hpf zebrafish exposed to genistein. Each symbol represents the median of 86 ± 16 mitochondria measured per larva (n=4). The capital letters indicate a significant difference from: C - controls (DMSO), E – positive controls (β-oestradiol). Asterisks specify statistical significance of the differences (*P≤0.05, Mann-Whitney U test).
4.2.3 The effects of genistein on the expression of vitellogenin 1 mRNA in 120 hpf zebrafish

The levels of vitellogenin 1 mRNA (vtg1) expression were measured in the 120 hpf zebrafish larvae exposed to 312.5, 625 and 1250 μg/L genistein with the use of real-time PCR. The embryos were exposed to the tested solutions starting from the 2-4 cell stage (0.75-1 hpf) up to 120 hpf. The levels of the vtg1 expression in the genistein exposed larvae were compared to the control group (0.015 DMSO) as well as to the positive control treatment group, i.e. the 20 μg/L β-oestradiol treated larvae.

There was a statistically significant difference (P<0.001, Welch’s ANOVA) in the level of the vtg1 across the treatment groups, i.e. 0.01% DMSO, 312.5 μg/L genistein, 625 μg/L genistein, 1250 μg/L genistein and 20 μg/L β-oestradiol. Moreover, there was a concentration-dependent increase in the level of vtg1 in the genistein-treated larvae (Figure 4.40). The specific comparison testing revealed that the vtg1 expression level was significantly higher in the larvae treated with either 625 μg/L or 1250 μg/L genistein than in the control larvae (P=0.007 and P<0.001, respectively; Games-Howell post-hoc test). The highest level of vtg1 was observed for the positive control treatment group which also was significantly different than the level of vtg1 in the controls (P<0.001, Games-Howell post-hoc test). Further pair-wise comparisons revealed that the vtg1 expression level in the 312.5 μg/L genistein and the 625 μg/L genistein treated larvae was significantly lower than in the larvae treated with the natural oestrogen
(P<0.001 and P=0.044, respectively; Games-Howell post-hoc test). However, the level of vtg1 in the 1250 μg/L genistein treatment group was similar to the level assessed for the larvae treated with 20 μg/L of the natural oestrogen (P=0.510, Games-Howell post-hoc test) as presented in Figure 4.40.

Figure 4.40. Vitellogenin 1 mRNA (vtg1) expression fold changes in the 120 hpf zebrafish larvae exposed to genistein. Data presented as boxplots (median and quartiles) with whiskers from minimum to maximum. The capital letters indicate a significant difference from: C - controls (DMSO), E – positive controls (β-oestradiol). Asterisks specify statistical significance of the differences (*P≤0.05, Games-Howell test).
4.3 Discussion

This study aimed to investigate the influence of genistein on the early development of zebrafish. Genistein is a naturally occurring compound found mostly in soy products and has been the subject of numerous studies because of its known oestrogenic activity. Little, however, is known about the developmental effects induced by exposure to this compound during vertebrate embryonic development (Sassi-Messai et al., 2009).

This study showed that genistein has toxic and teratogenic effects on the early development of zebrafish and affects morphology, heart rate, hatching success and survival of the treated embryos/larvae. This compound was also observed to affect the fine structure of the liver at early developmental stages. Furthermore, genistein showed clear oestrogenic effects as assessed based on the measurement of vitellogenin 1 mRNA levels in 120 hpf zebrafish larvae.

On the lethal and sub lethal effects of genistein on the zebrafish development

There are few reports on the effects of genistein on the early development of zebrafish. This study demonstrated concentration and time dependent toxicity of genistein on zebrafish development. 10 mg/L genistein constituted a lethal dose for the embryos at 24 hpf while 5 mg/L caused a dramatic decrease in the survival at 24 hpf followed by 100% mortality at 72 hpf. These observations contrast with the study by Kim et al. (2009) who reported that above 60% of the zebrafish treated with 6.75 mg/L genistein at 24 hpf were alive at 84 hpf. In another study 90% of the embryos exposed at 5 hpf to 4.7 mg/L genistein survived up to 96 hpf (Sassi-Messai et al., 2009). In addition, Sassi-Messai et al. did not observe any effects of ≤ 2.7 mg/L genistein (10 μM) on zebrafish survival prior to 105 hpf. Together these results indicate that the time point at which the exposure commences (0.75 – 1 hpf, 24 hpf and 5 hpf, respectively) may have a crucial impact on the toxicity of genistein.

The current study also demonstrated that genistein causes a concentration-dependent reduction in the heart rate in both 48 and 72 hpf zebrafish. Similar effects were reported by Kim et al. (2009) who observed heart rate at 54 hpf in zebrafish embryos exposed to 27, 13.5 and 6.75 mg/L genistein (i.e. 100, 50 and 25 μM) from 24 hpf onward. Due to a difference in the time of heart rate monitoring, a direct comparison of the results is hindered. Based on the comparison of the control embryos’ heart rate one can assume, however, that there were no major differences in the heart rate between the embryos/larvae used in the present work and the study of Kim et al. (135.07 ± 7.45 bpm at 48 hpf vs. 127.62 ± 6.34 bpm at 54 hpf, respectively). This relatively small divergence could be explained by a time-dependent reduction in the heart rate of developing zebrafish, as revealed in the current study by the comparison between 48 hpf (135.07 ± 7.45 bpm) and 72 hpf (127 ± 1.40 bpm) control zebrafish.

A reduction in heart rate caused by genistein could be related to its protein kinase-inhibiting properties affecting phosphorylation-dependent cardiac Ca\(^{2+}\) channels. However, as has been
previously demonstrated with the use of guinea pig myocytes, genistein can block Ca\textsuperscript{2+} channels (Belevych et al., 2002). This mechanism has been suggested to operate in the zebrafish embryos showing heart rate reduction when exposed to this compound (Kim et al., 2009).

This study showed a concentration-dependent reduction in hatching success of 72 hpf zebrafish larvae, which is in agreement with the report by Kim et al. (2009). However, while 2.5 mg/L genistein was observed to nearly completely inhibit the hatching of 72 hpf larvae in the present study, Kim et al. reported 43%, 12% and 0% hatching success for the 6.75, 13.5 and 27 mg/L concentrations of this phytoestrogen. These substantial differences in genistein’s effects can be, again, explained by a difference in the exposure starting point, i.e. 0.75-1 hpf vs. 24 hpf. This reasoning is further confirmed when the hatching success of control zebrafish larvae is compared between both studies. Kim et al. observed about 90% hatched larvae at 61 hpf while in this study hatching was monitored at 72 hpf and was 100%. Despite the disparity in the observation time point, one can assume that there were no fundamental differences in the hatching rate of larvae used in both experiments since at 72 hpf hatching success of the larvae observed by Kim et al. was probably also 100%. The fact that the same temperature (28 ± 0.5°C) was used through both experiments further justifies this reasoning because the temperature’s effects on the rate of zebrafish development and the toxicity of genistein can be dismissed.

Hatching delay or reduction could be due to the general underdevelopment of the genistein treated embryos/larvae. Inhibition of the cell growth or/and differentiation could occur because of genistein’s engagement in regulation of cell cycle checkpoints, its ability to modulate the transforming growth factor beta 1 signalling pathway and/or its anti-angiogenesis and antioxidant activities (Kim et al., 2009; Kim et al., 1998).

The morphological assessment of embryos/larvae treated with genistein revealed a dose-dependent decrease in the size of the head. Moreover, a characteristic dark appearance of the tissue was observed indicating neurotoxic effects at ≥ 2.5 mg/L genistein. These observations may indicate loss and apoptosis in neural cells in the brain which have been reported previously for 84 hpf zebrafish larvae exposed to 6.75 mg/L genistein (Kim et al., 2009). Furthermore, a concentration dependent apoptosis induced by genistein in the hindbrain and the anterior spinal cord of zebrafish embryos was reported by Sassi-Messai et al. (2009). In their study, acridine orange-stained apoptotic cells were observed in 24 hpf embryos treated with 676 μg/L genistein (2.5 μM) and the following TUNNEL analysis revealed apoptotic changes even at 0.5 μM concentration. One might therefore assume that all the concentrations of genistein tested in the present study may have led to apoptotic changes in the brain and underpinned the observed reduction in the size of heads or/and their dark appearance. Although no obvious alterations in the head morphology were observed in the larvae treated with 625 μg/L or 312.5 μg/L genistein (larvae used for the study on liver ultrastructure) lack of neurotoxic effects cannot be excluded. Yang et al. (2009) demonstrated, for example, that gene expression alterations caused by a toxicant in embryonic zebrafish may occur even at doses failing to cause any morphological or behavioural effects.
Another morphological alteration observed in the genistein treated larvae was pericardial oedema. The reduction in heart rate could be a reason for this malformation due to, for example, pooling of the blood in the cardiac chambers. It is, however, also possible that cardiac oedema was the primary effect of genistein exposure and led to the reduction in the heart rate. Cardiac oedema together with reduction or lack of major blood vessels have been reported for zebrafish embryos treated with genistein by Bakkiyanathan et al. (2010). Because of high phenotypic similarity to the VEGF-A loss of function model it is was suggested that genistein affects VEGF signalling pathways. The fact that VEGF receptors belong to the family of tyrosine kinases lends support to this hypothesis. The disturbance in the development of vasculature would have a global effect on the developing embryos/larvae which was generally seen in our study.

The morphological alterations induced by genistein in zebrafish embryos/larvae included also a decrease in the body pigmentation level. Genistein’s effects on heart rate could lead to slower blood flow resulting, for example, in the limited mobilization of yolk sac proteins and, consequently, reduced supply of these nutrients to the developing organs, such as skin. This reasoning is supported by the presence of enlarged yolk sacs/extensions observed in this study for the genistein treated embryos/larvae. Another reason for a disturbed distribution of necessary nutrients and slower development could be the inhibition of blood vessel formation which was previously reported for developing zebrafish embryos treated with genistein (Bakkiyanathan et al., 2010). Anti-proliferative properties of genistein could perhaps also play a role in the observed reduction in skin pigmentation since it could affect the division of skin melanophores.

Another feature commonly observed in this study for the genistein treated embryos/larvae was tail malformation. The concentrations of genistein causing this effect (≥ 2.5 mg/L) also inhibited hatching of the embryos which prevented the observation of the whole body axis. Body axis malformations were observed by Kim et al. (2009) in the zebrafish larvae exposed to 6.75 mg/L genistein starting at 24 hpf. A considerably later exposure starting point attenuated genistein’s toxicity and did not affect hatching rate to the degree observed in the present study. One might surmise, however, that the malformations observed in both studies are just different presentations of the same phenomenon. Body axis malformation was possibly the outcome of myocyte degeneration and apoptotic changes in the CNS (Kim et al., 2009). Impaired motility of the genistein treated embryos/larvae was noticed during the course of the present study and may further indicate that musculature and/or motoneural activity were affected by this compound.

On the effects of genistein on the liver ultrastructure of zebrafish larvae

The study of the zebrafish liver ultrastructure revealed that the hepatocyte mitochondria were clearly affected by genistein. At 625 μg/L genistein caused a significant reduction in the volume
fraction and a considerable decrease in the absolute volume of pooled mitochondria. This effect was linked with the reduction in the volume of individual mitochondria, as well as with reduced areas and minimal or/and maximal feret diameters of their profiles. Moreover, these mitochondria were noticeably less irregular in shape. Similar alterations in the mitochondrial shape were observed in the hepatocytes of the 312.5 μg/L genistein treated larvae, although neither a volume fraction nor the absolute volume of these organelles was changed. As discussed in Section 3.3, such alterations in the volume and shape of mitochondria may indicate that fission was a dominant occurrence in the observed cells. It is known, for example, that mitochondrial fission, i.e. breaking into smaller pieces, is observed early in programmed cell death (Hales, 2010) which, in turn, has been shown to be induced by genistein in various types of malignant cells (Kyle et al., 1997; Li et al., 1999). It was demonstrated by Baxa et al. (2005) that induction of apoptosis in T lymphoma cells by genistein occurs by mitochondrial depolarization with the involvement of the permeability transition pore (PTP). Since concentrations of genistein differed significantly between the present study and the study of Baxa et al. (≤2.5 μM vs. 15-30 μM, respectively) and no clear symptoms of apoptosis were seen in the zebrafish hepatocytes, the potential mitochondrial fission occurring in these cells seem to be related to more subtle processes. One of them could be perhaps genistein’s ability to affect mitochondrial proteins involved in mitochondrial fusion/fission. It was reported, for example, that mitochondria with reduced levels of OPA1 – a protein mediating mitochondrial fusion, are less likely to fuse and are eventually autophagocytosed (Twig et al., 2008).

Taking into consideration that morphological changes in mitochondria may indicate serious cellular disturbances, the fact that such changes were observed in the liver of a developing organism treated with environmentally relevant concentrations of genistein, should not be taken lightly. Future studies seem necessary to further investigate this phenomenon and uncover its potential consequences for the developing organisms.

The morphology of RER was also affected in the hepatocytes of the larvae treated with genistein. The enlarged RER volume fraction was observed in the hepatocytes of the larvae from the 625 μg/L treatment group. As already discussed in Section 3.3, RER in fish is involved in hepatic biotransformation processes (Lester et al., 1993) and consequently its increase may indicate adaptive responses to toxicity. In addition, more RER membranes might be a sign of higher metabolic activity and stimulation of hepatic protein synthesis (Braunbeck and Appelbaum, 1999).

Dilated and fragmented RER membranes were also observed in some hepatocytes of the genistein treated larvae. Similar effects were reported for the liver of zebrafish females exposed to 4-chloroaniline and were interpreted as general morphological evidence of the induction of biotransformation processes (Braunbeck et al., 1990). The fragmentation and vesiculation of RER membranes was also observed in the liver of rainbow trout treated with ochratoxin (Braunbeck, 1998). These changes together with degranulation of the membranes led to the transformation of RER system into an irregular network of short anastomosing cisternae and vesicles strongly resembling SER. According to Braunbeck, differentiation
between degranulated RER and true SER by means of electron microscopy was, in many cases, practically impossible. This could imply that a proliferation of SER could perhaps have occurred in the genistein-treated larvae. This, however, seems unlikely in the light of the fact that SER has not been observed in this study in control larvae, which is in accordance with previous reports (Burkhardt-Holm et al., 1999).

Sex-dependent morphological alterations were observed in the liver of zebrafish treated with 4-nitrophenol and included changes in endoplasmic reticulum (Braunbeck et al., 1989). While increased proliferation of SER was observed in male fish, a high degree of fenestration within cisternae of RER was seen in females. Thus, it could be suggested that the RER alterations observed in the present study in response to genistein might indicate ‘feminization’ of the liver. However, taking into account sexual immaturity of 120 hpf zebrafish, this explanation seems unlikely. It would be interesting, however, to investigate whether xeno- or phytoestrogens can affect the ultrastructure of zebrafish liver in a sex-dependent fashion with the use of sexually mature fish.

The results of the present study led us to surmise that genistein may also affect glycogen in the hepatocytes of zebrafish larvae. A significant increase or a trend toward increase was observed for the 312.5 μg/L or 625 μg/L genistein treated larvae. Studies on the influence of genistein on sugar metabolism have produced conflicting results. Some, for example, have observed a reduction in the amount of glycogen in the hepatocytes of the genistein treated rats (Nogowski et al., 2002). The study of Okazaki et al. (2002), however, revealed that general toxicological effects of repeated-28-day oral exposure to genistein included increased liver weights and glycogen deposition in periportal rat hepatocytes. A great variation in the amount and intracellular localization of glycogen observed in the present study in the zebrafish hepatocytes hinders drawing firm conclusions in this matter.

*On the effects of genistein on the vitellogenin 1 expression in 120 hpf zebrafish larvae*

Vitellogenin is the precursor of the egg yolk proteins and is produced by the liver of oviparous vertebrates under hormonal control. The amount of produced vitellogenin is positively correlated with the concentration of oestradiol, or any other compound able to mimic this natural oestrogen in binding to ERs. ER-dependent vitellogenesis may occur not only in females but also in males and juveniles which also possess these receptors in the hepatocytes. This phenomenon has been widely used as a biomarker of the exposure to oestrogenic substances (Sumpter and Jobling, 1995).

The present study was the first to investigate the oestrogenic potential of genistein to induce the expression of vitellogenin 1 mRNA (vtg1) in 120 hpf zebrafish larvae. A concentration-dependent up-regulation of vtg1 in the larvae was observed. The concentration-dependent increase in the level of this transcript was previously reported for adult male zebrafish treated with genistein (Kausch et al., 2008). While in their study 11-day exposure to 5000 μg/L
Genistein was required to induce \( vtg1 \) in a statistically significant way, both 625 and 1250 \( \mu g/L \) genistein significantly increased \( vtg1 \) in the present study. Based on these observations one might suggest that the sensitivity of zebrafish to oestrogenic potential of genistein is higher at earlier stages of development.

The observation of clear oestrogenic effects of genistein at 625 \( \mu g/L \) (2.31 \( \mu M \)) is particularly important since this concentration is physiologically achievable. The levels of 908 \( \mu g/L \) (median) and 684 \( \pm 443 \mu g/L \) (mean \( \pm \) SD) have been reported for the soy formula fed infants by Cao et al. (2009) and Setchell et al. (1997). Perturbing is also the fact that genistein at 1250 \( \mu g/L \) showed estrogenicity similar to that of the natural oestrogen- 17\( \beta \)-oestradiol, as assessed based on the \( vtg1 \) expression level. Particularly that the concentration of oestradiol used in the current study (20 \( \mu g/L \)) far exceeds the levels measured in healthy pre-menopausal women during luteinizing hormone peak which range from 372.87 to 1884.91 pM, i.e. 101.6 to 313.4 ng/L (Stricker et al., 2006). It is therefore possible that the oestrogenic potential of 625 \( \mu g/L \) genistein would be much greater than of 17\( \beta \)-oestradiol at physiological levels but further studies are necessary to confirm this assumption.

It should be noted, however, that genistein used in the present study was not glycoconjugated, as it is present in the plants, including soy (Dixon, 2004). Moreover, the concentrations reported for human tissues relate mostly to the free plus conjugated genistein levels and consequently may exceed the concentrations of unconjugated genistein. It cannot, however, be excluded that in the present study some molecules of genistein were bound to the serum proteins in zebrafish embryos/larvae. This could lead to the reduction in the amount of unconjugated genistein affecting the embryos/larvae. Moreover, for the first three days of exposure zebrafish were surrounded by chorions which, as may assume, could further decrease the actual concentrations of genistein acting on the developing embryos. Protective effects of the chorions were reported in the study of Fent and Meier (1994) who observed higher toxicity of triphenyltin to the European minnow larvae than to the embryos.

It is worth mentioning that large differences in the levels of \( vtg1 \) which were observed in the present study were not the result of experimental problems. High differences in the levels of this transcript were observed between individual adult zebrafish males treated with genistein in the study of Kausch et al. (2008). These observations are in accordance with the suggestion of Biales et al. (2007) that variability in vitellogenin expression is common to a number of aquatic vertebrates and indicate genetic causation.

In summary, this study showed that genistein at physiologically relevant concentrations can evoke significant changes in the processes regulated by the natural estrogen. These observations lead to concerns about the potential disturbances in hormonal homeostasis, particularly at early developmental stages, and support the suggestion of Xing et al. (2010) that until more data for humans become available, high soy diets should not be taken during the first trimester. Taking into account, that neonatal stages are also very sensitive to physiological disturbances, one might further suggest that soy-based formulae should be used with caution.
5 The Influence of Bisphenol A and Genistein in Combination on the Early Development of Zebrafish

5.1 Aim of study

The aim of this study was to investigate the effects of BPA and genistein in combination on the early development of zebrafish. Lethal and sublethal effects of the mixtures were observed for the 24, 48, 72, 96 and 120 hpf zebrafish embryos/larvae. In addition, the effects of BPA plus genistein on the liver ultrastructure of the 120 hpf zebrafish larvae were investigated. To assess the oestrogenic potential of the mixtures the vitellogenin 1 mRNA expression in 120 hpf zebrafish larvae was studied. All the mixtures’ effects were compared to the control group, i.e. the embryos/larvae reared in the appropriate solvent solution (0.1% or 0.01% DMSO). Additionally, the effects of the mixtures were compared to the results of the treatments with their individual components at corresponding concentrations. This procedure aimed to investigate the mode in which BPA and genistein affect each other (antagonistic, additive or synergistic) when in combination.

5.2 Results

5.2.1 Embryotoxic and teratogenic combined effects of BPA and genistein on the early development of zebrafish

In this section zebrafish embryos/larvae were exposed to the following mixtures: 4 mg/L BPA + 1.25 mg/L genistein, 4 mg/L BPA + 2.5 mg/L genistein, 8 mg/L BPA + 1.25 mg/L genistein, 8 mg/L BPA + 2.5 mg/L genistein and 16 mg/L BPA + 5 mg/L genistein. The exposure started at 2-4 cell stage of development (0.75-1 hpf) and continued until 120 hpf. Embryos and larvae were examined for survival and morphological defects at 24, 48, 72, 96 and 120 hpf. Heart rate at 48 and 72 hpf and hatching success at 72 hpf were also investigated. All the observations were compared to the effects of the corresponding, individual compounds of the mixtures.
Individual and combined effects of BPA and genistein on zebrafish survival

At 24 hpf there was a significant difference ($P \leq 0.001$) in the survival between the groups of the embryos treated with BPA and genistein individually and in combinations. 5 mg/L genistein was the only treatment with the individual EDC which caused a significant decrease in the embryos’ survival in comparison to the control group (ANOVA followed by Tukey’s post-hoc tests). In turn, 16 mg/L BPA + 5 mg/L genistein was the only treatment with combined BPA and genistein which significantly affected the survival of embryos ($P \leq 0.001$). Moreover, when the survival of the embryos from this combination treatment group was compared with the survival observed in the corresponding individual treatment groups (16 mg/L BPA and 5 mg/L genistein), significant differences were found in both cases (Figure 5.1, see also appendix B, Table 8.1). While 16 mg/L BPA caused $23.33 \pm 25.17\%$ mortality and 5 mg/L genistein caused $36.67 \pm 11.55\%$, the combination of these concentrations resulted in death of the $100.00 \pm 0.00\%$ of the treated population indicating at least additive, if not synergistic, effect.

24 hpf survival of zebrafish embryos exposed to individual and combined EDCs

Figure 5.1 Survival of 24 hpf zebrafish embryos exposed to BPA and genistein individually and in combinations. Graph plotted as means with standard deviation error bars. The capital letters signify survival significantly different from: C - controls, B – corresponding treatment with BPA only, G – corresponding treatment with genistein only. Asterisks indicate statistical significance of the differences (** $P \leq 0.01$ and *** $P \leq 0.001$, Tukey’s test).
At 48 hpf, as at 24 hpf, 16 mg/L BPA + 5 mg/L genistein was the only combination treatment group which affected survival of the treated embryos in a way significantly different from the controls and from the corresponding individual treatments, i.e. 16 mg/L BPA and 5 mg/L genistein (Figure 5.2).

![Graph showing 48 hpf survival of zebrafish embryos exposed to individual and combined EDCs](image)

**Figure 5.2** Survival of the 48 hpf zebrafish embryos exposed to BPA and genistein individually and in combinations. Graph plotted as means with standard deviation error bars. The capital letters signify survival significantly different from: C - controls, B – corresponding treatment with BPA only and G – corresponding treatment with genistein only. Asterisks indicate statistical significance of the differences (*** $P \leq 0.001$, Tukey's test).

At 72 hpf 5 mg/L genistein alone killed all the larvae, as did the combination of 16 mg/L BPA + 5 mg/L genistein (Figure 5.3). For all the remaining combination treatments the results were similar to 48 hpf and showed no indication of enhanced toxicity of BPA and genistein when in mixtures (ANOVA followed by a Tukey’s test, see also appendix B, Table 8.11).
72 hpf survival of zebrafish embryos exposed to individual and combined EDCs

![Graph showing survival (%)]

**Figure 5.3** Survival of the 72 hpf zebrafish larvae exposed to BPA and genistein individually and in combinations. Graph plotted as means with standard deviation error bars. The capital letters signify survival significantly different from: C – controls and B – corresponding treatment with BPA only. Asterisks indicate statistical significance of the differences (** P≤0.01 and *** P≤0.001, Tukey’s test).

At **96 hpf** not only 16 mg/L BPA + 5 mg/L genistein, but also 4 mg/L BPA + 2.5 mg/L genistein and 8 mg/L BPA + 2.5 mg/L genistein caused significant decrease in the survival of the exposed larvae when compared to the controls (ANOVA followed by a Tukey’s test) (Figure 5.4, see also appendix B, Table 8.1). The effects of these combined treatments did, however, not differ from the sole administration of genistein at matching concentrations. Moreover, for the 16 mg/L BPA + 5 mg/L genistein mixture, both its components caused similar effects when applied individually. As a result, no enhanced toxicity of the tested EDCs could be deduced based on the observation of the zebrafish larvae’ survival at 96 hpf.
96 hpf survival of zebrafish embryos exposed to individual and combined EDCs

Figure 5.4 Survival of the 96 hpf zebrafish larvae exposed to BPA and genistein individually and in combinations. Graph plotted as means with standard deviation error bars. The capital letters signify survival significantly different from: C-controls and B – corresponding treatment with BPA only. Asterisks indicate statistical significance of the differences (*** P≤0.001, Tukey’s test).

At 120 hpf, the new observation compared to the previous days was a significant decrease in the survival of the larvae exposed to the mixture of 8 + 1.25 mg/L BPA (Figure 5.5). This indicates a stronger effect of a combination treatment since sole treatments with either 8 mg/L BPA or 1.25 mg/L genistein did not affect survival of the larvae in a statistically significant fashion when compared to the controls (ANOVA followed by Tukey’s test). Further analysis revealed, however, that although this combined treatment affected survival of the larvae in a significantly different way than 1.25 mg/L genistein (P=0.010), its effect was not different from a sole administration of BPA at the 8 mg/L concentration.

All the mixtures containing 2.5 mg/L genistein also affected survival of 120 hpf larvae in a significant way, however, as on the previous days, this effect could be assigned to the sole toxicity of genistein. Based on these observations, no obvious additive or synergistic effects between the tested EDCs could be concluded.
Figure 5.5 Survival of the 120 hpf zebrafish larvae exposed to BPA and genistein individually and in combinations. Graph plotted as means with standard deviation error bars. The capital letters signify survival significantly different from: C—controls, B—corresponding treatment with BPA only and G—corresponding treatment with genistein only. Asterisks indicate statistical significance of the differences (** P≤0.01 and *** P≤0.001, Tukey’s test).

**Individual and combined effect of BPA and genistein on zebrafish morphology**

Morphological assessment of 24 hpf embryos revealed no major signs of increased toxicity of BPA and genistein when applied together (Figure 5.7). Only a few minor differences could be noticed when morphological scores were compared between the embryos treated with the mixtures and the groups exposed to the individual matching EDCs. For example, while there were no head morphology changes in either the 4 mg/L BPA (Figure 5.6 B) or 1.25 mg/L genistein treated embryos (Figure 5.6 E) (mean scores of 5.00), the larvae treated with their mixture (Figure 5.6 H) received a mean score of 4.88 for the head morphology. The 4 mg/L BPA + 1.25 mg/L genistein treated embryos had also slightly increased yolk sac (mean score=4.00) comparing to the embryos treated with the corresponding individual compounds (score=5.00 for either BPA or genistein treatment alone). Similarly, the mean total morphological score of the 4 mg/L BPA + 2.5 mg/L genistein treated embryos (Figure 5.6 I) was slightly lower (22.85) in comparison to the scores of the embryos treated solely with either BPA (score=25, Figure 5.6 B) or genistein (score=23.37, Figure 5.6 F). Analysis of the individual scores revealed that this difference resulted mainly from somewhat smaller heads (score=4.22) and slightly enlarged yolk extension (score=4.63) in the mixture group than in the individual EDCs groups (score=4.75 or 5.00 for the head and score=5.00 and 5.00 for the yolk extension, for genistein or BPA treated embryos, respectively). Slight antagonistic effects could however also be observed in this combination treatment group. While the mean score for the yolk sac was 3.75
for the genistein and 5.00 for the BPA treated embryos, this value was equal to 4.72 for the mixture exposed embryos.

All embryos exposed to the mixture of 16 + 5 mg/L BPA and genistein were dead and their bodies were severely malformed (lack or a critically reduced head size, lack of eyes, tail malformations or no tail, heart-region and yolk sac oedema) as can be seen in Figure 5.6 L. This clearly shows enhanced toxicity because there was above 50% survival in the BPA or genistein treated embryos, as shown in the previous section in Figure 5.1. Moreover, the malformations observed in the embryos treated with 16 mg/L BPA + 5 mg/L genistein were much more severe (the mean total morphological score=5) than those seen in the 16 mg/L BPA (score=21.17, Figure 5.6 D) or 5 mg/L genistein (score=14.42, Figure 5.6 G). Based on these observations one might surmise that BPA at 16 mg/L and genistein at 5 mg/L concentrations show enhanced teratogenic effect on zebrafish embryos when applied simultaneously.

Figure 5.6 Morphology of 24 hpf zebrafish embryos exposed to BPA and genistein individually and in mixtures. A – control (DMSO); B – 4 mg/L BPA; C – 8 mg/L BPA; D – 16 mg/L BPA; E – 1.25 mg/L genistein, F – 2.5 mg/L genistein; G – 5 mg/L genistein; H -4 mg/L BPA + 1.25 mg/L genistein; I - 4 mg/L BPA + 2.5 mg/L genistein; J – 8 mg/L BPA + 1.25 mg/L genistein; K - 8 mg/L BPA + 2.5 mg/L genistein; L - 16 mg/L BPA + 5 mg/L genistein. Arrows indicate reduced head, enlarged yolk sac, enlarged yolk extension and heart oedema.
Figure 5.7 Total morphological scores of 24 hpf embryos treated with BPA and genistein individually and in combinations. The percentage of the embryos scored was: 6.7% for 8 mg/L BPA, ≥10% for 1.25 mg/L genistein and 2.5 mg/L genistein and ≥20% for all the remaining treatment groups.

At 48 hpf there were some indicators of slightly enhanced teratogenicity of BPA and genistein when applied simultaneously based on the morphological scoring analysis of the treated embryos. Firstly, while the mean total morphological scores for the embryos treated with either 4 mg/L BPA (Figure 5.8 B) or 1.25 mg/L genistein (Figure 5.8 D) were 29.33 and 30.00, respectively, the embryos exposed to the combination of these two EDCs received the mean total score of 25.28 (Figure 5.9). Skin pigmentation level was one of the parameters which differed across groups: while for the BPA treated embryos pigmentation was scored as 4.92 and for the genistein only exposed embryos it was assessed as 5.00, in the embryos exposed to the mixture the pigmentation was observably reduced (3.65). Similarly there was an indication of the enhanced combined toxicity of BPA and genistein, when the morphology of yolk sacs and yolk extension was analysed. For the embryos exposed either to BPA or genistein, the mean scores were 4.67 and 5.00 for the yolk sac and 4.92 and 5.00 for the yolk extension, respectively. For the 4 mg/L BPA + 1.25 mg/L genistein treated embryos the yolk sac and yolk extension were enlarged and received mean scores of 3.69 and 3.67, respectively.

Addition of BPA slightly enhanced toxicity of 2.5 mg/L genistein based on the comparison of the mean total morphological scores of the embryos treated with 4 mg/L BPA + 2.5 mg/L genistein (Figure 5.8 G) or 8 mg/L BPA + 2.5 mg/L genistein (Figure 5.8 I) and the scores of the embryos exposed to the individual, corresponding compounds (Figure 5.8 B, C and E). Particularly affected by these mixtures was tail morphology (enhanced malformations) and skin pigmentation level (considerable decrease).
Antagonistic effects of BPA and genistein could also be observed in this study since there were fewer embryos presenting heart oedema when treated with 4 mg/L BPA + 2.5 mg/L genistein (a mean score of 3.33) than in the embryos exposed solely to 2.5 mg/L genistein (a score of 3.20).

Figure 5.8 Morphology of 48 hpf zebrafish embryos exposed to BPA and genistein individually and in mixtures. A - control (DMSO); B - 4 mg/L BPA; C - 8 mg/L BPA; D - 1.25 mg/L genistein; E - 2.5 mg/L genistein; F - 4 mg/L BPA + 1.25 mg/L genistein; G - 4 mg/L BPA + 2.5 mg/L genistein; H - 8 mg/L BPA + 1.25 mg/L genistein; I - 8 mg/L BPA + 2.5 mg/L genistein. Arrows indicate malformed tails, enlarged yolk sac or yolk extension. Stars indicate reduced skin pigmentation.
Similarly to the previous days, the morphological analysis of the 72 hpf larvae exposed to BPA and genistein individually and in combination revealed some indices of the slightly enhanced toxicity of two compounds when administered simultaneously (Figure 5.11). When the mean total morphological scores for the larvae treated with the mixtures were compared with the analogous scores for the larvae exposed to corresponding single compounds, the most noticeable differences were found for 4 mg/L BPA + 1.25 mg/L genistein (Figure 5.10 F) and 8 mg/L BPA + 1.25 mg/L (Figure 5.10 H). While 1.25 mg/L genistein had no obvious effects on the larval morphology (a score of 30.00, Figure 5.10 D) and the 4 mg/L BPA treated larvae (Figure 5.10 B) showed mild morphological alterations and were scored as 28.50, the score of 26.06 was given to the larvae exposed to 4 mg/L BPA + 1.25 mg/L genistein. Similarly to the 48 hpf results, the most noticeable differences were seen for the yolk sac, yolk extension (enlargement) and skin pigmentation (reduction). For the 8 + 1.25 treated larvae the head morphology and skin pigmentation constituted parameters whose scores differed most noticeable when compared to the single compound exposed larvae. While the reduction in the head size and pigmentation level in the mixture treated larvae were scored as 4.43 and 2.79, the analogous scores in the larvae exposed to BPA only were 4.86 and 3.71 and in the genistein solely exposed larvae there were no changes observed (both scores equal to 5). The analysis of the heart morphology revealed, however, slightly less toxic properties of the mixture. While the exposure to 8 mg/L BPA was observed to cause mild heart oedema (a mean score of 4.86) no effects on the heart morphology were observed in the 8 mg/L BPA + 1.25 mg/L genistein treated larvae (a score of 5.00).

In the larvae treated with 4 mg/L BPA + 2.5 mg/L genistein (Figure 5.10 G) or 8 mg/L BPA + 2.5 mg/L genistein (Figure 5.10 I) severe malformations affecting head (reduced size and necrotic...
brain tissue) and tail (kinked tails) were observed. In addition, heart and yolk oedema were a common feature for these treatment groups. These effects, however, were not different when compared to the morphological changes observed in the larvae treated with 2.5 mg/L genistein alone (Figure 5.10 E).

As the 5 mg/L concentration of genistein caused 100% mortality at 72 hpf, as did the combination with 16 mg/L BPA morphology could not be assessed in these larvae (Figure 5.11).

![Figure 5.10 Morphology of 72 hpf zebrafish larvae exposed to BPA and genistein individually and in mixtures. A - control (DMSO); B - 4 mg/L BPA; C - 8 mg/L BPA; D - 1.25 mg/L genistein; E - 2.5 mg/L genistein; F - 4 mg/L BPA + 1.25 mg/L genistein; G - 4 mg/L BPA + 2.5 mg/L genistein; H - 8 mg/L BPA + 1.25 mg/L genistein; I - 8 mg/L BPA + 2.5 mg/L genistein. Arrows indicate enlarged yolk sac, enlarged yolk extension and reduced head size. Stars indicate reduced skin pigmentation.](image-url)
At 96 hpf the toxicity of the tested mixtures containing 1.25 mg/L genistein increased comparing to the previous day showing time dependent toxicity (Figure 5.13 vs. Figure 5.11). This effect was correlated with the higher toxicity of the 1.25 mg/L genistein alone, which at 96 hpf caused total morphological changes scored as 27.88 while it caused no effect at 72 hpf.

Here again the mixture of 4 mg/L BPA + 1.25 mg/L genistein showed slightly enhanced toxicity when compared to the effects of its individual corresponding compounds. To elaborate, while the mean total morphological score for the 4 mg/L BPA treated larvae (Figure 5.12 B) was equal to 29.83 and for the 1.25 mg/L genistein treatment group (Figure 5.12 D) was 27.88, the morphology of the mixture exposed larvae was scored as 21.89 (Figure 5.12 F). The analysis of the individual scores revealed that this difference was mainly the result of the increased yolk sac and yolk extension, more severe heart malformations and further reduction in the skin pigmentation.

For the 8 mg/L BPA + 1.25 mg/L genistein treated larvae (Figure 5.12 H) the mean total morphological score was 18.09 and was lower than the scores given to the larvae treated with either BPA (a score of 20.07, Figure 5.12 C) or genistein (a score of 27.88, Figure 5.12 D) on its own. The main differences were observed in the head size and skin pigmentation level (increased reduction for both in the mixture treated larvae).

Similarly to the observations at 72 hpf, there were no clear indices of enhanced toxicity of the tested EDCs for the mixtures of 4 mg/L BPA + 2.5 mg/L genistein and 8 mg/L BPA + 2.5 mg/L genistein. Although the mixtures affected the morphology of exposed larvae in a most severe way (Figure 5.12 G and I) equally severe malformations were observed in the larvae exposed solely to 2.5 mg/L genistein (Figure 5.12 E).
Figure 5.12 Morphology of 96 hpf zebrafish larvae exposed to BPA and genistein individually and in mixtures. A - control (DMSO); B - 4 mg/L BPA; C - 8 mg/L BPA; D - 1.25 mg/L genistein; E - 2.5 mg/L genistein; F - 4 mg/L BPA + 1.25 mg/L genistein; G - 4 mg/L BPA + 2.5 mg/L genistein; H - 8 mg/L BPA + 1.25 mg/L genistein; I - 8 mg/L BPA + 2.5 mg/L genistein. Arrows indicate enlarged yolk sac, enlarged yolk extension, reduced head size and increased heart oedema. Stars indicate reduced skin pigmentation.

Figure 5.13 Total morphological scores of 96 hpf larvae treated with BPA and genistein individually and in combinations. The percentage of the larvae scored was ≥10% for the 1.25 mg/L genistein and 4 mg/L BPA, and ≥20% for all the remaining treatment groups.

At **120 hpf** over 60% of the larvae exposed to 4 mg/L BPA + 1.25 mg/L genistein had severe heart oedema, enlarged yolk sacs and yolk extensions as well as weaker body pigmentation and smaller heads when compared to the control group (Figure 5.14 E). Consequently the mean total morphological score (18.72) for this group was lower than for the controls (Figure 5.15). Moreover, these morphological alterations were to some extent more distinct than in
the 1.25 mg/L genistein (Figure 5.14 D) or 4 mg/L BPA solely treated larvae (Figure 5.14 B) which received the mean scores of 20.61 and 29.25, respectively.

Minimally increased toxicity of BPA and genistein when applied together was also observed for the mixture of 8 mg/L BPA + 1.25 mg/L genistein (Figure 5.14 F). The difference between the mean total morphological score of the mixture exposed larvae (15.78) was considerably lower than the score given to the genistein only treated larvae (score=20.61, Figure 5.14 D)) but only slightly lower than the score for the larvae exposed solely to BPA (score=16.94, Figure 5.14 C).

The larvae from all the remaining mixtures tested in this study were dead at this time point and received the mean total morphological scores indicating lethal malformations of the body (Figure 5.15).

Figure 5.14 Morphology of 120 hpf zebrafish larvae exposed to BPA and genistein individually and in mixtures. A - control (DMSO); B – 4 mg/L BPA; C – 8 mg/L BPA; D – 1.25 mg/L genistein; E - 4 mg/L BPA + 1.25 mg/L genistein; F - 8 mg/L BPA + 1.25 mg/L genistein Arrows indicate enlarged yolk sac, enlarged yolk extension, reduced head size and increased heart oedema. Stars indicate reduced skin pigmentation.
Individual and combined effect of BPA and genistein on the heart rate of 48 and 72 hpf zebrafish

There was a statistically significant difference in the heart rate across 48 hpf zebrafish exposed to all the tested treatments ($P \leq 0.001$ obtained with one-way ANOVA). A specific comparison between individual treatment groups (Tukey’s test) demonstrated that both the treatment with 8 mg/L BPA and the exposure to 2.5 mg/L genistein caused a significant decrease in the number of heart beats per minute when compared to the controls (Figure 5.16, see also appendix B, Table 8.12). From all the tested combinations of BPA and genistein only 4 mg/L BPA + 1.25 mg/L genistein showed no effect on heart rate in comparison to the control group. When heart rate was compared between the embryos exposed to the combinations of the EDCs and the embryos treated with the corresponding individual components of the mixtures, no indication of the enhanced toxicity of the combination treatments could be concluded.
Analysis of the heart rate of the 72 hpf zebrafish larvae treated with BPA or/and genistein showed that the treatments with 8 mg/L BPA, 2.5 mg/L genistein and with the mixtures containing either or both of them, affects zebrafish heart rate in a statistically significant manner when compared to the controls (ANOVA followed by Tukey’s test). When heart rate of the larvae exposed to the BPA and genistein combinations were compared with the heart rate of the larvae treated with analogous, individual EDCs, several significant differences were demonstrated (Figure 5.17, see also appendix B, Table 8.1). Both 4 + 2.5 and 8 + 2.5 mg/L BPA plus genistein affected heart rate in a manner significantly different than BPA at the corresponding concentrations. The comparison to the matching genistein concentrations revealed, however, that these effects were not different indicating that the toxicity of these mixtures was caused by genistein. In contrast, the combination of 8 mg/L + 1.25 mg/L BPA plus genistein influenced heart rate in a manner significantly different than genistein but not the BPA component of the mixture. Consequently, no evidence of increased toxicity of BPA and genistein in combination could be exposed based on heart rate of the 72 hpf zebrafish.
72 hpf heart rate of zebrafish embryos exposed to individual and combined EDCs

Figure 5.17 Heart rate of the 72 hpf zebrafish larvae treated with BPA and genistein individually and in combinations. Graph plotted as means with standard deviation error bars. The capital letters indicate heart rate significantly different from: C – controls, B – corresponding treatment with BPA only, G – corresponding treatment with genistein only. Asterisks indicate statistical significance of the differences (** P≤0.001, Tukey’s test).

Individual and combined effect of BPA and genistein on hatching success of 72 hpf zebrafish

The comparison of the hatching success of the 72 hpf larvae exposed to the tested solutions showed that 8 mg/L BPA, 2.5 mg/L genistein and all the mixtures containing one or both of them reduce the number of hatched larvae in a significant way (ANOVA followed by a Tukey’s test). Interestingly, although 4 mg/L BPA or 1.25 mg/L genistein had no effect on hatching success of 72 hpf zebrafish, a combination of these two compounds caused a significant reduction in the number of hatched larvae (P≤0.001) in contrast to a control group (Figure 5.18; see also appendix B, Table 8.13). Moreover, the effect of the 4 mg/L BPA + 1.25 mg/L genistein mixture (64.81 ± 13.98 % hatched larvae) was significantly different than the effects of the corresponding concentrations of BPA and genistein individually (96.30 ± 6.41 % and 100.00 ± 0.00%, respectively). This observation indicates an enhanced toxicity of BPA and genistein on the zebrafish hatching when applied simultaneously.

No other mixture tested in this study showed the effect significantly different from both of its components when administered individually. While 4 mg/L BPA + 2.5 mg/L genistein had significantly different effect on zebrafish hatching than 4 mg/L BPA, it was no different than a result of 2.5 mg/L genistein. In contrast, 8 mg/L BPA + 1.25 mg/L genistein resulted in decreased hatching success compared to 1.25 mg/L genistein, but not 8 mg/L BPA.
Hatching of zebrafish embryos exposed to individual and combined EDCs

Figure 5.18 Hatching success of the 72 hpf zebrafish larvae treated with BPA and genistein individually and in combinations. % hatching is the percentage of live larvae that hatched and survival was: 100% for 0 + 0 and 8 + 1.25, 96.7% for 0 + 1.25, 93.3% for 4 + 0, 0 + 2.5, 4 + 1.25 and 4 + 2.5 and 90% for 8 + 0 and 8 + 2.5 mg/L BPA + genistein. Graph plotted as means with standard deviation error bars. The capital letters indicate hatching success significantly different from: C - controls, B - corresponding treatment with BPA only, G - corresponding treatment with genistein only. Asterisks indicate statistical significance of the differences (*** P≤0.001, Tukey’s test).
5.2.2 The combined effects of BPA and genistein on the liver ultrastructure of larval zebrafish

In this study the fine structure of the liver in the 120 hpf zebrafish larvae treated with the mixtures of BPA and genistein were investigated. Following solutions were tested: 10 μg/L BPA + 312.5 μg/L genistein, 10 μg/L + 625 μg/L BPA genistein, 100 μg/L BPA + 312.5 μg/L genistein and 100 μg/L BPA + 625 μg/L genistein. Zebrafish embryos were placed in the tested solutions at 2-4 cell stage (0.75-1 hpf) and were exposed to them until 120 hpf. Morphometrical analysis and morphological description of the liver were carried out for every treatment group and compared to the controls, i.e. the larvae reared in 0.01% DMSO. In addition, the effects of the mixtures were compared with the effects of the corresponding, individual compounds, i.e. BPA or genistein at matching concentrations. The ultrastructure of the liver in control larvae and in the larvae treated with BPA only has been described in Section 3.2.2 while the characteristics of the liver of the genistein treated larvae have been presented in Section 4.2.2.

- The effects of individual and combined exposure to BPA and genistein on the liver ultrastructure of 120 hpf zebrafish – morphometric analysis

  - Volume of the hepatocytes

There was a statistically significant difference in the volume of hepatocytes across the treatment groups investigated in this study ($P=0.031$, Kruskal-Wallis test). Pair-wise comparisons (Mann-Whitney U test) between the larvae treated with oestrogenic compounds and the control group (503.92 ± 40.18 μm$^3$) revealed that the treatments with 10 μg/L BPA + 625 μg/L genistein and with 100 μg/L BPA + 312.5 μg/L genistein caused a significant reduction in the volume of the liver cells (381.08 ± 42.60 μm$^3$ and 373.93 ± 99.49 μm$^3$, respectively). Since no significant difference in the measured parameter was observed for any of the treatments with individual, corresponding components of the mixtures, the mutual enhancement of BPA and genistein cannot be excluded. Moreover, the effects of these combined treatments was the same as the effect of the treatment with 20 μg/L β-oestradiol ($P=1$ for both comparisons, Mann-Whitney U test). Further analysis showed that the effects observed for the treatments with 10 μg/L BPA + 625 μg/L genistein and 100 μg/L BPA + 312.5 μg/L genistein were statistically different than the effects of sole exposure to BPA but no different than the results of the treatments with genistein alone (Figure 5.19, see also appendix B, Tables 8.14 and 8.15).
Absolute volume of hepatocytes in 120 hpf zebrafish exposed to BPA and genistein individually and in combination

Figure 5.19 Absolute volumes of hepatocytes in 120 hpf zebrafish exposed to BPA and genistein individually and in mixtures. Data presented as boxplots (median and quartiles) with whiskers from minimum to maximum. The capital letters indicate a significant difference from: C - controls (DMSO), B – corresponding treatment with BPA only and E – positive controls (β-oestradiol). Asterisks specify statistical significance of the differences (*P≤0.05, Mann-Whitney U test).

- Nuclear volume of hepatocytes

Both the nuclear volume fraction and the absolute volume of the nuclei were similar when compared across the treatment groups (P=0.180 and P=0.257, respectively; Kruskal-Wallis test). Subsequent pair-wise comparisons revealed that 10 µg/L BPA + 625 µg/L genistein was the only treatment which caused significant increase in the nuclear volume fraction comparing to the control group (P=0.042, Mann-Whitney U test) as presented in Figure 5.20. This treatment, however, had no effect on the absolute volume of the nuclei in comparison to the controls (P=0.149, Mann-Whitney U test; Figure 5.21).

None of the mean absolute volumes of the nuclei in the zebrafish larvae treated with oestrogenic compounds, either individually or in combinations, was different from the volume of the nuclei in the hepatocytes of the larvae reared in DMSO solution (55.37 ± 7.20 µm³) when pair-wise comparisons were performed (Mann-Whitney U test) as presented in Figure 5.21 (see also appendix B, Tables 8.14 and 8.15).
Figure 5.20 Volume fractions of the nuclei in the hepatocytes of 120 hpf zebrafish exposed to BPA and genistein individually and in mixtures. Data presented as boxplots (median and quartiles) with whiskers from minimum to maximum. The capital letters indicate a significant difference from: C – controls (DMSO), E – positive controls (∆-oestradiol). Asterisks specify statistical significance of the differences (*P≤0.05, Mann-Whitney U test).
Absolute volume of nuclei in hepatocytes of 120 hpf zebrafish exposed to BPA and genistein individually and in combination

Figure 5.21 Absolute volumes of the nuclei in the hepatocytes of 120 hpf zebrafish exposed to BPA and genistein individually and in mixtures. Data presented as boxplots (median and quartiles) with whiskers from minimum to maximum. The capital letters indicate a significant difference from: B – corresponding treatment with BPA only. Asterisks specify statistical significance of the differences (*P≤0.05, Mann-Whitney U test).

- Pooled mitochondrial volume of hepatocytes

Both the volume fraction and the absolute volume of mitochondria were similar when compared across all the treatment groups (P=0.490 and P=0.053, respectively; Kruskal-Wallis test). Specific comparison testing of the mitochondrial volume fraction showed that the only treatments which resulted in the significant alteration when compared to the controls were 10 μg/L BPA and 625 μg/L genistein (P=0.043 and P=0.021, respectively; Mann-Whitney U test) as one see in Figure 5.22 (see also appendix B, Tables 8.14 and 8.15).

Further analysis revealed that the treatments with 10 μg/L BPA, 10 μg/L BPA + 312.5 μg/L genistein and 10 μg/L BPA + 625 μg/L genistein caused a significant decrease in the mean absolute volume of mitochondria in comparison to the controls (P=0.021, for all comparisons; Mann-Whitney U test; Figure 5.23). When the effects of the combinations were compared to the individual effects of BPA and genistein, no significant differences were revealed (Figure 5.23).
Volume fraction of pooled mitochondria in hepatocytes of 120 hpf zebrafish exposed to BPA and genistein individually and in combination

![Graph showing volume fraction of pooled mitochondria](image)

Figure 5.22 Volume fractions of pooled mitochondria in the hepatocytes of 120 hpf zebrafish exposed to BPA and genistein individually and in mixtures. Data presented as boxplots (median and quartiles) with whiskers from minimum to maximum. The capital letters indicate a significant difference from: C - controls (DMSO). Asterisks specify statistical significance of the differences (*P≤0.05, Mann-Whitney U test).
Individual mitochondrial volume of hepatocytes

The volume fraction of the individual mitochondria was similar across the treatment groups \((P=0.520, \text{Kruskal-Wallis test})\). Specific comparison testing showed that only exposure to the individual compounds (10 µg/L BPA, 312.5 µg/L genistein or 625 µg/L genistein) but none of the combination treatments changed the volume fraction in a significant way in comparison to the controls (Mann-Whitney U test, Figure 5.24).

When the mean absolute volume of individual mitochondria in the liver cells was compared across all the treatment groups no significant difference was found \((P=0.285, \text{Kruskal-Wallis test})\). A specific comparison of this parameter between the larvae treated with oestrogenic compound(s) and the controls revealed several differences. Mitochondria were significantly smaller in the zebrafish larvae treated with 10 µg/L BPA \((5.03 \pm 0.98 \mu m^3)\), 312.5 µg/L genistein \((4.91 \pm 0.51 \mu m^3)\) or 625 µg/L genistein \((4.44 \pm 0.60 \mu m^3)\) than in the larvae reared in 0.01% DMSO \((7.76 \pm 0.68 \mu m^3)\) as presented in Figure 5.25. Moreover, the exposure to the mixtures of 10 µg/L BPA + 625 µg/L genistein or 100 µg/L BPA + 312.5 µg/L genistein caused decrease in...
the volume of this organelle (5.05 ± 1.62 and 5.93 ± 2.73 µm³, respectively). Interestingly, although both 10 µg/L BPA and 312.5 µg/L genistein caused a significant decrease in the volume of mitochondria the combination of the two had no effect on the measured parameter (4.51 ± 2.74 µm³).

No differences were exhibited when the volume fraction or the absolute volume of the individual mitochondria were compared between the BPA or/and genistein treated larvae and the positive controls, i.e. the larvae treated with 20 µg/L β-oestradiol (Figure 5.24 and 5.25, see also appendix B, Tables 8.14 and 8.15).

![Volume fraction of individual mitochondria in hepatocytes of 120 hpf zebrafish exposed to BPA and genistein individually and in combination](image)

**Figure 5.24** Volume fractions of single mitochondrion in the hepatocytes of 120 hpf zebrafish exposed to BPA and genistein individually and in mixtures. Data presented as boxplots (median and quartiles) with whiskers from minimum to maximum. The capital letters indicate a significant difference from: C - controls (DMSO). Asterisks specify statistical significance of the differences (*P≤0.05, Mann-Whitney U test).
Absolute volume of individual mitochondria in hepatocytes of 120 hpf zebrafish exposed to BPA and genistein individually and in combination.

Figure 5.25 Absolute volumes of single mitochondrion in the hepatocytes of 120 hpf zebrafish exposed to BPA and genistein individually and in mixtures. Data presented as boxplots (median and quartiles) with whiskers from minimum to maximum. The capital letters indicate a significant difference from: C - controls (DMSO). Asterisks specify statistical significance of the differences (*P≤0.05, Mann-Whitney U test).

- Volume of rough endoplasmic reticulum in hepatocytes

The comparison of the volume fraction of RER across the treatment groups showed lack of a significant difference (P=0.061, Kruskal-Wallis test). Specific comparisons revealed that the treatment with 625 µg/L genistein significantly increased the volume fraction of RER in comparison to the controls (P=0.043, Mann-Whitney U test). Moreover, none of the mixtures containing 625 µg/L genistein had such an effect on the RER volume fraction based on the comparisons to the controls and to the sole genistein treatment (Figure 5.26, see also appendix B, Tables 8.14 and 8.15).

The mean absolute volume of rough endoplasmic reticulum was significantly different when compared across the treatment groups (P=0.007, Kruskal-Wallis test). Specific pair-wise comparison between the larvae treated with oestrogenic compound(s) and the controls (25.23 ± 4.26 µm³) revealed that both 10 µg/L BPA + 625 µg/L genistein and 100 µg/L BPA + 625 µg/L genistein caused a significant decrease in the RER volume (P=0.021 and 0.043, respectively, Mann-Whitney U test). Since sole exposure to 10 µg/L BPA, 100 µg/L BPA or 625 µg/L genistein had no effect on the RER volume, a joint interaction between these EDCs cannot be ruled out.
Moreover, when the volume of RER in the hepatocytes of larvae exposed to 100 µg/L BPA (43.63 ± 18.39 µm³) or 625 µg/L genistein (27.06 ± 4.10 µm³) were compared with the larvae treated with 100 µg/L BPA + 625 µg/L genistein (18.13 ± 3.23 µm³), statistically significant differences were revealed. This observation may indicate an enhanced effect of BPA and genistein when in combination.

Although a significant decrease in the RER volume in the larvae treated with the combination of 10 µg/L BPA + 625 µg/L genistein was different than the effect caused solely by 625 µg/L genistein (P=0.021), it was similar to what was observed in the larvae reared in 10 µg/L BPA (P=0.149; Figure 5.27). In addition, statistically different effects on the RER volume were demonstrated between 312.5 µg/L genistein vs. 10 µg/L BPA + 312.5 µg/L genistein, and 100 µg/L BPA in comparison to 100 µg/L BPA + 312.5 µg/L genistein.

Only the effect of the treatment with 100 µg/L BPA was significantly different than what was observed for the 20 µg/L β-oestradiol treated larvae. All the remaining treatments with BPA or/and genistein caused similar effects on the RER absolute volume as the treatment with the natural oestrogen (Figure 5.27).

![Volume fraction of RER in hepatocytes of 120 hpf zebrafish exposed to BPA and genistein individually and in combination](image)

**Volume fraction of RER in hepatocytes of 120 hpf zebrafish exposed to BPA and genistein individually and in combination**

- **C** - controls (DMSO), **G** – corresponding treatment with genistein only, **E** – positive controls (β-oestradiol).
- Asterisks specify statistical significance of the differences (*P≤0.05, Mann-Whitney U test).
Absolute volume of RER in hepatocytes of 120 hpf zebrafish exposed to BPA and genistein individually and in combination

Figure 5.27 Absolute volumes of rough endoplasmic reticulum in the hepatocytes of 120 hpf zebrafish exposed to BPA and genistein individually and in mixtures. Data presented as boxplots (median and quartiles) with whiskers from minimum to maximum. The capital letters indicate a significant difference from: C – controls (DMSO), B – corresponding treatment with BPA only, G – corresponding treatment with genistein only, E – positive controls (β-oestradiol). Asterisks specify statistical significance of the differences (*P≤0.05, Mann-Whitney U test).

Volume of glycogen

There was a statistically significant difference in the volume fraction and the mean absolute volume of glycogen when compared among all the treatment groups (P=0.029 and P=0.017, respectively; Kruskal-Wallis test). While the treatments with individual compounds, i.e. with 10 µg/L BPA, 100 µg/L BPA or 312.5 µg/L genistein significantly increased the volume fraction of glycogen in comparison to the control group, none of the mixtures had a similar effect (Mann-Whitney U test, Figure 5.28).

A specific comparison testing (Mann-Whitney U test) of the mean absolute volumes of glycogen revealed significantly more glycogen in the hepatocytes of the larvae treated with 10 µg/L BPA, 100 µg/L BPA or 312.5 µg/L genistein (20.60 ± 41.20, 93.51 ± 40.10 and 79.46 ± 40.82 µm³, respectively) than the liver cells of the control larvae (29.76 ± 19.58 µm³). Interestingly, the exposure to the possible combinations of these compounds, namely 10 µg/L BPA + 312.5 µg/L genistein and 100 µg/L BPA + 312.5 µg/L genistein, had no effect on the glycogen absolute volume. Moreover, neither the remaining combination treatments nor the exposure to the
natural oestrogen caused significant changes in the measured parameter when compared to the controls (Figure 5.29, see also appendix B, Tables 8.14 and 8.15).

Figure 5.28 Volume fractions of glycogen in the hepatocytes of 120 hpf zebrafish exposed to BPA and genistein individually and in mixtures. Data presented as boxplots (median and quartiles) with whiskers from minimum to maximum. The capital letters indicate a significant difference from: C – controls (DMSO), B – corresponding treatment with BPA only, G – corresponding treatment with genistein only, E – positive controls (β-oestradiol). Asterisks specify statistical significance of the differences (*P≤0.05, Mann-Whitney U test).
Absolute volume of glycogen in hepatocytes of 120 hpf zebrafish exposed to BPA and genistein individually and in combination

Figure 5.29 Absolute volume of glycogen in the hepatocytes of 120 hpf zebrafish exposed to BPA and genistein individually and in mixtures. Data presented as boxplots (median and quartiles) with whiskers from minimum to maximum. The capital letters indicate a significant difference from: C – controls (DMSO), B – corresponding treatment with BPA only, G – corresponding treatment with genistein only, E – positive controls (β-oestradiol). Asterisks specify statistical significance of the differences (*P≤ 0.05, Mann-Whitney U test).

Both the volume fraction and the mean absolute volume of bile canaliculi were similar when compared across the treatment groups (P=0.109 and P=0.146, Kruskal-Wallis test). The only treatment which caused a significant change in the volume fraction of bile canaliculi compared to the control group was 10 µg/L BPA (P=0.042, Mann-Whitney U test). Additional differences in the volume fraction of bile canaliculi were exhibited between the 10 µg/L BPA treated larvae and the larvae treated with the mixtures of 10 µg/L BPA + 312.5 µg/L genistein and 10 µg/L BPA + 625 µg/L genistein (Figure 5.30, see also appendix B, Tables 8.14 and 8.15).

Pair-wise comparisons of the bile canaliculi absolute volume between the EDC(s) treated larvae and the controls showed that neither BPA nor genistein on their own caused significant alterations. Only the treatment with 100 µg/L BPA + 312.5 µg/L genistein caused a significant decrease in the volume of bile canaliculi (2.80 ± 1.87 µm³) when compared to the control group (11.12 ± 7.33 µm³, P=0.043; Mann-Whitney U test). In addition, a significant difference in the measured parameter was observed between the larvae treated with 10 µg/L BPA and the
larvae exposed to the combination of 10 µg/L BPA + 625 µg/L genistein ($P=0.021$, Mann-Whitney U test; Figure 5.31).

![Volume fraction of bile canaliculi in hepatocytes of 120 hpf zebrafish exposed to BPA and genistein individually and in combination](image)

*Figure 5.30 Volume fractions of bile canaliculi in the livers of 120 hpf zebrafish exposed to BPA and genistein individually and in mixtures. Data presented as boxplots (median and quartiles) with whiskers from minimum to maximum. The capital letters indicate a significant difference from: C – controls (DMSO), B – corresponding treatment with BPA only. Asterisks specify statistical significance of the differences (*$P\leq0.05$, Mann-Whitney U test).*
Absolute volume of bile canaliculi in hepatocytes of 120 hpf zebrafish exposed to BPA and genistein individually and in combination

**compound(s) concentration [μg/L]**

Figure 5.31 Absolute volumes of bile canaliculi in the livers of 120 hpf zebrafish exposed to BPA and genistein individually and in mixtures. Data presented as boxplots (median and quartiles) with whiskers from minimum to maximum. The capital letters indicate a significant difference from: C - controls (DMSO), B – corresponding treatment with BPA only. Asterisks specify statistical significance of the differences (*P≤0.05, ** P≤0.01 and *** P≤0.001, Mann-Whitney U test).
The effects of individual and combined exposure to BPA and genistein on the liver ultrastructure of 120 hpf zebrafish – morphological assessment

The morphology of the liver in the 120 hpf larvae treated with BPA + genistein mixtures were compared to the morphology observed for the control group, i.e. 0.01% DMSO treated larvae which has been described in Section 3.2.3. Additional comparisons were made between the liver of the larvae treated with the mixtures and the larvae exposed to BPA or genistein on its own which were described in Section 3.2.3 and Section 4.2.2, respectively.

Mixtures of 10 µg/L BPA + 312.5 µg/L genistein or 10 µg/L BPA + 625 µg/L genistein did not cause any apparent alterations in the nuclear appearance when compared to controls. Most nuclei had low amount of heterochromatin, single or two nucleoli and moderately regular outline (Figure 5.32, 5.33 and 5.35). Mitochondria in both examined groups displayed a high variation of shapes, but small roundish forms were more numerous than larger, irregular ones (Figure 5.32 and 5.35). No difference in the size of mitochondria could be definitely concluded based solely on the observation analysis. However, the number of mitochondria was noticeably lower than in the control group (Figures 3.65-3.67). The amount of RER was similar when the 10 µg/L BPA + 312.5 µg/L genistein treated larvae were compared to controls. The exposure to 10 µg/L BPA + 625 µg/L genistein, however, somewhat reduced the number of the RER cisternae (Figure 5.35). Bile canaliculi were less often encountered and of much smaller profiles in response to the treatment with 10 µg/L BPA + 312.5 µg/L genistein (Figure 5.32 and 5.33) than in the control group (Figure 3.67). The exposure to 10 µg/L BPA + 625 µg/L genistein did not evoke substantial changes in the biliary tracts' appearance and the only difference was diminished variation of canalicular sizes comparing to the controls. A high inter-individual, as well as inter-cellular diversity in the amount of glycogen was observed for animals exposed to both of these mixtures when compared to controls. There seemed to be more macrophages, lysosomes and/or myelin whorls, the latter mainly within glycogen fields, in the zebrafish exposed to the EDCs mixtures than in the controls (Figures 5.32-5.35).
Figure 5.32 Liver of the 120 hpf zebrafish exposed to 10 µg/L BPA + 312.5 µg/L genistein. N – nucleus of the hepatocyte; M – mitochondria; → rough endoplasmic reticulum; B – bile canaliculus; G – glycogen. Notice myelin whorls (mw) within glycogen fields and bile canaliculi (insert). Scale bar –2 µm.

Figure 5.33 Liver of the 120 hpf zebrafish exposed to 10 µg/L BPA + 312.5 µg/L genistein. N – nuclei of hepatocytes; M – mitochondria; → rough endoplasmic reticulum; B – bile canaliculi; MN – nuclei of macrophages residing in the area near to the biliary passages. The borders between cells are less clear when compared to controls. Scale bar –2 µm.
Figure 5.34 Liver of 120 hpf zebrafish exposed to 10 µg/L BPA + 625 µg/L genistein. N – nucleus of the hepatocyte; M – mitochondria; → rough endoplasmic reticulum; BC – bile canaliculi; G – glycogen, MN – macrophage’s nucleus; Notice myelin whorls amidst glycogen granules and in the BC. Scale bar –2 µm.

Figure 5.35 Liver of the 120 hpf zebrafish exposed to 10 µg/L BPA + 625 µg/L genistein. N – nucleus of the hepatocyte; M – mitochondria, small roundish forms are common; → rough endoplasmic reticulum; G – glycogen; mw -myelin whorl. Scale bar –2 µm.
In general, a considerable inter – and intra-individual heterogeneity was observed in the morphology of the hepatocytes in the 100 µg/L BPA + 312.5 µg/L genistein or 100 µg/L BPA + 625 µg/L genistein treated larvae. This was most particularly visible for RER and glycogen in the hepatocytes of the 100 µg/L BPA + 312.5 µg/L genistein treated larvae and for mitochondria in zebrafish exposed to either of these mixtures. While some hepatocytes were observed to have ‘normal’ amounts of RER (Figure 5.37), other showed substantial paucity of this organelle (Figures 5.38 and 5.41). The stacks of more than three RER membranes were observed occasionally and smaller aggregations or even individual RER cisternae were dominant (Figures 5.36 and 5.40). Mitochondria in the hepatocytes of the larvae from both treatment groups showed a wide variation of sizes and shapes (Figure 5.36-5.42), albeit small and round forms were by far the most common. In some hepatocytes the amount of these small mitochondria was clearly augmented while in others only a few were seen. The treatment with 100 µg/L BPA + 312.5 µg/L genistein or 100 µg/L BPA + 625 µg/L genistein did not have any obvious effects on the nuclear morphology since most of the nuclei looked similar to those observed in the liver of the control group (Figures 5.36, 5.39 and 5.41). The treatment with tested mixtures, however, to some degree influenced the hepatocytes’ membranes as the borders between the cells were often hard to discern (Figure 5.36, 5.37 and 5.40). Moreover, the hepatocytes were sometimes observed to differ in the electron density of their cytoplasmic compartments. In the larvae exposed to 100 µg/L BPA + 312.5 µg/L genistein bile canaliculi were encountered less frequently and were of smaller sizes when compared to the controls (Figure 5.38). Similar, but less sharpened difference was observed for the larvae reared in 100 µg/L + 625 µg/L genistein. On the whole, there has been a somewhat increased frequency of unusual figures such as myelin-like membranes amidst glycogen or in the bile canaliculi (Figures 5.37 and 5.40). A large multivesicular body (Figure 5.38), several lipid droplets (Figure 5.39) and a group of lymphocytes in a single blood vessel (Figure 5.41) – hence unusual structures in comparison to other treatment groups, were observed in the livers of the larvae treated with these BPA and genistein mixtures. Macrophages were repeatedly observed, mainly in the vicinity to the bile structures.
Figure 5.36 Liver of the 120 hpf zebrafish exposed to 100 µg/L BPA + 312.5 µg/L genistein. N – nuclei of the hepatocytes; M – mitochondria or reduced size and number; → rough endoplasmic reticulum. Notice lack of clear intercellular borders. Scale bar –2 µm.

Figure 5.37 Liver of the 120 hpf zebrafish exposed to 100 µg/L BPA + 312.5 µg/L genistein. N – nucleus of one of the hepatocytes; Nu – nucleolus; M – mitochondria; → rough endoplasmic reticulum; mw - myelin whorl. Notice electron-dense vesicles of presumable lysosomes (*). Scale bar –2 µm.
Figure 5.38 Liver of the 120 hpf zebrafish exposed to 100 µg/L BPA + 312.5 µg/L genistein. M – mitochondria; B – bile canaliculus; MV – a multivesicular body. Notice paucity of RER. Scale bar –2 µm.

Figure 5.39 Liver of the 120 hpf zebrafish exposed to 100 µg/L BPA + 312.5 µg/L genistein. N – nuclei of the hepatocytes; M – mitochondria; LD – lipid droplets. Scale bar –2 µm.
Figure 5.40 Liver of the 120 hpf zebrafish exposed to 100 µg/L BPA + 625 µg/L genistein. N – nucleus of a hepatocyte; M – mitochondria; → rough endoplasmic reticulum; B – bile canaliculi; G – glycogen, notice a myelin whorl (mw) and increased amount of presumable lysosomes (*). Scale bar –2 µm.

Figure 5.41 Liver of the 120 hpf zebrafish exposed to 100 µg/L BPA + 625 µg/L genistein. N – nucleus of a hepatocyte; M – mitochondria; → rough endoplasmic reticulum; S – sinusoid with lymphocytes (L). Notice myelin bodies in the bile canaliculus (insert). Scale bar –2 µm.
The most noticeable effect of 17β-oestradiol on the hepatocytes’ morphology was reduction in the variation of sizes and shapes of mitochondria when compared to the controls. Although the longitudinal, sometimes twisted mitochondria were present, small and round/oval forms were dominant (Figures 5.42 - 5.44). The differences in the appearance of the other observed parameters were not obvious. There was a degree of variation between the hepatocytes of individual larvae as well as within the same liver. RER in some cells was outstandingly abundant and organized in parallel stacks (Figure 5.43) while in others could be limited to a few membranes scattered throughout the cytoplasm (Figure 5.44). Some of the RER membranes showed indicators of entering into the process of fragmentation and dilation, however it was not an often phenomenon and not as obvious as observed for some previously described treatments. Glycogen in some hepatocytes was organized in clear, moderate-size aggregations (Figure 5.42) while in other, often neighbouring cells this storage product was not observed (Figure 5.44). No difference could be deduced based on the observation of the hepatocytes nuclei morphology in comparison to the control group (Figures 5.42 – 5.44). Macrophages were observed but their number did not differ significantly when compared to controls.

Figure 5.42 Liver of the 120 hpf zebrafish exposed to 20 µg/L 17β-oestradiol. N – nucleus of a hepatocyte; M – mitochondria; → RER; G – glycogen. Scale bar – 2 µm.
Figure 5.43 Liver of the 120 hpf zebrafish exposed to 20 µg/L 17β-oestradiol. N – nucleus of a hepatocyte; M – mitochondria of mostly small sizes; → RER, notice its increased amount. Scale bar –2 µm.

Figure 5.44 Liver of the 120 hpf zebrafish exposed to 20 µg/L 17β-oestradiol. N – nuclei of the hepatocytes; M – mitochondria; → RER of reduced amount; SD – space of Disse with microvilli. Insert –dilated cisterns of RER. Scale bar –2 µm.
Shape analysis of the mitochondrial profiles in the hepatocytes of the 120 hpf zebrafish larvae exposed to BPA or/and genistein.

Area of the mitochondrial profiles

When the areas of the mitochondrial profiles in the hepatocytes were compared among all the treatment groups (DMSO, 17β-oestradiol, 10 µg/L BPA, 100 µg/L BPA, 312.5 µg/L genistein, 625 µg/L genistein, 10 µg/L BPA + 312.5 µg/L genistein, 10 µg/L BPA + 625 µg/L genistein, 100 µg/L BPA + 312.5 µg/L genistein and 100 µg/L BPA + 625 µg/L genistein) no statistically significant difference was found (P=0.319, Kruskal-Wallis test). Several differences, however, were revealed when the mitochondrial profiles were compared between the larvae exposed to oestrogenic substance(s) and the control group (Mann-Whitney U test). There was a significant reduction in the areas of the mitochondrial profiles in the hepatocytes of the 10 µg/L BPA, 100 µg/L BPA, 312.5 µg/L genistein and 625 µg/L genistein treated larvae (Figure 5.45). Similarly, the mitochondrial profiles of significantly reduced areas were observed in the positive control group, i.e. in the hepatocytes of the 20 µg/L 17β-oestradiol treated larvae (Figure 5.45). None of the tested mixtures altered areas of the mitochondrial profiles when compared to the controls, although there was a clear trend (P=0.058) towards reduced areas in the 100 µg/L BPA + 312.5 µg/L genistein treatment group. In addition, the effects of the mixtures and of the treatments with individual compounds with the exception of 10 µg/L BPA were similar to the effects of the positive control treatment group (Figure 5.45).

The comparison of the mitochondrial profiles’ areas distribution between the larvae treated with oestrogenic compound(s) and the controls (Chi-squared test) revealed significant differences for the following treatment groups: 10 µg/L BPA, 100 µg/L BPA, 312.5 µg/L genistein and 625 µg/L genistein (Figure 5.46). In all cases there were more mitochondrial profiles of smaller area range and fewer larger profiles compared to the control group.
Area of mitochondrial profiles in the hepatocytes of the 120 hpf zebrafish exposed to BPA or/and genistein

Figure 5.45 Areas of the mitochondrial profiles in the hepatocytes of the 120 hpf zebrafish larvae exposed to BPA and genistein individually and in mixtures. Each symbol represents the median of 86 ± 16 mitochondria measured per larva (n=4). The capital letters indicate a significant difference from: C - controls (DMSO), E – positive controls (β-oestradiol). Asterisks specify statistical significance of the differences (*P≤0.05, Mann-Whitney U test).

Figure 5.46 Distribution of the areas of the mitochondrial profiles in the hepatocytes of the 120 hpf zebrafish larvae exposed to BPA and genistein individually and in mixtures. Data presented as the mean (n=4) of the percentages of the mitochondrial profiles of a given area range. Asterisks indicate values significantly different from the controls (*P≤0.05 and **P≤0.01, Chi-squared test).
Circularity of the mitochondrial profiles

The circularity of the mitochondrial profiles in the hepatocytes of the 120 hpf zebrafish larvae was similar amidst all the treatment groups \((P=0.343,\) Kruskal-Wallis test). Moreover, none of the individual or combined treatments with BPA and genistein altered the circularity of the mitochondrial profiles in a significant way when compared to the controls (Mann-Whitney U). The most noticeable trend towards increased circularity \((P=0.081)\) comparing to the controls was revealed for the 625 \(\mu\)g/L genistein treated larvae (Figure 5.47).

The comparison of the distributions of the mitochondrial profiles circularity \((\text{Chi-squared test})\) revealed statistically significant differences for both the treatments with 100 \(\mu\)g/L BPA and \(\beta\)-oestradiol when compared to the control larvae. There was a higher percentage of the mitochondrial profiles of increased circularity (the value approaching 1) in the hepatocytes of the larvae from the 100 \(\mu\)g/L BPA treatment group in comparison to the controls. In turn, there were more mitochondrial profiles deviating from the circular shape in the 17\(\beta\)-oestradiol treatment group comparing to the control larvae (Figure 5.48).

Figure 5.47 Circularity of the mitochondrial profiles in the hepatocytes of the 120 hpf zebrafish exposed to BPA or/and genistein. Each symbol represents the median of 86 ± 16 mitochondria measured per larva \((n=4)\). The capital letters indicate a significant difference from: G – corresponding treatment with genistein only, E – positive controls (\(\beta\)-oestradiol). Asterisks specify statistical significance of the differences (*)\(P\leq0.05\), Mann-Whitney U test).
Figure 5.48 Distribution of the circularity of the mitochondrial profiles in the hepatocytes of the 120 hpf zebrafish larvae exposed to BPA and genistein individually and in mixtures. Data presented as the mean (n=4) of the percentages of the mitochondrial profiles of a given circularity range. Asterisks indicate values significantly different from the controls (*P≤0.05 and *** P≤0.001, Chi-squared test).

- Maximum feret diameter of the mitochondrial profiles

When the medians of the maximum feret diameters of the mitochondrial profiles in the hepatocytes of the zebrafish larvae were compared between the treatment groups no statistically significant difference was found (P=0.222, Kruskal-Wallis test). Several significant differences, however, were revealed with the subsequent specific comparisons (Mann-Whitney U test) between the profiles observed in the oestrogenic compound treated larvae and the controls. Significant reduction in the maximum feret diameter of the mitochondrial profiles was observed in the result of the treatment with 10 µg/L BPA, 312.5 µg/L genistein and 625 µg/L genistein (Figure 5.49). An additional difference in the maximum feret diameter of mitochondrial profiles was found between the 10 µg/L treated larvae and the 10 µg/L BPA + 625 µg/L genistein treatment group. There were no differences in maximum feret diameter of the mitochondrial profiles observed in the BPA or/and genistein treated larvae and the positive control treatment group (Mann-Whitney U test).

Several statistically significant differences were exhibited when the distributions of the mitochondrial profiles’ maximum feret diameters were analysed. BPA at both tested concentration as well as genistein at 312.5 and 625 µg/L significantly altered the distribution of the maximum feret diameter in comparison to the control group (Chi-squared test). A noticeable indication of reduction (0.05 > p < 0.1) in the maximum feret diameter of the
mitochondrial profiles was also observed for the 10 µg/L BPA + 312.5 µg/L genistein as well as 100 µg/L + 312.5 µg/L genistein treatment groups (Figure 5.50).

Max feret diameter of mitochondrial profiles in the hepatocytes of the 120 hpf zebrafish exposed to BPA or/and genistein

Figure 5.49 Maximum feret diameters of the mitochondrial profiles in the hepatocytes of the 120 hpf zebrafish larvae exposed to BPA and genistein individually and in mixtures. Each symbol represents the median of 86 ± 16 mitochondria measured per larva (n=4). The capital letters indicate a significant difference from: C - controls (DMSO), B – corresponding treatment with BPA only, G – corresponding treatment with genistein only. Asterisks specify statistical significance of the differences (*P≤0.05, Mann-Whitney U test).
Figure 5.50 Distribution of the maximum feret diameters of the mitochondrial profiles in the hepatocytes of the 120 hpf zebrafish larvae exposed to BPA and genistein individually and in mixtures. Data presented as the mean percentage (n=4) of the mitochondrial profiles of a given length range. Asterisks indicate values significantly different from the controls (** P≤0.01 and *** P≤0.001, Chi-squared test).

- Minimum feret diameter of the mitochondrial profiles

Minimum feret diameters of the mitochondrial profiles in the hepatocytes of the zebrafish larvae from all the treatment groups were similar (P=0.363, Kruskal-Wallis test). The specific comparison of the individual treatments with the control group (Mann-Whitney U test) revealed that there were significant differences in this parameter for all the treatments with single oestrogenic compounds, i.e.: 10 µg/L BPA, 100 µg/L BPA, 312.5 µg/L genistein, 625 µg/L genistein as well as 20 µg/L β-oestradiol (Figure 5.51). All of these treatments as well as the treatment with 100 µg/L BPA + 312.5 µg/L genistein had significant effect on the distribution of the mitochondrial profiles’ minimum feret diameters comparing to the controls (Chi-squared test) as one can see in Figure 5.52.
Min feret diameter of mitochondrial profiles in the hepatocytes of the 120 hpf zebrafish exposed to BPA or/and genistein

Figure 5.51 Minimum feret diameters of the mitochondrial profiles in the hepatocytes of the 120 hpf zebrafish larvae exposed to BPA and genistein individually and in mixtures. Each symbol represents the median of 86 ± 16 mitochondria measured per larva (n=4). The capital letters indicate a significant difference from: C - controls (DMSO). Asterisks specify statistical significance of the differences (*P≤0.05, Mann-Whitney U test).

Figure 5.52 Distribution of the minimum feret diameters of the mitochondrial profiles in the hepatocytes of the 120 hpf zebrafish larvae exposed to BPA and genistein individually and in mixtures. Data presented as the mean percentage (n=4) of the mitochondrial profiles of a given length range. Asterisks indicate values significantly different from the controls (*P≤0.05, ** P≤0.01 and *** P≤0.001, Chi-squared test).
Major to minor axis ratio of the mitochondrial profiles

The major to minor axis ratio of the mitochondrial profiles observed in the zebrafish larvae’ hepatocytes was similar ($P=0.295$) when compared among all the treatment groups (Kruskal-Wallis test). A pair-wise comparison of the mitochondrial profiles (Mann-Whitney U test) between the oestrogenic compound(s) treated larvae and the controls revealed that 10 µg/L BPA + 625 µg/L genistein was the only treatment which changed the aspect ratio in a significant way ($P=0.028$, Figure 5.53). When the effect of this mixture was compared to the effects of its individual components, significant difference was revealed only for genistein ($P=0.042$).

The analysis of the distribution of the aspect ratios revealed a significant difference in the mitochondrial profiles ($P\leq0.005$, Chi-square test) between the larvae treated with 20 µg/L 17β-oestradiol and the controls (Figure 5.54).

**Figure 5.53** Major to minor axis ratios of the mitochondrial profiles in the hepatocytes of the 120 hpf zebrafish individually and in mixtures. Each symbol represents the median of 86 ± 16 mitochondria measured per larva (n=4). The capital letters indicate a significant difference from: C – controls (DMSO), G – corresponding treatment with genistein only, E – positive controls (β-oestradiol). Asterisks specify statistical significance of the differences (*$P\leq0.05$, Mann-Whitney U test).
Figure 5.54 Distribution of the major to minor axis ratios of the mitochondrial profiles in the hepatocytes of the 120 hpf zebrafish larvae exposed to BPA and genistein individually and in mixtures. Data presented as the mean percentage (n=4) of the mitochondrial profiles of a given aspect ratio range. Asterisks indicate values significantly different from the controls (** P≤0.01, Chi-squared test).
5.2.3 The combined effects of BPA and genistein on the expression of vitellogenin 1 mRNA in 120 hpf zebrafish

In this study the estrogenicity of BPA and genistein in combination was assessed based on its effect on the expression of vitellogenin 1 mRNA (vtg1) in the 120 hpf zebrafish larvae. All the embryos used in this study were exposed to the tested solutions from 0.75-1 hpf (2-4 cell stage) until 120 hpf. The expression levels of vtg1 in the larvae exposed to the BPA + genistein mixtures were compared to the control group (0.01% DMSO) as well as to the larvae treated with the natural oestrogen (20 µg/L β-oestradiol). In addition, the vtg1 levels were compared between the BPA plus genistein treated larvae and the larvae exposed to the corresponding, individual components of the mixtures.

There was a significant difference in the level of vtg1 in 120 hpf zebrafish larvae across all the treatment groups (P≤0.001, one-way ANOVA). Moreover, all the mixtures containing ≥312.5 µg/L genistein, namely 10 µg/L BPA + 625 µg/L genistein, 100 µg/L BPA + 625 µg/L genistein and 2000 µg/L BPA + 1250 µg/L genistein caused a significant increase in the expression level of vtg1 compared to the controls (P≤0.001 for all comparisons, Tukey’s test). Neither 10 µg/L BPA + 312.5 µg/L genistein nor 100 µg/L BPA + 312.5.5 affected the expression of vtg1 in a statistically significant way when compared to the controls (P=0.253 and P=0.201, respectively; Tukey’s test).

The vtg1 expression level in the larvae treated with 10 µg/L BPA + 312.5 µg/L genistein, 10 µg/L BPA + 625 µg/L genistein and 100 µg/L BPA + 312.5 µg/L genistein was significantly different than in the larvae treated with the natural oestrogen (P≤0.034 for all comparisons, Tukey’s test). However for the 100 µg/L BPA + 625 µg/L genistein and 2000 µg/L BPA + 1250 µg/L genistein treated larvae the level of vtg1 was similar to the positive control group (P=0.237 and P=1.00, respectively, Tukey’s test) as presented in Figure 5.55.

Except for 10 µg/L BPA + 312.5 µg/L genistein, the effects of all the tested mixtures on the vtg1 were significantly different than the effects of BPA only at corresponding concentrations. No differences, however, were revealed when the levels of vtg1 in the larvae treated with the mixtures were compared to the corresponding genistein only treatment groups (Figure 5.55).
Vitellogenin expression in zebrafish exposed to BPA, genistein, and their mixture

Figure 5.55 Vitellogenin 1 mRNA (vtg1) expression fold changes in the 120 hpf zebrafish larvae exposed to BPA and genistein individually and in mixtures. The capital letters indicate a significant difference from: C – controls (DMSO), B – corresponding treatment with BPA only, E – positive controls (β-oestradiol). Asterisks specify statistical significance of the differences (*P≤0.05, Tukey’s test).
5.3 Discussion

Following the study on the individual effects of BPA and genistein, the effects of both EDCs in combination on the development of zebrafish embryos/larvae was investigated.

Although considerable progress has been made in assessing the combined effects of chemicals in recent years, the effects of mixtures still remain one of the most daunting challenges in environmental toxicology (Sun et al., 2009). One of the problems in this field is weak understanding of the mixtures and the use of conflicting definitions for the chemical interactions. For example, an interaction classified as synergism by some authors may be considered as additive by others. Based on the review of the studies focused on chemical mixtures, Hertzberg and MacDonell (2002) stated that the problem of inconsistent terminology plagues mixture and interactions science.

One of the reasons for this confusion might be inappropriate use of the intuitively appealing and popular approach of effect summation (Kortenkamp and Altenburger, 1998). In effect summation, the two agents A and B are assumed to act additively, if their combined effect, $E_{A+B}$, equals the sum of the effects of the single compounds, $E_A + E_B$. If the effect of the mixture exceeds that predicted by summation, it is interpreted as synergism, while if it is smaller, the interaction is classified as antagonistic. The application of effect summation is, however, limited to combination of compounds producing linear dose-response curves (Berenbaum, 1989). Taking into consideration that linear dose-response curves are rarely seen in toxicology (Kortenkamp and Altenburger, 1998) and that non-monotonic dose responses are often produced by EDCs (Welshons et al., 2003), the summation effect approach could not have been uncritically used for analysis of the results from the current study. Particularly that some mixture toxicologists consider this approach as inappropriate (Howard and Webster, 2013).

There are two other approaches used in toxicology to determine a reference point for the assessment of combination effects in terms of synergism, additivity and antagonism, namely the concentration addition (CA) --and the independent action (IA). Similarly to the summation effect approach, CA or IA could not have been uncritically used in the present work for several reasons.

Taking into consideration that both BPA and genistein can bind and act through ERs, the main assumption of the IA model, i.e. different, and thus, independent actions of the compounds, was not met in the present study, excluding the use of the IA.

The CA approach, in turn, assumes similarity of action for the mixture’s components (Kortenkamp et al., 2007) and has been widely used in the studies on endocrine disruptor mixtures (Kortenkamp, 2007). In fact, this approach has been proposed by the US Environmental Protection Agency as the default in assessing chemical interactions (EPA, 2000). The CA model, however, was also not fully applicable for the present study. Firstly, in the studies based on the concept of CA the tested mixtures are composed of chemicals at equitoxic
concentrations. The composition of the tested mixture is therefore dependent on and dictated by the potential of its individual compounds to cause a given response/endpoint. The goal of the present study was, however, not to identify the modes of action between BPA and genistein per se, but to examine the outcome of the exposure to their mixtures in living and developing organism. Furthermore, for this study the mixtures representing those possible to occur in real life, i.e. at a specific ratio of both compounds, were of greatest interest. This study has demonstrated that genistein was several times more toxic than BPA, as revealed by the survival, heart rate, hatching success and morphology of zebrafish embryos/larvae treated with either compound. Consequently, to meet the CA approach requirement of the compounds equitoxicity, much higher concentrations of BPA than genistein would have to be used to make the mixtures. This, however, is not the case for the environmentally relevant mixtures of these EDCs. BPA concentrations ranging from 0.2 to 9.2 μg/L were reported for unconjugated BPA in the blood of human foetuses at term (Schonfelder et al., 2002b), while the range of 0 to 842 μg/kg was measured for canned foods (Sajiki et al., 2007). Thus both 10 μg/L and 100 μg/L represent environmentally relevant doses of BPA and were included in the current study. For genistein, 908 μg/L (median) and 684 ± 443 μg/L (mean ± SD) were reported for the soy formula fed infants (Cao et al., 2009; Setchell et al., 1997) thus genistein's concentrations tested in the present study, i.e. 312.5 μg/L (1.16 μM) and 625 μg/L (2.31 μM) can represent real life scenario. The mixtures composed of BPA at 10 or 100 μg/L and genistein at 312.5 or 625 μg/L are therefore environmentally relevant and at the same time, far from being equitoxic. Simultaneous exposure to both compounds is very feasible due to the wide distribution of both compounds in the environment. Humans may be simultaneously exposed to BPA and genistein, for example, consuming soy products stored in the cans lined with BPA-containing resins or heated up in the polycarbonate plastics containing BPA.

Another problem with utilization of the CA approach was its assumption of similar mode of action, i.e. that one chemical can be replaced totally or in part by an equal fraction of an equieffective concentration of another compound without diminishing of the overall combined effect (Kortenkamp et al., 2007). Even though both BPA and genistein are oestrogenic compounds due to their ability to bind to ERs, it is possible that each of them is also able to act in ER-independent fashion. Genistein has been shown to modulate signalling pathways involving growth factors through inhibition of tyrosine kinases and have antiangiogenic and antioxidant properties (Kim et al., 2009; Kim et al., 1998). BPA, in turn, is suspected to cause neurotoxic effects in rats through ER-independent mechanism involving the activation of extracellular signal-regulated kinase (ERK) and inhibition of anti-apoptotic nuclear factor kappa B (NF-kappa B) (Lee et al., 2007). Based on this information one might surmise that not only “similar” pathways can be affected by these two compounds. Only the study on the BPA or/and genistein effects on the expression of vitellogenin 1 mRNA meets the assumption of the “similarity” because vitellogenin transcription is an ER-dependent process and hence it has been proposed as a biomarker of oestrogenic exposure (Sumpter and Jobling, 1995). In the remaining studies presented in this work the observed endpoints, i.e. survival, heart rate,
hatching success, morphology and the ultrastructure of the liver, could potentially also be affected by ER-independent and hence “not similar” mechanisms.

Although the present study is not based upon a perfect mathematical approach, the interpretation of the observed results was based on logical assumptions. The term “additivity” was used not in the mathematical sense but to describe the cases in which chemicals “acted together” to produce effects without enhancing or diminishing each other’s action (Kortenkamp, 2007). Consequently, when the toxicity of the mixtures was somewhat enhanced in comparison to their components acting individually the action was considered as additive. The term “synergistic”, in turn, was used only when the frequency or intensity of a given endpoint was clearly higher than what was expected from the “acting together” of the individual components. Antagonistic interactions were diagnosed if the effect of the mixture was less toxic than the effect of the most toxic of its components.

Several observations of the present study indicated enhanced toxicity of BPA and genistein when applied simultaneously. These included, for example, increased mortality of the 16 mg/L BPA + 5 mg/L genistein treated embryos at 48 hpf or the mortality of 120 hpf larvae treated with 8 mg/L BPA + 1.25 mg/L genistein. These observations indicate additivity between BPA and genistein. The analysis of embryonic/larval morphology also revealed additivity of the tested EDCs. It was generally observed that the morphological alterations in the embryos/larvae treated with the mixtures were more pronounced than for those treated with the individual compounds. Very often it was a matter of increased toxicity of the more toxic component in the presence of the other compound like, e.g. when the malformations following the treatment with 2.5 mg/L genistein in 24 hpf embryos were somewhat enhanced in the presence of 4 mg/L BPA, the latter showing no effects on its own. The additivity of the two compounds was also revealed with the observation of altered phenotype of the embryos treated with 4 mg/L BPA + 1.25 mg/L genistein since there were no effects for either compound individually. One of the observed phenotypic alterations was a slight decrease in the head size of the mixture treated larvae. Head size reduction was also observed for the sole treatment with ≥2.5 mg/L genistein and this effect was further increased upon co-exposure with 4 mg/L BPA. One might surmise therefore that the addition of BPA enhanced the toxicity of genistein at 1.25 mg/L. This, however, is not the only possible explanation. Even though no apparent changes in the head morphology were seen for BPA at 4 or 8 mg/L concentrations, a slight reduction in the size and darkened appearance of the tissue were noticed for the 16 mg/L BPA treated embryos. BPA at ≥40 mg/L was reported to cause changes in the brain of developing rat embryos (Xing et al., 2010). The neurotoxic effects of this EDC were also seen in the developing zebrafish by Lam et al. (2011) who reported inhibited growth and branching of the spinal cord axons in the transgenic fish Tg(Nkx2.2a-mEGFP) treated with 5000 µg/L. Based on these reports it is possible that BPA in the mixture of 4 mg/L BPA + 1.25 mg/L not only enhanced the neurotoxic effects of genistein but actively participated in the processes leading to disturbances in the brain development. Such a scenario would be in agreement with the
report of Xing et al. (2010) who saw dramatically enhanced toxicity of BPA by different doses of genistein based on the observations of the CNS malformations in embryonic rats.

The observed effects indicate that BPA and genistein can act in additive fashion. This is in agreement with previous studies in which additivity has been frequently reported in respect to the oestrogenic endocrine disruptors (Kortenkamp, 2007). These additive effects point towards the predominance of the common molecular pathways for BPA and genistein. Knowing oestrogenic properties of both BPA and genistein, ER-dependent mechanisms can be considered as those common pathways.

No obvious enhancement of BPA and genistein effects was also observed in the present study indicating lack of additivity. This was particularly true for the majority of the mixtures consisting of BPA and genistein, both at NOECs. The additivity should not, however, be excluded for these mixtures because NOECs cannot be equated with zero-effect levels (Kortenkamp et al., 2007). Consequently addition of not evident effects leading to the increased effects but still below the threshold of identification can be taking place. Lack of clear enhancement of toxicity was also seen for the mixtures containing at least one component at concentrations causing severe effects when used on its own. It seems possible also in these cases that the toxicity enhancement was too small to be identified. Another reason behind the lack of enhanced effectiveness of these mixtures could be the saturability of ERs. Assuming that both mixture components act in ER-dependent fashion, and knowing that binding of a ligand to its receptor shows the property of saturation, i.e. that once all receptors are occupied there is no further increase in their number with the increase of ligand concentration (Welshons et al., 2003), no increase in the effect would be possible.

Based on the observations from the present study, it also seems possible that the label “no-interaction” could be given due to the lack of statistical proof for additivity. The study on the vitellogenin 1 mRNA (vtg1) expression provides basis for such reasoning. No statistically significant enhancement was observed in the oestrogenic potential of the mixtures based on the comparisons of the vtg1 levels in the larvae treated with BPA and genistein in combinations vs. treatments with individual components. There has been no study to date on the effects of exactly the same mixture on the expression of vitellogenin transcript (vtg) or protein (Vtg) in zebrafish or other fish species. Several studies, however, tested other mixtures of oestrogenic EDCs and observed additive effects when assessing vitellogenin induction as an endpoint. These studies were generally based on the CA model and tested the mixtures consisting of the compounds at equitoxic concentrations (Thorpe et al., 2003; Thorpe et al., 2001). Additivity was also reported by Sun et al. (2009) who studied plasma vitellogenin induction in male adult Japanese medaka (Oryzias latipes) treated with the binary mixtures of oestradiol, 4-tert-nonylphenol and BPA. The study of Sun et al. was based on the factorial analysis and without use of the CA model, hence is similar to the present work. Based on these reports and also on the observations from other parts of the present study we surmise that high variability in the vtg1 expression levels may be the reason why additivity between BPA and genistein was not statistically significant. High biological variation in fish vitellogenin levels have been previously
reported (Biales et al., 2007) and high standard deviations in the measured mRNA or protein can be found in the literature (Kausch et al., 2008; Muncke and Eggen, 2006). Even though not statistically significant, the mean levels of vtg1 in the mixtures treated larvae were consistently higher than the sum of the levels measured in the corresponding treatments with either BPA or genistein. For example, while the 2000 μg/L BPA or 1250 μg/L genistein treated larvae had 24.33 ± 28.54 - and 501.27 ± 201.07 – fold increased levels of vtg1 compared to the controls, 1032.97 ± 1088.67-fold increase was observed in the larvae treated with their mixture.

It cannot be, however, ruled out that lack of interactions is the actual reason for which no statistically significant changes were observed in the vtg1 levels between the larvae treated with the mixtures and those exposed solely to genistein. Jukosky et al. (2008) observed lower vitellogenic induction of the mixtures of nonylphenol, 17α-ethinylestradiol and 17β-oestradiol comparing to equipotent concentrations of 17β-oestradiol alone using medaka (Oryzias latipes). They suggested that the potency of the mixtures was lower than that of 17β-oestradiol alone. It seems therefore possible that analogous decrease in the potency of the BPA and genistein mixtures was responsible for the lack of clear additive effects in the present study.

The effects pointing to antagonistic interactions were also observed in the current study. Such interactions between the chemical compounds could be explained with a competitive site theory (Duan et al., 2008). Since both BPA and genistein can act through ERs, it seems possible that these two compounds will compete to bind to the limited number of these active sites. A great difference in the toxic potential of BPA and genistein may underpin antagonistic effects when the toxicity of sole genistein treatments is alleviated in the presence of high concentrations of less toxic BPA. Numerous BPA molecules have a higher chance to occupy the limited number of ERs and thus the probability of genistein to act through these pathways is decreased. Higher incidence of heart oedema in the 48 hpf zebrafish treated with 2.5 mg/L genistein than in the embryos exposed to 4 mg/L BPA + 1.2 mg/L genistein might result from such mechanisms. This hypothesis, however, does not explain the observations of decreased toxicity of BPA in the presence of genistein. The observations of mild heart oedema in the 8 mg/L BPA treated larvae and no obvious heart malformations in the 8 mg/L BPA + 1.25 mg/L genistein treatment group is an example of such phenomenon. It is possible, however, that ER-independent effects of genistein such as tyrosine kinase inhibition, can play a role in the alleviation of the ER-dependent toxicity of BPA. Further studies are necessary to investigated molecular mechanisms leading to the observed effects on the zebrafish phenotype.

Some of the ultrastructural changes in the liver of BPA or/and genistein treated larvae also indicated antagonistic interactions between these compounds. For example, individual treatment with both BPA and genistein affected size and shape of mitochondria to a much higher degree than when used in combinations. Similarly, while there was a significant increase in the amount of glycogen in the larvae treated with individual EDCs, there were no differences in the mixture exposed larvae when compared to controls. Taking into consideration that the liver is a central metabolic organ, antagonistic interactions, if true, could be related to the BPA or/and genistein metabolism. Three out of five mixtures of oestrogenic chemicals studied by
Silva et al. (2011) with the use of a breast cancer cell proliferation assay (E-screen) showed less than additive effects based on the CA model. It was suggested by these authors that the deviations were due to increased metabolism of steroidal oestrogen in the mixture setting. It is therefore conceivable that differential activation of metabolizing enzymes or efflux pumps by one of the components of the BPA and genistein mixtures might lead to the removal of the other compound but this hypothesis needs to be confirmed experimentally. Particularly that the analysis of the results obtained in the study on the liver ultrastructure is very problematic. The main reason for this difficulty is lack of studies exploring the effects of combined EDCs on this organ and, consequently, lack of certainty in respect to what is “expected” for the mixture treatment in respect to individual organelles.

To complicate matters further, not only additivity or antagonism, but also effects indicating synergism between BPA and genistein could be observed in the present study. Clearly higher mortality of the 24 hpf zebrafish exposed to 16 mg/L BPA + 5 mg/L genistein (100.00 ± 0.00 %) than of the embryos treated solely with BPA (23.33 ± 25.17 %) or genistein (36.67 ± 11.55 %) point towards such interactions. Because of high variation of this endpoint it could, however, be argued that this observation represents additive rather than synergistic effect. Nevertheless, the analysis of hatching success of larvae treated with BPA or/and genistein strongly indicates synergism. While all the larvae (100%) exposed to 1.25 mg/L genistein and 96.30 ± 6.41% of the population treated with 4 mg/L BPA were hatched, the exposure to the mixture caused a noticeable inhibition of this process (64.81 ±13.98%).

Synergistic interactions between BPA and genistein have been previously reported by Xing et al. (2010) who observed the development of in vitro cultured post-implantation rat embryos treated with both EDCs individually and in mixtures. A significant synergistic interaction was observed in this study for 12 out of 20 analysed end points. The dominance of synergistic interactions in the study of Xing et al. and their rarity in the present study may perhaps be the reflection of great discrepancy in the concentrations of BPA and genistein. These differences in the concentrations resulted from clearly higher sensitivity of zebrafish embryos to both BPA and genistein when compared to rat embryos. While inhibition of embryonic development was observed for rat embryos beginning at 32 mg/L BPA and 10 mg/L genistein, indicators of adverse effects in zebrafish development were seen for 4 mg/L BPA and 1.25 mg/L genistein. Interestingly, the toxicity of genistein was about three times higher than BPA in both studies, regardless of the species.

An indicator of synergistic effects of BPA and genistein could also be found in the liver ultrastructure of the zebrafish larvae treated with 100 μg/L BPA + 312.5 μg/L genistein. The volume of RER in this treatment group was significantly lower compared to controls and to both corresponding treatments with individual compounds. Taking into account that the reaction of endoplasmic reticulum is of outstanding importance for the diagnosis of the toxic action of chemicals in fish liver (Braunbeck et al., 1990) this alterations may be of considerable importance. However, as already mentioned, the analysis of the data on the liver ultrastructure is very difficult. That is why the main conclusion from the liver phenotype observations is that
BPA and genistein, both individually and in combinations, lead to alterations in the hepatocytes of zebrafish larvae which, although difficult to interpret when it comes to the mode of action between chemicals, might indicate that the physiology of this organ may be compromised. Complex physiological studies are therefore necessary to link observed phenotypic changes with the potentially disturbed mechanisms and enable drawing undeniable conclusions on the mode of action between BPA and genistein when acting on the liver in developing zebrafish.

In addition to the fact that all possible combined effects, i.e. additivity, antagonism and synergism, were observed in this study for BPA and genistein mixtures, these effects seem also to be organ-dependent. An observation pointing to such possibility is the effect of the 4 mg/L BPA + 2.5 mg/L genistein mixture on the 24 hpf embryos. While additive effects were observed for this mixture based on the assessment of the head morphology, antagonistic interactions were detected in the yolk sac size. These observations are in agreement with the report of Jordan et al. (2012) who demonstrated that unique metabolites for each organ may cause organ-specific effects of a single mixture.

To summarize, all the classic types of toxic interactions, namely additivity, synergy and antagonism, could be identified between BPA and genistein in the present study. These effects were dependent on the individual compounds’ concentrations as well as the observed endpoints. No single conclusion can therefore be made on how these two compounds act on the developing organism when applied simultaneously. Lack of a mathematical approach which could be uncritically used in the present study hindered the analysis of the obtained results in respect to the identification of the modes of action for BPA and genistein in combination. Other systems, particularly those based on in vitro models and with the use of straightforward endpoints (e.g. cell proliferation) and equitoxic mixtures, would undoubtedly allow for easier interpretation of the data. Such systems, however, do not give information on sensitive developmental stages, omit the absorption, distribution, metabolism, excretion, and cannot reveal tissue-specific effects. All these processes are particularly important when assessing the effects of chemical mixtures. The composition of the mixtures reaching the organ of interest in the living organism might be quite different from the one applied. That is why the present study examined the more complex in vivo situation.

Therefore, although the interpretation of chemical interactions (additivity, synergism and antagonism) is hindered when using in vivo models, the effects of the mixtures are more representative of environmental exposure to chemical compounds.
6 General Discussion and Future Work

6.1 General Discussion

- On the importance of the studies on chemical mixtures

There are around 70,000 chemicals available on market today and about 1500 new ones are introduced every year (Bertollini and Danzon, 2004). Given the myriad array of the chemicals and the multitude of sources we are exposed to mixtures rather than individual compounds.

It is simply impossible to study the effects of all the possible combinations of the chemicals released into the environment. For example, with 70,000 chemicals there would be $5.13 \times 10^{13}$ possible three compound-mixtures alone. Consequently it is important to make careful judgments when deciding on what mixtures should be investigated. One of the central criteria is the question on how likely it is for the exposure to a given mixture to occur (Boobis, 2012).

One of the aims of the present study was to investigate the effects of the BPA and genistein mixtures on the developing zebrafish. Humans may be exposed to the combined BPA and genistein throughout life, including the vulnerable period of intrauterine development.

Not only humans, but also wildlife can be co-exposed to both compounds. Given that most of the chemicals used by people in everyday life are released into the water systems, aquatic organisms are particularly exposed to the chemical mixtures, including those containing BPA and genistein.

- On zebrafish as a model system for the studies on chemical mixtures

Zebrafish (Danio rerio) is a most useful model to study the effects of the mixtures as:

- it shares many similarities with mammals due to a conserved vertebrate developmental program;
- many of the genes/molecules essential in the toxicological responses in humans are also found in zebrafish (Lam et al., 2011);
- responses to some chemicals, drugs and environmental toxicants produce similar pathological consequences to those encountered in mammals (Hill et al., 2005; Lam et al., 2008);
- it represents the inhabitants of perhaps the most polluted niche of today’s world;
- it combines the benefits of both in vivo and in vitro systems.
Testing chemicals within the context of whole-organism metabolism allows the observation of the effects resulting from multiple mechanisms and specific toxicokinetics (Shelby et al., 1996). This seems to be particularly important when studying the chemical mixtures since their composition undergoes considerable modifications due to these processes. Consequently the mixtures reaching the organ(s) of interest might be quite different from the ones formulated in the laboratory.

Working with this model allows testing of the mixtures’ influence on the specific stages in the development which is not possible when using in vitro systems. In addition, due to external development of the embryos/larvae the continuous observation of the teratogenic effects of EDC mixtures is possible during the most sensitive period of life. The implementation of a scoring system makes possible the comparison of the phenotypes between the treatment groups, as showed in this study. It also enables the specific interactions between the mixtures’ components to be revealed. Hatching and heart rate of embryos/larvae are sensitive endpoints of the sublethal of the EDC mixtures and also enable revealing detection of additive, synergistic or antagonistic effects between the compounds.

In vivo studies, although more representative in terms of what might be expected, may give indications of only gross effects and therefore reveal little about mechanisms of action (Shelby et al., 1996). In contrast, in vitro test systems permit the investigation of the underlying mechanisms of action by detecting effects on molecular scale (Muncke et al., 2007; Segner et al., 2003b). Molecular scale screening is, however, also possible when using zebrafish embryos/larvae. Muncke and Eggen (2006) have proposed the assays with the use of developing zebrafish for testing molecular effects in the subacute toxicity range and named this test MolDarT (from molecular scale screening based on DarT - the embryo test with Danio rerio). MolDarT offers great potential for the studies on the EDC mixtures as has been demonstrated in the present study by testing vitellogenin 1 mRNA expression in the larvae treated with BPA or/and genistein. The current study was, in fact, the first to investigate the estrogenicity of EDC mixtures based on the up-regulation of vitellogenin using MolDarT.

On the liver as a valid endpoint in the studies of EDC mixtures

The present study is the first systematic attempt to examine the effect of EDC mixtures on liver ultrastructure in the developing zebrafish. Previous studies on the endocrine disruptors have mostly focused on different aspects of the reproductive systems. The reports on the antithyroidal actions of some EDCs have broadened the research in endocrine disruption on the neurological or neuroendocrine actions of these compounds.

However, as observed by Guillette (2006), the focus on oestrogenic, antioestrogenic and antithyroidal effects of EDCs has produced a dogma that research on these compounds is largely about receptor binding or mechanisms based on sex steroid or thyroid action. This narrow focus has hampered our understanding of the full extent of the actions of EDCs. The
The liver is a vital organ and failure of its functions is life threatening. Cancer of the liver (hepatocellular carcinoma) is the fifth most common cancer and ranks as the third leading cause of cancer mortality worldwide (El-Serag and Rudolph, 2007). Even though liver function is essential for hormonal homeostasis, this organ is much neglected in the studies related to endocrine disruption. The concentration of free sex hormones (active forms) in the blood is controlled by the hepatic metabolism, as well as by the amount of sex-hormone binding protein (SHBP) synthesized in the liver. Thus, it seems possible that the abnormalities in the function of this organ can affect the hormonal milieu of the organism.

This reasoning leads to conviction that more studies should be devoted to this organ in relation to EDCs. Liver-related studies (hepatic metabolism of hormones and its effect on hormonal milieu) have also been outlined as one of areas where future work could produce insight into mechanisms of endocrine disruption (Guillette, 2006). Therefore, one of the main aims of the present study was to investigate whether two of well known environmental estrogens may affect the ultrastructure of this organ in the developing organism.

This study demonstrated that exposure to EDCs, individually and in mixtures, affects the ultrastructure of the liver in zebrafish larvae. The observed alterations let us surmise that BPA and/or genistein, even at environmentally relevant concentrations, may affect the function of this organ during the most vulnerable period of life, i.e. early development. The central observation of this study was altered mitochondrial morphology in the hepatocytes of zebrafish larvae following EDC(s) treatment. Those alterations were even observed as a result of the treatments with physiologically achievable concentrations, i.e. 10 µg/L BPA or 312.5 µg/L genistein. As discussed previously (Sections 3.3 and 4.3) such changes in the mitochondrial morphology might be related to oxidative stress or indicate the disturbances in the fusion and fission of these organelles. It has been proposed that oxidative stress might be a crucial factor in the developmental origins of adult disease (Luo et al., 2006). It is of crucial importance, therefore, to further investigate whether the alterations in mitochondrial morphology were associated with oxidative stress.

There is one additional reason for which more attention should be paid to the liver in the endocrine disruption field. Previous studies revealed expression of a large number of sex-dependent genes in the liver of mouse and rats (Waxman and Holloway, 2009). This sexual dimorphism is thought to have a major impact on hepatic physiology, inflammatory responses, diseased states and the metabolism of steroids, drugs and environmental chemicals. Furthermore, a recent study by Zhang et al. (2011a) revealed that there are 1,249 sex-biased genes in human liver, with 70% showing higher expression in females. These findings established that human hepatic sex differences are widespread and affect diverse cell metabolic processes. It would thus be very interesting to investigate whether the exposure to oestrogenic endocrine disruptors during early development can alter the hepatic profile of the
gene expression. Particularly that several key nuclear receptor pathways, including ER signalling, were among the top pathways associated with sex-biased gene expression in human liver (Zhang et al., 2011a). Zebrafish embryos/larvae seem to offer a great potential for the studies focused on the alterations in the metabolizing enzymes profiles caused by EDCs because transcript profiling by microarray and quantitative PCR revealed that the majority of zebrafish cytochrome P450 genes (CYP) are expressed in embryos (Goldstone et al., 2010).

To summarize, the present study showed that zebrafish is a potentially useful model for studies on EDCs mixtures. The action of these compounds can be studied in this model at physiological, anatomical and molecular levels. The implementation of stereological techniques offers great potential to reveal ultrastructural alterations and points to the possible physiological effects caused by these chemicals. In addition, the morphometrically obtained data enables objective description, comparisons between the treatment groups, i.e. between the mixtures and corresponding individual compounds, as well as statistical analyses. This study also demonstrated that all types of chemical interactions, i.e. antagonism, additivity and synergism are possible for two compounds when using in vivo model. These interactions are clearly dependent on the mixture components’ concentrations, the endpoint studied and the time of the observation.
6.2 Future work

Future work on this model should include the following:

- The investigation of the three-dimensional (3D) microstructure of the liver. Since one of the limitations of the present study was a two dimensional approach in the investigation of the mitochondrial morphology, advanced stereological methods could be used to learn more about the 3D structure of these organelles. Second order stereology which includes methods to quantify the spatial arrangement or spatial pattern of tissue components over a limited range of length scales (Reed et al., 2010) offers some tools to explore this matter. These methods enable the assessment of the degree of positive or negative correlation of a feature with itself, or with another well-defined feature, over a range of distance (Reed et al., 2010).

- The examination of oxidative stress indices and antioxidant parameters in the BPA or/and genistein treated zebrafish larvae. In their study Wu et al. (2011a) demonstrated that BPA and nonylphenol, individually and in mixtures, enhanced the production of hydroxyl radicals and lipid peroxidation in the 168 hpf zebrafish larvae. In addition, these treatments caused the inhibition of antioxidant enzymes such as catalase, superoxide dismutase, glutathione peroxidise, glutathione reductase or glutathione-S-transferase. The analogous studies could thus be performed on the 120 hpf, BPA or/and genistein treated larvae. Because the dissection of the liver is impossible at this stage due to a small body size, whole body homogenates would have to be used. Consequently the effects of the tested compounds would be tested in respect to the whole larvae and not their specific organs. Immunohistochemical localization and mensuration of the ROS scavengers levels on paraffin of frozen sections of zebrafish larvae could be performed to study organ-specific (including liver), ROS-related effects of BPA or/and genistein. At least two antibodies with reactivity towards zebrafish, i.e. anti-catalase and anti-superoxide dismutase are available on the market (Abcam).

- The investigation on IGF-1 receptors or/and IGF-1 synthesis in the zebrafish treated with BPA or/and genistein. IGF-1 is one of the hormones produced by the liver. Previous studies have demonstrated that heterozygous knockout6 female mice \(\text{Igf}1^{-/-}\) with 50% reduction in the level of IGF-1 receptor, displayed greater resistance to oxidative stress (Holzenberger et al., 2003). Abnormal regulation of blood glucose was also observed in these mice and was linked to the fact that IGF-1 receptor is involved in the pancreatic control of glucose homeostasis (Kulkarni et al., 2002). Indices of both increased oxidative stress and disturbed carbohydrate metabolism were observed in the present study. It would be therefore interesting to study whether these changes were related to the alterations in the IGF-1

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6Homozygus null mutants \(\text{Igf}1^{-/-}\) died at birth (Holzenberger et al., 2003).
receptors or and IGF-1 levels. Real time PCR and Western blot could be used to try to answer these questions.

- The exploration of the mechanisms behind the observed alterations in the mitochondrial morphology. One of the approaches to elucidate this matter could be to study the levels of the proteins involved in the mitochondrial fusion and fission. The report by Wu et al. (2011b) serves as a basis for such study. They observed oxidative stress triggered fragmentation of mitochondria in human lung adenocarcinoma cells (ASTC-a-1) and African green monkey SV40-transformed kidney fibroblast cells (COS-7). This fragmentation was abolished by exposure to dehydroascorbic acid, a reactive oxygen species scavenger, indicating that oxidative stress can induce mitochondrial fragmentation. Further investigation revealed that mitochondrial fragmentation was a result of inhibited fusion and enhanced fission. These observations led Wu et al. to conclude that mitochondrial oxidative stress mediated through Drp1 (the profission, dynamin-related protein 1) and Mfn2 (the profusion protein, mitofusin 2) causes an imbalance in mitochondrial fission–fusion. It would be therefore interesting to find out whether the Drp1 and Mfn2 were affected in the BPA or and genistein treated larvae showing significant reduction in the size of individual mitochondria. Real time PCR could be used to investigate the expression levels (mRNA) of these proteins in the treated larvae. Western blot, i.e. protein level analysis of Mfn2 and Drp1 could also be carried out because specific antibodies for these proteins and with reactivity towards zebrafish are available on the market.
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8 Appendices

8.1 Appendix A – supplementary protocols

Bisphenol A solutions

MW = 228.29 g/mol; Solubility = 50 mg/ml (219.02 mM)

1. 20 mg BPA + 1 ml DMSO → 20 g/L (87.61 mM); 100 % DMSO.
   Aliquoted as 42 µl.
2. Dilute 625 x → 32 mg/L (140.17 µM) BPA [40 µl of ↑ + 24.96 ml H₂O]; 0.16 % DMSO
3. Dilute 2 x → 16 mg/L (70.08 µM) BPA; 0.08 % DMSO
4. Dilute 2 x → 8 mg/L (35.04 µM) BPA; 0.04 % DMSO
5. Dilute 2 x → 4 mg/L (17.52 µM) BPA; 0.02 % DMSO
6. Dilute 2 x → 2 mg/L (8.76 µM) BPA; 0.01 % DMSO
7. Dilute 2 x → 1 mg/L (4.38 µM) BPA; < 0.01 % DMSO
8. Dilute 2 x → 500 µg/L (2.19 µM) BPA; < 0.01 % DMSO
9. Dilute 5 x → 100 µg/L (438 nM) BPA; < 0.01 % DMSO
10. Dilute 10 x → 10 µg/L (43.8 nM) BPA; < 0.01 % DMSO

Genistein solutions

MW = 270.24 g/mol; Solubility = 27 mg/ml (100 mM)

1. 20 mg genistein + 1 ml DMSO → 20 g/L (74.01 mM); 100 % DMSO.
   Aliquoted as 25 µl.
2. Dilute 1000 x → 20 mg/L (74 µM) genistein [24 µl of ↑ + 23.976 ml H₂O]; 0.1 % DMSO
3. Dilute 2 x → 10 mg/ml (37 µM) genistein; 0.05 % DMSO
4. Dilute 2 x → 5 mg/ml (18.5 µM) genistein; 0.025 % DMSO
5. Dilute 2 x → 2.5 mg/ml (9.25 µM) genistein; 0.0125 % DMSO
6. Dilute 2 x → 1.25 mg/ml (4.625 µM) genistein; P< 0.01 % DMSO
7. Dilute 2 x → 625 µg/ml (2.313 µM) genistein; P< 0.01 % DMSO
8. Dilute 2 x → 312.5 µg/ml (1.156 µM) genistein; P< 0.01 % DMSO

7 According to OECD DMSO concentration should not exceed 0.01% (100 µl/L).
17β-oestradiol solutions

MW = 272.38 g/mol; Solubility = 20 mg/ml (73.43 mM)

1. 10 mg oestradiol + 1 ml DMSO → 10 g/L (36.71 mM); 100 % DMSO. Aliquoted as 12 µl.
2. Dilute 500 x → 20 mg/L (73.43 µM ) oestradiol [10 µl of ↑ + 4.99 ml H₂O]; 0.2 % DMSO
3. Dilute 1000 x → 20 µg/L (73.43 nM) oestradiol; P< 0.01 % DMSO

Mixtures of Bisphenol A and genistein (BPA & GEN)

1. Mix 50 : 50 of 32 mg/L BPA : 10 mg/L genistein → 16 mg/L BPA + 5 mg/L genistein; 0.105 % DMSO
2. Mix 50 : 50 of 16 mg/L BPA : 5 mg/L genistein → 8 mg/L BPA + 2.5 mg/L genistein; 0.05 % DMSO
3. Mix 25 : 25 : 50 of 16 mg/L BPA : 5 mg/L genistein : H₂O → 8 mg/L BPA + 1.25 mg/L genistein; 0.046 % DMSO
4. Mix 25 : 50 : 25 of 16 mg/L BPA : 5 mg/L genistein : H₂O → 4 mg/L BPA + 2.5 mg/L genistein; 0.03 % DMSO
5. Mix 25 : 25 : 50 of 16 mg/L BPA : 5 mg/L genistein : H₂O → 4 mg/L BPA + 1.25 mg/L genistein; 0.026 % DMSO
6. Mix 12.5 : 25 : 62.5 of 16 mg/L BPA : 5 mg/L genistein : H₂O → 2 mg/L BPA + 1.25 mg/L genistein; 0.016 % DMSO
7. Mix 10 : 25 : 65 of 1 mg/L BPA : 2.5 mg/L genistein : H₂O → 100 µg/L BPA + 625 µg/L genistein; P< 0.01 % DMSO
8. Mix 10 : 12.5 : 77.5 of 1 mg/L BPA : 2.5 mg/L genistein : H₂O → 100 µg/L BPA + 312.5 µg/L genistein; P< 0.01 % DMSO
9. Mix 1 : 25 : 74 of 1 mg/L BPA : 2.5 mg/L genistein : H₂O → 10 µg/L BPA + 625 µg/L genistein; P< 0.01 % DMSO
10. Mix 1 : 12.5 : 86.5 of 1 mg/L BPA : 2.5 mg/L genistein : H₂O → 10 µg/L BPA + 312.5 µg/L genistein; P< 0.01 % DMSO
PROTOCOL 2

Processing for transmission electron microscopy – necessary solutions

Sodium cacodylate buffer (500ml of 0.2 M solution)

- Dissolve 21.4 g sodium cacodylate (MW=214 g) in 400 ml millipore grade water
- Adjust the pH to 7.4 with 1 M HCl and make up to 500 ml with millipore water in a volumetric flask to give a 0.2 M solution
- Store at 4°C for up to 1 month in a screw top bottle which is clearly labelled and dated
- Dilute 50:50 to make 0.1 M buffer when required

! This contains arsenic, is a suspected carcinogen and mutagen and has cumulative toxicity; therefore, it should be handled with appropriate precautions.

Paraformaldehyde (50 ml of 10% solution)

- Add 40 ml of millipore grade water to 5 g paraformaldehyde powder in a 100-ml beaker
- Heat and stir on a magnetic stirring hotplate on a fairly high setting (≤60°C) until a milky suspension is formed
- Do not boil as it polymerizes at 100 °C
- Add a few drops (1-3) of 1N NaOH until the suspension is completely clear
- Make up to a final volume of 50 ml with millipore grade water and allow to cool
- Filter
- This should always be made up fresh on the day of fixation

! This is mutagenic and must be prepared in a fume hood as it is particularly irritating to the respiratory system.

Glutaraldehyde (25 % solution)

- It is bought as a 25% solution and stored at 4 °C for up to 3 months or at -20 °C up to a year;

! Glutaraldehyde is extremely irritating to the eyes and is also suspected mutagen/teratogen

Glutaraldehyde and paraformaldehyde fixative (for 100 ml fixative)

- Add 10 ml 25% glutaraldehyde, 25 ml 10% paraformaldehyde and 15 ml water to 50 ml 0.2 M sodium cacodylate buffer
- Consists of 2.5% Glutaraldehyde and 2.5% paraformaldehyde in 0.1 M sodium cacodylate buffer
- Always make fresh on the day of fixation and use at room temperature.

**Osmium tetroxide**

- Prepare a 2% solution of osmium tetroxide by dissolving the contents of 1 ampoule of OsO4 (0.25 g) in 12.5 ml of Millipore H2O (2 ampoules in 25 ml is commonly used)
- Dissolution is best done overnight in a brown or opaque wide mouthed bottle in a refrigerator, wrapped in tin foil and clearly labelled as Osmium with a warning of toxicity, and dated
- Final working solution of osmium tetroxide, which is 1%, is prepared by mixing the equal volumes of 2% solution and 0.2 M Sodium cacodylate buffer pH 7.2-7.4 (10 ml of 2% OsO4 and 10 ml of 0.2 M sodium cacodylate buffer is commonly used)

! The main hazard from osmium tetroxide is caused by the vapour, which is an extreme irritant and which can affect the vision so control of the vapour is very important. If it is spilled outside the fume hood, the area should be evacuated until well ventilated.

**Low viscosity Agar Resin**

- This is bought in kit form and prepared by thoroughly mixing the pre-weighed components:
  - LV Resin 48 g
  - VH1 Hardener 16 g
  - VH2 Hardener 36 g
  - LV Accelerator 2.5 g

- Care must be taken with the measuring and mixing process otherwise blocks of an inconsistent hardness will result

! It has none of the known carcinogenic effects of Spurr, but as with all resins, proper handling precautions such as the use of gloves and a fume hood or a well-ventilated area should be observed.

**Propylene oxide**

! Care must be taken with this substance, as it is extremely inflammable. It is also a teratogen/mutagen and causes cancer in laboratory animals. Contact with skin can cause delayed blistering so suitably resistant gloves must be worn.
8.2 Appendix B – supplementary data tables

Table 8.1 Specific comparisons of the survival of the 24, 48, 72, 96 and 120 hpf zebrafish exposed to 2, 4, 8, 16 and 32 mg/L BPA from 0.75 – 1 hpf until 120 hpf.

<table>
<thead>
<tr>
<th>Tukey’s Multiple Comparison Test</th>
<th>24 hpf</th>
<th>48 hpf</th>
<th>72 hpf</th>
<th>96 hpf</th>
<th>120 hpf</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1% DMSO vs. 2 mg/L</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>0.1% DMSO vs. 4 mg/L</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>0.1% DMSO vs. 8 mg/L</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>P&lt;0.05</td>
<td>ns</td>
</tr>
<tr>
<td>0.1% DMSO vs. 16 mg/L</td>
<td>ns</td>
<td>P&lt;0.01</td>
<td>P&lt;0.001</td>
<td>P&lt;0.001</td>
<td>P&lt;0.001</td>
</tr>
<tr>
<td>0.1% DMSO vs. 32 mg/L</td>
<td>P&lt;0.001</td>
<td>P&lt;0.001</td>
<td>P&lt;0.001</td>
<td>P&lt;0.001</td>
<td>P&lt;0.001</td>
</tr>
<tr>
<td>2 mg/L vs. 4 mg/L</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>2 mg/L vs. 8 mg/L</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>2 mg/L vs. 16 mg/L</td>
<td>ns</td>
<td>P&lt;0.01</td>
<td>P&lt;0.001</td>
<td>P&lt;0.001</td>
<td>P&lt;0.001</td>
</tr>
<tr>
<td>2 mg/L vs. 32 mg/L</td>
<td>P&lt;0.001</td>
<td>P&lt;0.001</td>
<td>P&lt;0.001</td>
<td>P&lt;0.001</td>
<td>P&lt;0.001</td>
</tr>
<tr>
<td>4 mg/L vs. 8 mg/L</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>4 mg/L vs. 16 mg/L</td>
<td>ns</td>
<td>P&lt;0.05</td>
<td>P&lt;0.001</td>
<td>P&lt;0.001</td>
<td>P&lt;0.001</td>
</tr>
<tr>
<td>4 mg/L vs. 32 mg/L</td>
<td>P&lt;0.001</td>
<td>P&lt;0.001</td>
<td>P&lt;0.001</td>
<td>P&lt;0.001</td>
<td>P&lt;0.001</td>
</tr>
<tr>
<td>8 mg/L vs. 16 mg/L</td>
<td>ns</td>
<td>ns</td>
<td>P&lt;0.05</td>
<td>P&lt;0.001</td>
<td>P&lt;0.001</td>
</tr>
<tr>
<td>8 mg/L vs. 32 mg/L</td>
<td>P&lt;0.001</td>
<td>P&lt;0.001</td>
<td>P&lt;0.001</td>
<td>P&lt;0.001</td>
<td>P&lt;0.001</td>
</tr>
<tr>
<td>16 mg/L vs. 32 mg/L</td>
<td>P&lt;0.001</td>
<td>P&lt;0.001</td>
<td>P&lt;0.001</td>
<td>ns</td>
<td>ns</td>
</tr>
</tbody>
</table>

Table 8.2 BPA’s LC50s for the 24, 48, 72, 96 and 120 hpf zebrafish.

<table>
<thead>
<tr>
<th>BPA [mg/L]</th>
<th>BPA [µM]</th>
<th>Log conc [µM]</th>
<th>24 hpf mortality [%]</th>
<th>48 hpf mortality [%]</th>
<th>72 hpf mortality [%]</th>
<th>96 hpf mortality [%]</th>
<th>120 hpf mortality [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>8.76</td>
<td>0.9425</td>
<td>3.33</td>
<td>3.33</td>
<td>3.33</td>
<td>3.33</td>
<td>3.33</td>
</tr>
<tr>
<td>4</td>
<td>17.52</td>
<td>1.2435</td>
<td>6.66</td>
<td>6.66</td>
<td>6.66</td>
<td>6.66</td>
<td>6.66</td>
</tr>
<tr>
<td>8</td>
<td>35.04</td>
<td>1.5446</td>
<td>3.33</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>13.3</td>
</tr>
<tr>
<td>16</td>
<td>70.09</td>
<td>1.8456</td>
<td>23.33</td>
<td>23.33</td>
<td>33.33</td>
<td>90</td>
<td>100</td>
</tr>
<tr>
<td>32</td>
<td>140.17</td>
<td>2.1466</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td><strong>LC50 [µM]</strong></td>
<td></td>
<td>73.47</td>
<td>69.63</td>
<td>64.73</td>
<td>43.15</td>
<td>39.20</td>
<td></td>
</tr>
<tr>
<td><strong>LC50 [mg/L]</strong></td>
<td></td>
<td>16.77</td>
<td>15.89</td>
<td>14.78</td>
<td>9.85</td>
<td>8.95</td>
<td></td>
</tr>
</tbody>
</table>

LC50 values were calculated using Probit Analysis (SPSS Statistics).
Table 8.3 Mean heart rate (bpm) of the 48 and 72 hpf zebrafish exposed to 2, 4, 8 and 16 mg/L BPA.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Heart rate at 48 hpf Mean ± SD (bpm)</th>
<th>Heart rate at 72 hpf Mean ± SD (bpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1% DMSO</td>
<td>135.07 ± 7.45</td>
<td>127.57 ± 1.40</td>
</tr>
<tr>
<td>2 mg/L BPA</td>
<td>130.00 ± 13.54</td>
<td>128.17 ± 3.33</td>
</tr>
<tr>
<td>4 mg/L BPA</td>
<td>115.77 ± 19.03</td>
<td>118.93 ± 6.88</td>
</tr>
<tr>
<td>8 mg/L BPA</td>
<td>82.73 ± 13.85</td>
<td>88.00 ± 7.36</td>
</tr>
<tr>
<td>16 mg/L BPA</td>
<td>22.33 ± 6.03</td>
<td>22.67 ± 2.57</td>
</tr>
</tbody>
</table>

Table 8.4 Specific comparisons of the heart rate of the 48 and 72 hpf zebrafish exposed to 2, 4, 8 and 16 mg/L BPA.

Tukey's Multiple Comparison Test  | 48 hpf | 72 hpf |
--------------------------------|--------|--------|
0.1% DMSO vs. 2 mg/L BPA       | ns     | ns     |
0.1% DMSO vs. 4 mg/L BPA       | ns     | ns     |
0.1% DMSO vs. 8 mg/L BPA       | P<0.01 | P<0.001|
0.1% DMSO vs. 16 mg/L BPA      | P<0.001| P<0.001|
2 mg/L vs. 4 mg/L BPA          | ns     | ns     |
2 mg/L vs. 8 mg/L BPA          | P<0.01 | P<0.001|
2 mg/L vs. 16 mg/L BPA         | P<0.001| P<0.001|
4 mg/L vs. 8 mg/L BPA          | ns     | P<0.001|
4 mg/L vs. 16 mg/L BPA         | P<0.001| P<0.001|
8 mg/L vs. 16 mg/L BPA         | P<0.001| P<0.001|

Table 8.5 Specific comparison of the hatching success of the 72 hpf zebrafish exposed to 2, 4, 8 and 16 mg/L BPA.

Tukey's Multiple Comparison Test  | 72 hpf |
--------------------------------|--------|
0.1% DMSO vs. 2 mg/L BPA       | ns     |
0.1% DMSO vs. 4 mg/L BPA       | ns     |
0.1% DMSO vs. 8 mg/L BPA       | P<0.001|
0.1% DMSO vs. 16 mg/L BPA      | P<0.001|
2 mg/L vs. 4 mg/L BPA          | ns     |
2 mg/L vs. 8 mg/L BPA          | P<0.001|
2 mg/L vs. 16 mg/L BPA         | P<0.001|
4 mg/L vs. 8 mg/L BPA          | P<0.001|
4 mg/L vs. 16 mg/L BPA         | P<0.001|
8 mg/L vs. 16 mg/L BPA         | ns     |
Table 8.6 Specific comparisons of the survival of the 24, 48, 72, 96 and 120 hpf zebrafish exposed to 1.25, 2.5, 5 and 10 mg/L genistein from 0.75 – 1 hpf until 120 hpf.

<table>
<thead>
<tr>
<th>Tukey’s Multiple Comparison Test</th>
<th>24 hpf</th>
<th>48 hpf</th>
<th>72 hpf</th>
<th>96 hpf</th>
<th>120 hpf</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1% DMSO vs. 1.25 mg/L</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>P&lt;0.001</td>
</tr>
<tr>
<td>0.1% DMSO vs. 2.5 mg/L</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>P&lt;0.001</td>
<td>P&lt;0.001</td>
</tr>
<tr>
<td>0.1% DMSO vs. 5 mg/L</td>
<td>P&lt;0.001</td>
<td>P&lt;0.001</td>
<td>P&lt;0.001</td>
<td>P&lt;0.001</td>
<td>P&lt;0.001</td>
</tr>
<tr>
<td>0.1% DMSO vs. 10 mg/L</td>
<td>P&lt;0.001</td>
<td>P&lt;0.001</td>
<td>P&lt;0.001</td>
<td>P&lt;0.001</td>
<td>P&lt;0.001</td>
</tr>
<tr>
<td>1.25 mg/L vs. 2.5 mg/L</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>P&lt;0.001</td>
<td>P&lt;0.001</td>
</tr>
<tr>
<td>1.25 mg/L vs. 5 mg/L</td>
<td>P&lt;0.001</td>
<td>P&lt;0.001</td>
<td>P&lt;0.001</td>
<td>P&lt;0.001</td>
<td>P&lt;0.001</td>
</tr>
<tr>
<td>1.25 mg/L vs. 10 mg/L</td>
<td>P&lt;0.001</td>
<td>P&lt;0.001</td>
<td>P&lt;0.001</td>
<td>P&lt;0.001</td>
<td>P&lt;0.001</td>
</tr>
<tr>
<td>2.5 mg/L vs. 5 mg/L</td>
<td>P&lt;0.001</td>
<td>P&lt;0.001</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>2.5 mg/L vs. 10 mg/L</td>
<td>P&lt;0.001</td>
<td>P&lt;0.001</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>5 mg/L vs. 10 mg/L</td>
<td>P&lt;0.001</td>
<td>P&lt;0.001</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
</tr>
</tbody>
</table>

Table 8.7 Genistein’s LC50s for the 24, 48, 72, 96 and 120 hpf zebrafish.

<table>
<thead>
<tr>
<th>Genistein [mg/L]</th>
<th>Genistein [µM]</th>
<th>Log conc [µM]</th>
<th>24 hpf mortality [%]</th>
<th>48 hpf mortality [%]</th>
<th>72 hpf mortality [%]</th>
<th>96 hpf mortality [%]</th>
<th>120 hpf mortality [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.25</td>
<td>4.62</td>
<td>0.6646</td>
<td>3.33</td>
<td>3.33</td>
<td>3.33</td>
<td>3.33</td>
<td>3.33</td>
</tr>
<tr>
<td>2.5</td>
<td>9.25</td>
<td>0.9661</td>
<td>3.33</td>
<td>3.33</td>
<td>3.33</td>
<td>6.66</td>
<td>76.66</td>
</tr>
<tr>
<td>5</td>
<td>18.5</td>
<td>1.2672</td>
<td>36.66</td>
<td>43.33</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>10</td>
<td>37</td>
<td>1.5682</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>LC50 [µM]</td>
<td>18.46</td>
<td>17.73</td>
<td>11.89</td>
<td>7.55</td>
<td>6.52</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LC50 [mg/L]</td>
<td>4.99</td>
<td>4.79</td>
<td>3.21</td>
<td>2.04</td>
<td>1.76</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

LC50 values were calculated using Probit Analysis (SPSS Statistics)

Table 8.8 Mean heart rate (bpm) of the 48 and 72 hpf zebrafish exposed to 1.25, 2.5 and 5 mg/L genistein.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Heart rate at 48 hpf Mean ± SD (bpm)</th>
<th>Heart rate at 72 hpf Mean ± SD (bpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1% DMSO</td>
<td>135.07 ± 7.45</td>
<td>127.57 ± 1.40</td>
</tr>
<tr>
<td>1.25 mg/L genistein</td>
<td>132.07 ± 5.27</td>
<td>129.87 ± 0.32</td>
</tr>
<tr>
<td>2.5 mg/L genistein</td>
<td>101.43 ± 14.87</td>
<td>36.83 ± 5.95</td>
</tr>
<tr>
<td>5 mg/L genistein</td>
<td>42.43 ± 2.68</td>
<td>0.00 ± 0.00</td>
</tr>
</tbody>
</table>
Table 8.9 Specific comparisons of the heart rate of the 48 and 72 hpf zebrafish exposed to 1.25, 2.5 and 5 mg/L genistein.

<table>
<thead>
<tr>
<th>Tukey’s Multiple Comparison Test</th>
<th>48 hpf</th>
<th>72 hpf</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1% DMSO vs. 1.25 mg/L genistein</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>0.1% DMSO vs. 2.5 mg/L genistein</td>
<td>P&lt;0.01</td>
<td>P&lt;0.001</td>
</tr>
<tr>
<td>0.1% DMSO vs. 5 mg/L genistein</td>
<td>P&lt;0.001</td>
<td>P&lt;0.001</td>
</tr>
<tr>
<td>1.25 mg/L vs. 2.5 mg/L genistein</td>
<td>P&lt;0.01</td>
<td>P&lt;0.001</td>
</tr>
<tr>
<td>1.25 mg/L vs. 5 mg/L genistein</td>
<td>P&lt;0.001</td>
<td>P&lt;0.001</td>
</tr>
<tr>
<td>2.5 mg/L vs. 5 mg/L genistein</td>
<td>P&lt;0.001</td>
<td>P&lt;0.001</td>
</tr>
</tbody>
</table>

Table 8.10 Specific comparison of the hatching success of the 72 hpf zebrafish exposed to 1.25 and 2.5 mg/L genistein.

<table>
<thead>
<tr>
<th>Tukey’s Multiple Comparison Test</th>
<th>72 hpf</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1% DMSO vs. 1.25 mg/L genistein</td>
<td>ns</td>
</tr>
<tr>
<td>0.1% DMSO vs. 2.5 mg/L genistein</td>
<td>P&lt;0.001</td>
</tr>
<tr>
<td>1.25 mg/L vs. 2.5 mg/L genistein</td>
<td>P&lt;0.001</td>
</tr>
</tbody>
</table>
Table 8.11 Specific comparisons of the survival of the 24, 48, 72, 96 and 120 hpf zebrafish exposed to BPA and genistein individually and in mixtures from 0.75-1 hpf until 120 hpf.

<table>
<thead>
<tr>
<th>Tukey's Multiple Comparison Test</th>
<th>24 hpf</th>
<th>48 hpf</th>
<th>72 hpf</th>
<th>96 hpf</th>
<th>120 hpf</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1% DMSO vs. 4 mg/L BPA</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>0.1% DMSO vs. 8 mg/L BPA</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>0.1% DMSO vs. 16 mg/L BPA</td>
<td>ns</td>
<td>ns</td>
<td>P&lt;0.001</td>
<td>PP&lt;0.001</td>
<td>PP&lt;0.001</td>
</tr>
<tr>
<td>0.1% DMSO vs. 1.25 mg/L GEN</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>0.1% DMSO vs. 2.5 mg/L GEN</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>P&lt;0.001</td>
<td>PP&lt;0.001</td>
</tr>
<tr>
<td>0.1% DMSO vs. 5 mg/L GEN</td>
<td>P&lt;0.01</td>
<td>P&lt;0.001</td>
<td>P&lt;0.001</td>
<td>P&lt;0.001</td>
<td>P&lt;0.001</td>
</tr>
<tr>
<td>0.1% DMSO vs. 4+1.25 mg/L BPA+GEN</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>0.1% DMSO vs. 4+2.5 mg/L BPA+GEN</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>P&lt;0.001</td>
<td>P&lt;0.001</td>
</tr>
<tr>
<td>0.1% DMSO vs. 8+1.25 mg/L BPA+GEN</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>P&lt;0.01</td>
<td>ns</td>
</tr>
<tr>
<td>0.1% DMSO vs. 8+2.5 mg/L BPA+GEN</td>
<td>ns</td>
<td>ns</td>
<td>P&lt;0.001</td>
<td>P&lt;0.001</td>
<td>P&lt;0.001</td>
</tr>
<tr>
<td>0.1% DMSO vs. 16+5 mg/L BPA+GEN</td>
<td>P&lt;0.001</td>
<td>P&lt;0.001</td>
<td>P&lt;0.001</td>
<td>P&lt;0.001</td>
<td>P&lt;0.001</td>
</tr>
<tr>
<td>4 mg/L BPA vs. 4+1.25 mg/L BPA+GEN</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>4 mg/L BPA vs. 4+2.5 mg/L BPA+GEN</td>
<td>ns</td>
<td>ns</td>
<td>P&lt;0.001</td>
<td>P&lt;0.001</td>
<td>ns</td>
</tr>
<tr>
<td>8 mg/L BPA vs. 8+1.25 mg/L BPA+GEN</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>8 mg/L BPA vs. 8+2.5 mg/L BPA+GEN</td>
<td>ns</td>
<td>ns</td>
<td>P&lt;0.001</td>
<td>P&lt;0.001</td>
<td>ns</td>
</tr>
<tr>
<td>16 mg/L BPA vs. 16+5 mg/L BPA+GEN</td>
<td>P&lt;0.001</td>
<td>P&lt;0.001</td>
<td>P&lt;0.001</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>1.25 mg/L GEN vs. 4+1.25 mg/L BPA+GEN</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>1.25 mg/L GEN vs. 8+1.25 mg/L BPA+GEN</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>P&lt;0.01</td>
<td>ns</td>
</tr>
<tr>
<td>2.5 mg/L GEN vs. 4+2.5 mg/L BPA+GEN</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>2.5 mg/L GEN vs. 8+2.5 mg/L BPA+GEN</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>5 mg/L GEN vs. 16+5 mg/L BPA+GEN</td>
<td>P&lt;0.001</td>
<td>P&lt;0.001</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
</tr>
</tbody>
</table>
Table 8.12 Specific comparisons of the heart rate of the 48 and 72 hpf zebrafish exposed to BPA and genistein individually and in mixtures.

<table>
<thead>
<tr>
<th>Tukey’s Multiple Comparison Test</th>
<th>48 hpf</th>
<th>72 hpf</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1% DMSO vs. 4 mg/L BPA</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>0.1% DMSO vs. 8 mg/L BPA</td>
<td><em>P&lt;0.001</em></td>
<td><em>P&lt;0.001</em></td>
</tr>
<tr>
<td>0.1% DMSO vs. 1.25 mg/L GEN</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>0.1% DMSO vs. 2.5 mg/L GEN</td>
<td><em>P&lt;0.01</em></td>
<td><em>P&lt;0.001</em></td>
</tr>
<tr>
<td>0.1% DMSO vs. 4+1.25 mg/L BPA+GEN</td>
<td><em>P&lt;0.001</em></td>
<td><em>P&lt;0.001</em></td>
</tr>
<tr>
<td>0.1% DMSO vs. 4+2.5 mg/L BPA+GEN</td>
<td><em>P&lt;0.01</em></td>
<td><em>P&lt;0.001</em></td>
</tr>
<tr>
<td>0.1% DMSO vs. 8+1.25 mg/L BPA+GEN</td>
<td><em>P&lt;0.001</em></td>
<td><em>P&lt;0.001</em></td>
</tr>
<tr>
<td>0.1% DMSO vs. 8+2.5 mg/L BPA+GEN</td>
<td><em>P&lt;0.001</em></td>
<td><em>P&lt;0.001</em></td>
</tr>
<tr>
<td>4 mg/L BPA vs. 4+1.25 mg/L BPA+GEN</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>4 mg/L BPA vs. 4+2.5 mg/L BPA+GEN</td>
<td>ns</td>
<td><em>P&lt;0.001</em></td>
</tr>
<tr>
<td>8 mg/L BPA vs. 8+1.25 mg/L BPA+GEN</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>8 mg/L BPA vs. 8+2.5 mg/L BPA+GEN</td>
<td>ns</td>
<td><em>P&lt;0.001</em></td>
</tr>
<tr>
<td>1.25 mg/L GEN vs. 4+1.25 mg/L BPA+GEN</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>1.25 mg/L GEN vs. 8+1.25 mg/L BPA+GEN</td>
<td><em>P&lt;0.01</em></td>
<td><em>P&lt;0.001</em></td>
</tr>
<tr>
<td>2.5 mg/L GEN vs. 4+2.5 mg/L BPA+GEN</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>2.5 mg/L GEN vs. 8+2.5 mg/L BPA+GEN</td>
<td><em>P&lt;0.05</em></td>
<td>ns</td>
</tr>
</tbody>
</table>

Table 8.13 Specific comparison of the hatching success of the 72 hpf zebrafish exposed to BPA and genistein individually and in mixtures.

<table>
<thead>
<tr>
<th>Tukey’s Multiple Comparison Test</th>
<th>Significance</th>
</tr>
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<tbody>
<tr>
<td>0.1% DMSO vs. 4 mg/L BPA</td>
<td>ns</td>
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<tr>
<td>0.1% DMSO vs. 8 mg/L BPA</td>
<td><em>P&lt;0.001</em></td>
</tr>
<tr>
<td>0.1% DMSO vs. 1.25 mg/L GEN</td>
<td>ns</td>
</tr>
<tr>
<td>0.1% DMSO vs. 2.5 mg/L GEN</td>
<td><em>P&lt;0.001</em></td>
</tr>
<tr>
<td>0.1% DMSO vs. 4+1.25 mg/L BPA+GEN</td>
<td><em>P&lt;0.001</em></td>
</tr>
<tr>
<td>0.1% DMSO vs. 4+2.5 mg/L BPA+GEN</td>
<td><em>P&lt;0.001</em></td>
</tr>
<tr>
<td>0.1% DMSO vs. 8+1.25 mg/L BPA+GEN</td>
<td><em>P&lt;0.001</em></td>
</tr>
<tr>
<td>0.1% DMSO vs. 8+2.5 mg/L BPA+GEN</td>
<td><em>P&lt;0.001</em></td>
</tr>
<tr>
<td>4 mg/L BPA vs. 4+1.25 mg/L BPA+GEN</td>
<td><em>P&lt;0.001</em></td>
</tr>
<tr>
<td>4 mg/L BPA vs. 4+2.5 mg/L BPA+GEN</td>
<td><em>P&lt;0.001</em></td>
</tr>
<tr>
<td>8 mg/L BPA vs. 8+1.25 mg/L BPA+GEN</td>
<td>ns</td>
</tr>
<tr>
<td>8 mg/L BPA vs. 8+2.5 mg/L BPA+GEN</td>
<td>ns</td>
</tr>
<tr>
<td>1.25 mg/L GEN vs. 4+1.25 mg/L BPA+GEN</td>
<td><em>P&lt;0.001</em></td>
</tr>
<tr>
<td>1.25 mg/L GEN vs. 8+1.25 mg/L BPA+GEN</td>
<td><em>P&lt;0.001</em></td>
</tr>
<tr>
<td>2.5 mg/L GEN vs. 4+2.5 mg/L BPA+GEN</td>
<td>ns</td>
</tr>
<tr>
<td>2.5 mg/L GEN vs. 8+2.5 mg/L BPA+GEN</td>
<td>ns</td>
</tr>
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</table>
Table 8.14 Specific comparison of the absolute volumes and volume fractions of the parameters analysed in the liver of 120 hpf zebrafish.

<table>
<thead>
<tr>
<th></th>
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<th></th>
<th></th>
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<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>0.01% DMSO vs. 10 μg/L BPA</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>≤0.05</td>
<td>≤0.05</td>
<td>≤0.05</td>
<td>≤0.05</td>
<td>ns</td>
<td>≤0.05</td>
<td>≤0.05</td>
<td>≤0.05</td>
<td>ns</td>
<td></td>
</tr>
<tr>
<td>0.01% DMSO vs. 100 μg/L BPA</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>≤0.05</td>
<td>≤0.05</td>
<td>≤0.05</td>
<td>ns</td>
<td></td>
</tr>
<tr>
<td>0.01% DMSO vs. 312.5 μg/L GEN</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>≤0.05</td>
<td>ns</td>
<td>≤0.05</td>
<td>≤0.05</td>
<td>ns</td>
<td>≤0.05</td>
<td>≤0.05</td>
<td>≤0.05</td>
<td>ns</td>
<td></td>
</tr>
<tr>
<td>0.01% DMSO vs. 625 μg/L GEN</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>≤0.05</td>
<td>ns</td>
<td>≤0.05</td>
<td>≤0.05</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.01% DMSO vs. 10 μg/L BPA + 312.5 μg/L GEN</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>≤0.05</td>
<td>ns</td>
<td>≤0.05</td>
<td>≤0.05</td>
<td>ns</td>
<td>≤0.05</td>
<td>≤0.05</td>
<td>≤0.05</td>
<td>ns</td>
<td></td>
</tr>
<tr>
<td>0.01% DMSO vs. 10 μg/L BPA + 625 μg/L GEN</td>
<td>≤0.05</td>
<td>≤0.05</td>
<td>ns</td>
<td>≤0.05</td>
<td>≤0.05</td>
<td>≤0.05</td>
<td>≤0.05</td>
<td>ns</td>
<td>≤0.05</td>
<td>≤0.05</td>
<td>≤0.05</td>
<td>ns</td>
<td></td>
</tr>
<tr>
<td>0.01% DMSO vs. 100 μg/L BPA + 312.5 μg/L GEN</td>
<td>≤0.05</td>
<td>≤0.05</td>
<td>ns</td>
<td>≤0.05</td>
<td>≤0.05</td>
<td>≤0.05</td>
<td>≤0.05</td>
<td>ns</td>
<td>≤0.05</td>
<td>≤0.05</td>
<td>≤0.05</td>
<td>ns</td>
<td></td>
</tr>
<tr>
<td>0.01% DMSO vs. 100 μg/L BPA + 625 μg/L GEN</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>≤0.05</td>
<td>≤0.05</td>
<td>≤0.05</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>0.01% DMSO vs. 20 μg/L 17ß-oestradiol</td>
<td>≤0.05</td>
<td>≤0.05</td>
<td>ns</td>
<td>≤0.05</td>
<td>≤0.05</td>
<td>≤0.05</td>
<td>≤0.05</td>
<td>ns</td>
<td>≤0.05</td>
<td>≤0.05</td>
<td>≤0.05</td>
<td>ns</td>
<td></td>
</tr>
<tr>
<td>10 μg/L BPA vs. 10 μg/L BPA + 312.5 μg/L GEN</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>≤0.05</td>
<td>ns</td>
<td>ns</td>
<td></td>
<td></td>
</tr>
<tr>
<td>312.5 μg/L GEN vs. 10 μg/L BPA + 312.5 μg/L GEN</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>≤0.05</td>
<td>ns</td>
<td>ns</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 μg/L BPA vs. 10 μg/L BPA + 625 μg/L GEN</td>
<td>≤0.05</td>
<td>≤0.05</td>
<td>ns</td>
<td>≤0.05</td>
<td>≤0.05</td>
<td>≤0.05</td>
<td>≤0.05</td>
<td>ns</td>
<td>≤0.05</td>
<td>≤0.05</td>
<td>≤0.05</td>
<td>ns</td>
<td></td>
</tr>
<tr>
<td>625 μg/L GEN vs. 10 μg/L BPA + 625 μg/L GEN</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>≤0.05</td>
<td>≤0.05</td>
<td>≤0.05</td>
<td>ns</td>
<td></td>
</tr>
<tr>
<td>100 μg/L BPA vs. 100 μg/L BPA + 312.5 μg/L GEN</td>
<td>≤0.05</td>
<td>≤0.05</td>
<td>ns</td>
<td>≤0.05</td>
<td>≤0.05</td>
<td>≤0.05</td>
<td>≤0.05</td>
<td>ns</td>
<td>≤0.05</td>
<td>≤0.05</td>
<td>≤0.05</td>
<td>ns</td>
<td></td>
</tr>
<tr>
<td>312.5 μg/L GEN vs. 100 μg/L BPA + 312.5 μg/L GEN</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>≤0.05</td>
<td>ns</td>
<td>ns</td>
<td></td>
<td></td>
</tr>
<tr>
<td>100 μg/L BPA vs. 100 μg/L BPA + 625 μg/L GEN</td>
<td>≤0.05</td>
<td>≤0.05</td>
<td>ns</td>
<td>≤0.05</td>
<td>≤0.05</td>
<td>≤0.05</td>
<td>≤0.05</td>
<td>ns</td>
<td>≤0.05</td>
<td>≤0.05</td>
<td>≤0.05</td>
<td>ns</td>
<td></td>
</tr>
<tr>
<td>625 μg/L GEN vs. 100 μg/L BPA + 625 μg/L GEN</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>≤0.05</td>
<td>≤0.05</td>
<td>≤0.05</td>
<td>ns</td>
<td></td>
</tr>
</tbody>
</table>

Absolute volumes (Vol) and volume fractions (Vv) of the hepatocytes (Hept), nuclei of the hepatocytes (Nucl), mitochondria of the hepatocytes (Mito), RER of the hepatocytes, glycogen (Glyc) and bile canaliculi (BC). The effects of the treatments were compared to the control group (DMSO). The effects of the mixtures were also compared to the individual components’ effects.
Table 8.15 Summary of the results on the liver ultrastructure of the zebrafish larvae treated with BPA or/and genistein at 72 hpf and 120 hpf.

<table>
<thead>
<tr>
<th>Age</th>
<th>72 hours post-fertilization</th>
<th>120 hours post-fertilization</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Compound</strong></td>
<td>BPA</td>
<td>BPA</td>
</tr>
<tr>
<td><strong>Concentration</strong></td>
<td>10 µg/L</td>
<td>100 µg/L</td>
</tr>
<tr>
<td><strong>Vol hepatocyte</strong></td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td><strong>Vol nucleus</strong></td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td><strong>Vv pooled mito</strong></td>
<td>light green</td>
<td>light green</td>
</tr>
<tr>
<td><strong>Vol pooled mito</strong></td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td><strong>Vv single mito</strong></td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td><strong>Vol single mito</strong></td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td><strong>Vol RER</strong></td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td><strong>Vv bile cc</strong></td>
<td>light green</td>
<td>light green</td>
</tr>
<tr>
<td><strong>Vol bile cc</strong></td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td><strong>Vv glycogen</strong></td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td><strong>Vol glycogen</strong></td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td><strong>MP area</strong></td>
<td>light green</td>
<td>light green</td>
</tr>
<tr>
<td><strong>MP max feret</strong></td>
<td>light green</td>
<td>light green</td>
</tr>
<tr>
<td><strong>MP min feret</strong></td>
<td>light green</td>
<td>light green</td>
</tr>
<tr>
<td><strong>MP aspect ratio</strong></td>
<td>light green</td>
<td>light green</td>
</tr>
</tbody>
</table>

Absolute volumes (Vol) and volume fractions (Vv) of the hepatocytes, nuclei, mitochondria (mito), RER, bile canaliculi (bile cc) and glycogen, as well as the descriptors of the mitochondrial profiles (MP) – area, circularity, maximum and minimum feret diameters and major to minor axis ratio (AR) were compared between the BPA or/and genistein treated larvae and the controls. Colours indicate: dark purple – significantly more (p<0.05) than controls; light purple – noticeably more (p<0.1) than controls; dark green - significantly less (p<0.05) than controls; light green - noticeably less (p<0.1) than controls; blue - similar to controls (p>0.1).
Table 8.16 Inter-experimental comparisons of the fold changes in the vtg1 expression in the 120 hpf zebrafish larvae treated with BPA and genistein, individually and in mixtures.

<table>
<thead>
<tr>
<th>TREATMENT</th>
<th>Fold-changes</th>
<th>MEAN</th>
<th>SD</th>
<th>SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Exp.1</td>
<td>Exp.2</td>
<td>Exp.3</td>
<td>Exp.4</td>
</tr>
<tr>
<td>DMSO</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>17βE2</td>
<td>1650.51</td>
<td>488.26</td>
<td>551.13</td>
<td>1460.30</td>
</tr>
<tr>
<td>10 µg/L BPA</td>
<td>0.55</td>
<td>1.24</td>
<td>0.62</td>
<td>0.88</td>
</tr>
<tr>
<td>100 µg/L</td>
<td>0.31</td>
<td>1.19</td>
<td>0.60</td>
<td>0.59</td>
</tr>
<tr>
<td>2000 µg/L BPA</td>
<td>2.42</td>
<td>66.21</td>
<td>16.65</td>
<td>12.06</td>
</tr>
<tr>
<td>312.5 µg/L GEN</td>
<td>1.71</td>
<td>6.52</td>
<td>2.91</td>
<td>9.39</td>
</tr>
<tr>
<td>625 µg/L GEN</td>
<td>32.93</td>
<td>155.24</td>
<td>248.03</td>
<td>97.09</td>
</tr>
<tr>
<td>1250 µg/L GEN</td>
<td>279.07</td>
<td>667.99</td>
<td>383.09</td>
<td>674.92</td>
</tr>
<tr>
<td>10 µg/L BPA + 312.5 µg/L GEN</td>
<td>4.32</td>
<td>33.29</td>
<td>3.20</td>
<td>5.94</td>
</tr>
<tr>
<td>10 µg/L BPA + 625 µg/L GEN</td>
<td>3.90</td>
<td>291.63</td>
<td>177.88</td>
<td>90.57</td>
</tr>
<tr>
<td>100 µg/L BPA + 312.5 µg/L GEN</td>
<td>1.53</td>
<td>37.51</td>
<td>11.19</td>
<td>6.01</td>
</tr>
<tr>
<td>100 µg/L BPA + 625 µg/L GEN</td>
<td>44.72</td>
<td>66.67</td>
<td>161.72</td>
<td>444.21</td>
</tr>
<tr>
<td>2000 µg/L BPA + 1250 µg/L GEN</td>
<td>1051.35</td>
<td>2565.99</td>
<td>228.05</td>
<td>286.50</td>
</tr>
</tbody>
</table>
Table 8.17 Inter-experimental comparison of the cycle thresholds (CT) for the vtg1 in the in the 120 hpf zebrafish larvae treated with BPA and genistein, individually and in mixtures.

<p>| GOI   | Treatment [µg/L] | Cr1 | Cr2 | Cr3 | Cr4  | Cr1 AVG | Cr2 AVG | Cr3 AVG | Cr4 AVG | Cr1 SD  | Cr2 SD  | Cr3 SD  | Cr4 SD  |
|-------|-----------------|-----|-----|-----|------|---------|---------|---------|---------|---------|---------|---------|---------|---------|
|       | DMSO            |     |     |     |      |         |         |         |         |         |         |         |         |         |
| ef1a  | 17βE2           | 17.30 | 15.64 | 15.46 | 16.44 | 17.38 | 15.74 | 15.44 | 16.53 | 0.07 | 0.08 | 0.03 | 0.11 |
| ef1a  | 10 BPA          | 16.59 | 16.37 | 15.62 | 16.82 | 16.59 | 16.24 | 15.63 | 16.81 | 0.02 | 0.15 | 0.01 | 0.03 |
| ef1a  | 10 BPA          | 16.62 | 16.26 | 15.62 | 16.84 |         |         |         |         |         |         |         |         |         |
| ef1a  | 100 BPA         | 16.66 | 16.23 | 15.42 | 15.92 | 16.66 | 15.42 | 15.94 | 0.02 | 0.02 | 0.01 | 0.03 |
| ef1a  | 100 BPA         | 16.65 | 16.26 | 15.42 | 15.93 |         |         |         |         |         |         |         |         |         |
| ef1a  | 100 BPA         | 16.68 | 16.25 | 15.41 | 15.97 |         |         |         |         |         |         |         |         |         |
| ef1a  | 2000 BPA        | 16.04 | 15.85 | 15.41 | 16.10 | 16.06 | 15.87 | 15.40 | 16.05 | 0.04 | 0.29 | 0.01 | 0.07 |
| ef1a  | 625 GEN         | 16.27 | 15.79 | 15.67 | 16.36 | 16.21 | 15.73 | 15.62 | 16.33 | 0.09 | 0.05 | 0.05 | 0.02 |
| ef1a  | 625 GEN         | 16.26 | 15.69 | 15.61 | 16.33 |         |         |         |         |         |         |         |         |         |
| ef1a  | 625 GEN         | 16.11 | 15.71 | 15.57 | 16.31 |         |         |         |         |         |         |         |         |         |
| ef1a  | 1250 GEN        | 16.28 | 16.02 | 15.34 | 16.34 | 16.30 | 16.05 | 15.33 | 16.38 | 0.08 | 0.04 | 0.01 | 0.04 |
| ef1a  | 1250 GEN        | 16.39 | 16.10 | 15.33 | 16.41 |         |         |         |         |         |         |         |         |         |
| ef1a  | 1250 GEN        | 16.23 | 16.04 | 15.31 | 16.39 |         |         |         |         |         |         |         |         |         |
| ef1a  | 10 BPA+312.5 GEN| 16.37 | 16.04 | 15.23 | 16.06 | 16.45 | 16.10 | 15.26 | 16.09 | 0.10 | 0.06 | 0.03 | 0.03 |
| ef1a  | 10 BPA+312.5 GEN| 16.41 | 16.16 | 15.27 | 16.09 |         |         |         |         |         |         |         |         |         |
| ef1a  | 10 BPA+312.5 GEN| 16.56 | 16.11 | 15.27 | 16.12 |         |         |         |         |         |         |         |         |         |
| ef1a  | 10 BPA+625 GEN  | 16.21 | 15.96 | 15.46 | 16.00 | 16.27 | 15.87 | 15.45 | 15.98 | 0.14 | 0.14 | 0.00 | 0.02 |
| ef1a  | 10 BPA+625 GEN  | 16.43 | 15.94 | 15.45 | 15.97 |         |         |         |         |         |         |         |         |         |
| ef1a  | 100 BPA+312.5 GEN| 16.49 | 15.81 | 15.71 | 15.76 | 16.52 | 15.75 | 15.73 | 15.76 | 0.07 | 0.10 | 0.07 | 0.03 |
| ef1a  | 100 BPA+312.5 GEN| 16.47 | 15.63 | 15.81 | 15.73 |         |         |         |         |         |         |         |         |         |
| ef1a  | 100 BPA+312.5 GEN| 16.60 | 15.80 | 15.67 | 15.79 |         |         |         |         |         |         |         |         |         |
| ef1a  | 100 BPA+625 GEN  | 16.51 | 16.65 | 15.41 | 16.16 | 16.40 | 16.27 | 15.35 | 16.11 | 0.12 | 0.34 | 0.05 | 0.06 |
| ef1a  | 100 BPA+625 GEN  | 16.43 | 16.12 | 15.32 | 16.12 |         |         |         |         |         |         |         |         |         |
| ef1a  | 100 BPA+625 GEN  | 16.26 | 16.02 | 15.32 | 16.04 |         |         |         |         |         |         |         |         |         |
| ef1a  | 2000 BPA+1250 GEN| 16.52 | 16.28 | 15.40 | 16.36 | 16.71 | 16.37 | 15.52 | 16.40 | 0.17 | 0.08 | 0.11 | 0.06 |
| ef1a  | 2000 BPA+1250 GEN| 16.84 | 16.39 | 15.61 | 16.37 |         |         |         |         |         |         |         |         |         |
| ef1a  | 2000 BPA+1250 GEN| 16.77 | 16.44 | 15.55 | 16.47 |         |         |         |         |         |         |         |         |         |
| ef1a  | NTC             | undt | undt | undt | undt |         |         |         |         |         |         |         |         |         |
| ef1a  | NTC             | undt | undt | undt | undt |         |         |         |         |         |         |         |         |         |
| ef1a  | NTC             | undt | undt | undt | undt |         |         |         |         |         |         |         |         |         |
| vtg1  | DMSO            | 27.63 | 27.50 | 26.65 | 28.45 | 28.03 | 27.80 | 26.83 | 28.80 | 0.47 | 0.30 | 0.25 | 0.38 |
| vtg1  | DMSO            | 27.91 | 27.79 | 26.73 | 28.76 |         |         |         |         |         |         |         |         |         |</p>
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