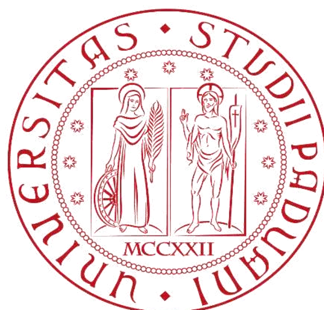

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DIPARTIMENTO DI SCIENZE DEL FARMACO

CORSO DI LAUREA MAGISTRALE IN CHIMICA E TECNOLOGIA FARMACEUTICHE

TESI DI LAUREA

NEUROBEHAVIOURAL EFFECTS OF 17-α-ETHINYLESTRADIOL ON ZEBRAFISH LARVAE AND OPTIMIZATION OF FEEDING PROTOCOLS

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Declaration

“I declare that this thesis is my original work. All literature and other sources, that I used during my work are stated in the literature list and cited properly. This work has not been used to achieve same or another degree.”

Dichiarazione

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Morgana Bonina

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Riassunto

L'estrogeno sintetico 17-α-etinilestradiolo (EE2), ingrediente attivo nella maggior parte dei contraccettivi orali, rientra nella classe dei composti chimici che alterano il sistema endocrino (*Endocrine disrupting chemicals*). Le elevate concentrazioni di EE2 rilevate nell'ambiente acquatico risultano dannose per il regolare funzionamento del sistema riproduttivo di specie vertebrate ed invertebrate, causando infertilità, malformazioni e cambiamenti del comportamento sessuale. Poiché gli effetti dell'EE2 sul comportamento riproduttivo delle specie acquatiche sono stati ampiamente investigati, abbiamo deciso di focalizzare la seguente ricerca sulle conseguenze comportamentali non legate alla riproduzione. Il modello animale testato è lo zebrafish (*Danio rerio*), tra i più impiegati nella ricerca neurocomportamentale. Le larve zebrafish sono state trattate con tre diverse concentrazioni di EE2 (0.5, 5, 50 ng/L) e due periodi di esposizione differenti, uno di breve durata (da 4 a 7 giorni post fertilizzazione) e uno di lunga durata (da 4 a 14 giorni post fertilizzazione). Inoltre, la reversibilità degli effetti nel tempo è stata esaminata a seguito di un periodo di sospensione dalla somministrazione del composto. I due saggi comportamentali proposti, lo *spontaneous swimming assay* e il *bouncing ball assay*, hanno permesso di analizzare, rispettivamente, la naturale attività locomotoria e la risposta di evitamento delle larve. L'EE2, alle concentrazioni di 0.5 e 5 ng/L, ha indotto un significativo decremento del parametro "iniziazioni" nello *spontaneous swimming assay* dopo un breve periodo di esposizione. Per ciò che concerne il *bouncing ball assay*, solo le concentrazioni di 5 e 50 ng/L nel trattamento di lunga durata hanno dimostrato avere un impatto negativo sulla risposta di evitamento. Non è stato riscontrato nessun prolungamento degli effetti a seguito del periodo di interruzione dal trattamento. Inoltre, l'esposizione al composto non ha indotto alcuna evidenza di tossicità per lo sviluppo delle larve.

In conclusione, possiamo affermare che l'EE2 rappresenta un pericolo per la sopravvivenza degli zebrafish in quanto l'alterazione dei parametri comportamentali presi in esame risulta di vitale importanza nel controllo delle manovre anti-predatorie. Investigazioni future sono ritenute necessarie per determinare più approfonditamente gli effetti ed il meccanismo d'azione dell'EE2.

In aggiunta agli studi sopracitati, è stata condotta una seconda ricerca con l'obiettivo principale di ottimizzare i protocolli di alimentazione comunemente utilizzati nell'allevamento dei pesci zebrafish, da sempre un ostacolo in termini di costi e manodopera per i laboratori di ricerca. Sono stati esaminati gli effetti di due diversi protocolli alimentari, comprendenti cibi secchi e

vivi (*Artemia*), sulla sopravvivenza delle larve. I risultati ottenuti non hanno mostrato una differenza significativa tra i protocolli usati.

Abstract

The synthetic estrogen 17-α-ethinylestradiol (EE2), active ingredient in many oral contraceptives, belongs to the class of endocrine disrupting chemicals (EDCs). The high concentrations of EE2 detected in the aquatic environment have been shown to be harmful to the regular functioning of the reproductive system of vertebrate and invertebrate species, causing infertility, malformations and changes in sexual behaviour. Since the effects of EE2 on the reproductive behavior of aquatic species have been extensively investigated, we decided to focus the following research on non-reproductive behavioral consequences. The animal model tested is zebrafish, among the most used in neurobehavioral research. Zebrafish larvae *(Danio rerio*) were treated with three different concentrations of EE2 (0.5, 5, 50 ng/L) and two different exposure periods, one of short duration (4 to 7 days post-fertilization) and one of long duration (4 to 14 days post-fertilization). In addition, the reversibility of effects over time was examined following a period of suspension from the administration of the compound. The two behavioral assays proposed, the spontaneous swimming assay and the bouncing ball assay, allowed to analyze, respectively, the natural locomotor activity and the avoidance response of larvae. EE2, at concentrations of 0.5 and 5 ng/L, induced a significant decrease of the "initiations" parameter in the spontaneous swimming assay after a short period of exposure. Regarding the bouncing ball assay, concentrations of 5 and 50 ng/L in the long duration treatment impacted negatively on the avoidance response. No prolongation of effects was found after the period of interruption from the treatment. Furthermore, exposure to the compound did not induce any evidence of toxicity to the development of larvae.

In conclusion, we can state that EE2 represents a danger to the survival of zebrafish as the alteration of the behavioral parameters examined is of vital importance in the control of anti-predatory maneuvers. Further investigations are necessary to determine the effects and mechanism of action of EE2 on zebrafish model.

In addition to the aforementioned studies, second research was conducted with the aim of optimizing the feeding protocols commonly used in zebrafish rearing, which have always been an obstacle in terms of costs and manpower for research laboratories. The effects of two different feeding protocols, including a mix of dry and live food (*Artemia*), on larval survival were examined. The results did not show a significant difference on larvae survival between the protocols used.

Table of contents

1. Introduction 1

1.1 Zebrafish 1

1.1.1 Principal features 1

1.1.2 Animal model in biomedical research 2

1.1.3 Husbandry 4

1.2 Feeding 5

1.2.1 Artemia 6

1.2.2 Paramecium 7

1.3 Endocrine disrupting chemicals (EDCs) 8

1.4. 17-α-Ethinylestradiol (EE2) 9

1.4.1. Description 9

1.4.2 17-α-Ethinylestradiol, an estrogenic chemical 10

1.4.3. Mechanism of action 12

1.4.4. Neurobehavioural effects 13

2. Aim of the thesis 15

3. Experimental Part 17

3.1 Zebrafish 17

3.1.1 Care and maintenance 17

3.1.2 Breeding protocol and eggs' collection 17

3.2 Husbandry of larvae 18

3.2.1 Artemia hatching system 19

3.3 Drugs, solvents and solutions 20

3.4 Range of concentrations 20

3.5 Equipment 20

3.6 Programs 21

3.7 Drug treatments 21

3.8 Behavioural assays 22

3.8.1 Spontaneous swimming assay 22

3.8.2 Bouncing ball assay 23

3.9 Statistical analysis 24

4. Results 25

4.1. Survival time 25

4.2 Behavioural analysis 26

4.2.1 Spontaneous swimming assay 27

4.2.2 Bouncing ball assay 33

4.3 Survival time of husbandry experiment 34

5. Discussion 35

5.1 EE2 exposure did not affect larvae survival 35

5.2 17-α-Ethinylestradiol effects on spontaneous swimming behaviour 36

6. Conclusion 41

7.Abbreviations 43

8.List of figures 45

9.List of Tables 47

8. References 49

1. Introduction

1.1 Zebrafish

1.1.1 Principal features

Zebrafish (*Danio rerio*) is a small fresh-water tropical fish, native to Southeast Asia (rivers, canals, etc.) belonging to the subphylum *Vertebrata*, in particular to the subclass *Teleostei* (Basu & Sachidanandan, 2013). It is considered a key model species in molecular and biological research to investigate the mechanisms involved in several human diseases (Lieschke & Currie, 2007). Compared to rodents, zebrafish require an easier husbandry of larvae and adults, smaller space to work, cost-effective and optimized experimental protocols, which facilitate the performance of pharmacological and genetical screenings. Furthermore, they are characterized by a fast growth rate, usually after 3 months post fertilization are capable of reproducing, and they are easily recognisable; males are bigger and more yellow or pink tinged than females, which are fatter (Figure 1).

Figure 1. Adult female and male zebrafish. Adapted from: www.worldlifeexpectancy.com

For optimal breeding it is common to set the water tanks temperature at 28±1°C and to follow a controlled light-dark cycle (14:10h). Females usually produce 100-200 eggs per day, which

are externally fertilized by males (Basu & Sachidanandan, 2013). Since the discovery of 50-80% sequence identity with the human genome, many genetic techniques - cloning, mutagenesis, transgenesis and mapping approaches - have been developed, making them a genetically tractable vertebrate model system for biomedical studies. Furthermore, the rapid development, the transparency of embryos/larvae and the small size are all optimal characteristics to investigate human disease and to screen therapeutic drugs (Lawrence, 2007). The increasing interest in using zebrafish as a model animal in neuroscience is also due to the high morphological similarity between their central nervous system and the human brain. Indeed, several regions of their brain (cerebellum, optic and tectum, medulla, hypothalamus) are structurally close to the human ones (Stewart et al., 2014; Korolenko et al., 2017). The neurogenesis process begins 10 hours post fertilization (hpf) and it will continue until the 3 days post fertilization (dpf), except for the blood brain barrier which will be completely formed at 10 dpf (Stewart et al., 2014; Korolenko et al., 2017). Typical body movements and touch-responsiveness commence at 17 hpf. After hatching (52 hpf), they acquire the ability to swim to pursue food particles only at 4 dpf (Stewart et al., 2014; Korolenko et al., 2017).

1.1.2 Animal model in biomedical research

Zebrafish possess several advantages over other animal models in biomedical research (Figure 2), especially for the investigation of developmental and mental disorders. Despite rodents have been widely employed in laboratory during the past decades, several aspects limit their use in large-scale genetic and therapeutic studies. Many researchers turned to zebrafish, which represents nowadays the leading animal model system to investigate the molecular mechanisms of human genetic diseases and to identify new potential therapeutic strategies (Access, 2021). Neuroanatomical and neurochemical similarities reported between zebrafish and humans allowed investigators to study a wide range of neurological and psychiatric disorders such as Alzheimer's disease (AD), Parkinson's disease (PD), affective disorders and schizophrenia (Saleem & Kannan, 2018).

Zebrafish are widely used to assess developmental neurotoxicity, especially as an *in vivo* model. Even if they represent a more complex model system than others, such as Drosophila melanogaster, toxicity experiments turn out to be cheaper and quicker. Substance’s

administration also results to be a simple process. Indeed, zebrafish are immersed in chemical solutions and the compounds can penetrate the embryo's external membrane by passive diffusion (Amora & Giordani, 2018). Furthermore, the transparency of the embryos allows to visualize individual genes, through fluorescence or dyeing procedures, making use of non-invasive imaging techniques and it allows to perform easier genetic manipulations. Another important characteristic is their small size which is ideal for high-throughput screening of active compounds (Saleem & Kannan, 2018).

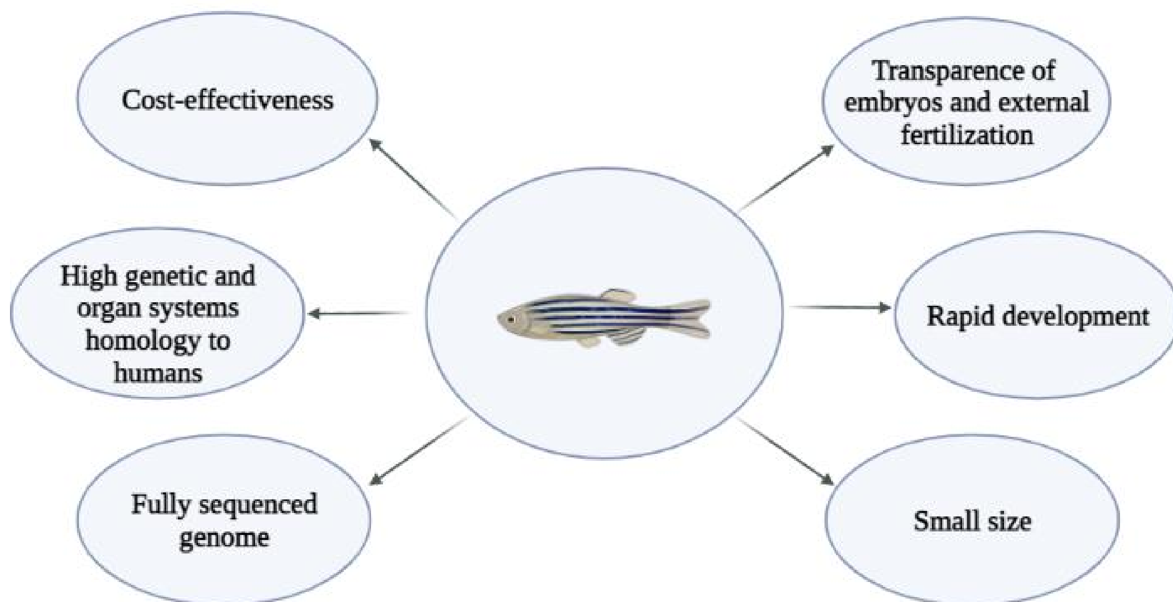


Figure 2. Advantages of using zebrafish as an animal model in biomedical research. Created with BioRender.com

Zebrafish is also emerging as useful species in neurobehavioral research, offering the opportunity to investigate brain mechanisms and possible therapies for neurological disorders. Several zebrafish brain area show homologous functions to those of humans: the telencephalic area, memory-associated, and the habenula, involved in adaptive fear responses and anti-predatory behaviours, resemble the hippocampus and the amygdala in humans, respectively. Finally, they are characterized by exceptional cognitive abilities; their behaviour is significantly affected by external stimuli and environmental factors, displaying evident responses to pharmacological manipulations (Saleem & Kannan, 2018). The recent classification and characterization of a broad range of behaviours, such as aggression, anxiety, memory, object discrimination and color preference, and the tracing of specific behavioral endpoints leads to an improvement in drug screening and behavioral phenomics (Cachat et al., 2010) (Fontana et

al., 2018). Due to the complexity of the neuronal mechanisms involved, the creation of validate and reliable experimental models still represents a challenge in novel pharmacological therapies research (Fontana et al., 2018).
All the advantages mentioned above make zebrafish an important biomedical model, although there are several indisputable limitations on its use: dissimilarities between their respiratory system and the one of humans (Teame et al., 2019); difficult drug dose absorbed detection (dependent on individual surface area and gill activity); obstacles in pharmacokinetic studies (Fontana et al., 2018).

1.1.3 Husbandry

The interest on zebrafish as an experimental animal model in many fields of research is continuously increasing. In order to meet this growing demand, the number of rearing facilities is rising and most laboratories are investing more on their maintenance and cure (Craig et al., n.d.). Usually, every laboratory follows its own protocol because of the lack of sufficient studies on novel husbandry techniques and nutritional regimens, especially during the larval and juvenile stage. Standardized conditions could lead to a significant improvement on growth, welfare and survival, affecting also the reproductive performances of fish (C. Certal, 2016).
The rearing conditions should reflect those of their natural environment as closely as possible. Several studies conducted in zebrafish habitats suggest which are the optimal physio-chemical properties of water. Indeed, they seem to prefer still or slow moving water with pH level around 8 and a slightly high clarity (~> 35 cm) (Lawrence, 2007).

1.2 Feeding

Knowledge of dietary habits of wild zebrafish is relevant to improve feeding protocols, as they should respect their specific digestive physiology and eating behaviour (Lawrence, 2007). Zebrafish consume a wide range of food as they are omnivorous. Their feeding is based mainly on zooplankton, phytoplankton and insects, although worms and small crustaceans can be included (Nakayama et al., 2018).

In the laboratory, the most challenging period of zebrafish rearing is the first-feeding phase as the maximal digestible food size is around 100 μm and preys must respect this range (Lawrence, 2007). The approaches currently used are various; most of them are based only on live food such as Paramecium or Artemia, others on processed dry food or both the two. Usually, during the first days post-fertilization (4-7 dpf), larval nutrition is based on the nutrients from the yolk sac. After this period, it is common to feed them with paramecium or rotifers approximately until 15 dpf. Then, most of breeders continue the feeding with a combination of food, usually Artemia (*Artemia nauplii*), and processed dry food (C. Certal, 2016).

As already mentioned, specific dietary requirements are not still delineated, and self-manufactured diets can produce unsatisfactory and variable results. Recently studies have shown how the role of live feeds is becoming crucial. Despite the optimization of dry feed-based protocols, the rates of growth and survival are still low compared to those with live feed (Nakayama et al., 2018). Among the many advantages of live feeds there are certainly the high digestibility and bioavailable source of nutrition, the engagement of prey capture behaviour and the numerous nutritional properties included (rich amino acid profile) (Delbos, 2012). Unfortunately, their use could increase the probability of pathogens growth, as they work as vectors. It is important to point out that using only this type of food source, without any other supplements, can lead to reduced growth and development on fish (C. Certal, 2016).

1.2.1 Artemia

Artemia sp. (Figure 3), popularly known as brine shrimps, is the most used live food for zebrafish culture, together with rotifers and paramecium. These small crustacean, high-salinity environments inhabitants, are characterized by a short lifecycle, high fecundity and can be easily obtained by cysts hatching (Léger et al., 1987). Considering the simple and low-cost equipment required for assembling an Artemia hatchery, many laboratories possess their own culture to guarantee a continuous food intake to the fish. Furthermore, cysts have a very long shelf life without losing their hatching capacity when stored in vacuum or in the refrigerator, contributing to avoid waste. An important problem to overcome is the salt contamination. Indeed, newly hatched Artemia eggs need to be washed with freshwater before feeding fish. As they cannot survive for long time in these condition, new batches of nauplii must be prepared daily, making the hatching system a time-consuming process for small laboratories (Nakayama et al., 2018) . Food organisms should meet certain physical requirements to be accepted. Nauplii are easily caught by fish as they do not possess an efficient escape response (Léger et al., 1987). Moreover, their bright colour and continuous movement stimulate the predatory response of fish. Regarding the size, ingestibility strongly depends on it. It has been demonstrated by Vanhaecke and Sorgeloos (1980) that naupliar size and volume change among different strains, leading to a reasoned selection among the various commercial cysts as zebrafish larvae cannot ingest them easily (Léger et al., 1987). Indeed, Artemia nauplii are not recommended for the initial period of feeding due to their size (500×100–150 µm) and swimming speed could represent an obstacle for larvae (Delbos, 2012).

Figure 3. Newly hatched artemia nauplii. Adapted from: (Delbos, 2012)

1.2.2 Paramecium

Paramecia (Paramecium sp.) are unicellular organisms (Figure 4), which range from 50 to 300 um in size -depending on the species- normally found in freshwater environments. Their small size makes them an ideal live feed for first-feeding larvae, so that the optimization of culture protocols is constantly increasing (Delbos, 2012).

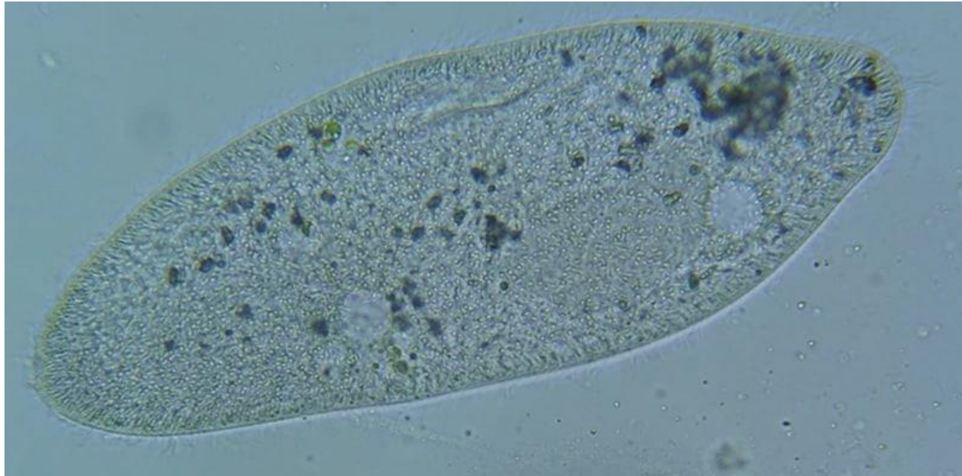


Figure 4. Paramecium under the microscope. Adapted from: https://microscopeclarity.com

However, paramecia cultures are unsuitable for small academic laboratories as they required a laborious maintenance and can easily become a source of pathogens and parasites. Indeed, subculturing and various filtration and sterilization steps need to be repeated periodically (Nakayama et al., 2018).

1.3 Endocrine disrupting chemicals (EDCs)

The number of chemicals released in the aquatic environment has increasing rapidly during the last decades, especially due to the increment of industrialization (Bashir et al., 2020).

EDCs (endocrine disrupting chemicals) are probably among the most widespread classes of contaminants generating a great concern in the global community. These compounds are known for interfering with the functions of both vertebrate and several invertebrate hormone systems (Guillette & Gunderson2001; Fenske, Concato, Vanin, Tamagno, de Oliveira Sofiatti, et al., 2020). Indeed, they exert their action by blocking or mimicking the effects of endogenous hormones (estrogens/androgens), altering their synthesis or metabolism, and modifying hormone receptor levels (Sonnenschein & Soto, 1998). Although it was originally thought that EDCs interfered with the endocrine system mainly by nuclear receptors such as estrogen receptor, androgen receptors, progesterone receptors and thyroid receptors (ERs), today investigators demonstrated that the mechanisms involved are more extensive (Diamanti-Kandarakis et al., 2009).

Dioxins/PCBs, DDT/DDE, bisphenol A, phthalates, alkylphenols, and phytoestrogens are just some examples of the wide range of chemicals belonging to this heterogeneous class, characterized by a highly variability in terms of structure and mechanism of action (Diamanti-Kandarakis et al., 2009). The sources of exposure are different and vary around the world as they are used in a wide range of fields such as chemical, agricultural industries or consumer products (Sonnenschein & Soto, 1998). People who work with industrial chemicals or pesticides are at high risk compared to others, but exposure occurs also through easier ways: drinking contaminated water, breathing contaminated air, or ingesting food (Diamanti-Kandarakis et al., 2009). Because of their long half-life and difficult decomposition, they could be not metabolized, or be metabolized and turn into more toxic forms. Considering that EDCs are not the only environmental contaminants, the consequences of exposure may be due to additive or synergic actions from different compounds or different classes of EDCs (Diamanti-Kandarakis et al., 2009). Since the aquatic environment is the last site reached by these contaminants (Sonnenschein & Soto, 1998), many researchers investigated on the EDCs-related health risks in wildlife, focusing on the biological mechanisms by which they interfere with the normal endocrine system activity and the possible consequences. Many studies demonstrated that their presence in the aquatic environment is correlated to several reproductive and behavioural disruptions in fish populations, such as altered mating and swimming activity

(Sárria et al., 2011), induction of vitellogenesis (Tyler C.R., 1998) and fertility reduction (Volkova et al., 2015). Furthermore, they also affect the human health, altering reproductive processes, promoting cancer growth (breast and testicular), dysfunctions of neuronal and immune system (Barreiros et al., 2016; H. R. Lee et al., 2013).

1.4. 17-α-Ethinylestradiol (EE2)

1.4.1. Description

IUPAC Name: (13*R*,17*S*)-17-ethynyl-13-methyl-7,8,9,11,12,14,15,16-octahydro-6*H*-cyclopenta[a]phenanthrene-3,17-diol. (PubChem release 2019.06.18)

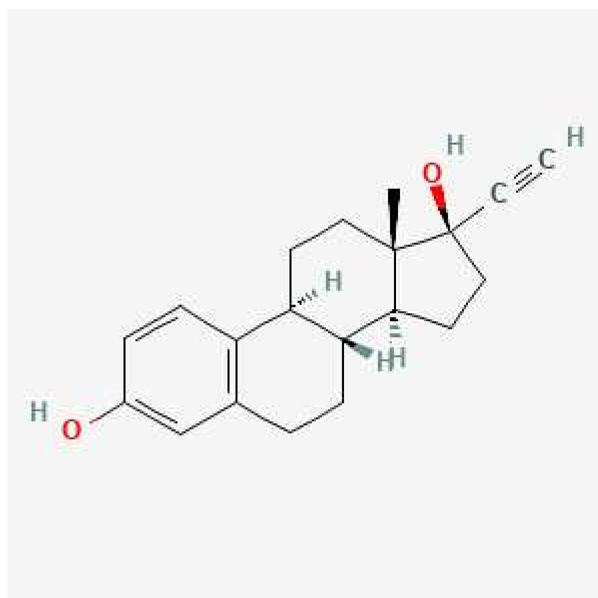


Figure 5. Chemical structure of 17-α-ethinylestradiol. (https://pubchem.ncbi.nlm.nih.gov)

Molecular formula: $C_{20}H_{24}O_2$
Molecular weight: 296.4
Solubility: Low solubility in water.
Chemical and physical properties: Clear, colourless liquid, air and light sensitive.
(National Center for Biotechnology Information (2021). PubChem Compound Summary for CID 131770026, 17-alpha-Ethinyl estradiol.)

17-α-ethinylestradiol (Figure 5) is a synthetic form of estrogen widely employed in several oral hormonal contraceptives, generally in combination with progestins. Previously, its use was principally destined for the palliative therapy of patients with advanced prostate cancer and for the treatment of moderate to severe vasomotor symptoms correlated to gynecological disorders. Nowadays it is also included in menopausal hormone therapy.
(National Center for Biotechnology Information (2021). PubChem Compound Summary for CID 5991, Ethinylestradiol)
EE2 has an estrane steroid nucleus, commonly present in all estrogens, containing 18-carbon atoms arranged in four rings. The main difference in terms of structure from estradiol is the ethynyl group at C_{17}, which leads to a relevant increasing of the estrogenic activity. (Merchenthaler, 2018) Indeed, compared to the natural form 17-β-estradiol, EE2 shows a higher binding affinity to estrogen receptors (ER) (Fenske et al., 2020).

1.4.2 17-α-Ethinylestradiol, an estrogenic chemical

Estrogenic chemicals are probably the most studied EDCs, not only because of the high levels detected in the environment but principally because of the several negative effects on aquatic populations that have been discovered over the last decades (Foster & Brown, 2018). This subgroup includes the synthetic estrogen 17-α-ethinylestradiol (EE2), an active ingredient in many oral contraceptives, considered the most bioactive estrogen in the aquatic environment (Kolpin et al., 2002). The amount of active compound released into the environment through wastewaters is worrying as wildlife and humans may be exposed (Fenske et al., 2020). For this reason, the European Union included it among the “emerging aquatic pollutants” (Barreiros et al., 2016). The potential sources of contamination of EE2 in the environment are various (Figure 6), but the main source is the human urine, through which the excess compound is excreted and reach the aquatic systems (Foster & Brown, 2018). Considering that the EE2 excretion of a single human being is approximately 4.5 and 6 μg daily (feces and urine), the total amount of estrogen discharged annually is estimated to be around 4.4 kg per million inhabitants (Barreiros et al., 2016). After the excretion process in the human body, EE2 is present as an inactive conjugate form. Once it reaches the sewage treatment plants, it is reconverted to its active form by bacteria and released in rivers, streams etc. becoming a risk for the aquatic populations. Although the concentrations detected in surface waters are lower than those of endogenous

estrogens, recent *in vivo* studies demonstrated that its biological activity is 10 to 50 times higher than that of E2 and E1 due to its elevated bioconcentration and longer half-life time (Segner et al., 2003).

The EE2 concentrations detected in effluents and influents of waste and surface waters of different countries are highly variable, ranging from 0.2 ng/L to 200 ng/L (Ying et al., 2002). Traces were found also in sludge and sediment samples. The low solubility in water (4.8 mg/l) and high octanol-water partition coefficient (4.12) of EE2 compared to the other estrogen hormones (Table 1), are related to its high sorption potential on soil or sediment and explain how it can persist in the environment for long periods (Barreiros et al., 2016).

Chemical Name	Water solubility (mg/l at 20⁰)	Log K_{ow}	Vapor pressure (mm Hg)
Estrone (E1)	13	3.43	$2.3 \times 10{-}10$
17-β-Estradiol (E2)	13	3.94	$2.3 \times 10{-}10$
Estriol (E3)	13	2.81	$6.7 \times 10{-}15$
17-α-Ethinylestradiol (EE2)	4.8	4.15	$4.5 \times 10{-}11$

Table 1. Physico-chemical proprerties of estrogen hormones. Based on: Pojana et al. (2007) and Ying et al. (2002).

Furthermore, contamination of the environment by EE2 may also occur through livestock wastewater as EE2 is used to treat many diseases and through runoff from manure and sewage sludge, used in agricultural activities (Aris et al., 2014).

Despite the several consequences related to its exposure, reproductive system disruptions are the most studied. For this purpose, zebrafish (*Danio rerio*) is the most performing animal model, presenting an high resemblance between its biological processes and those of mammals (Panula, 2010). Furthermore, zebrafish is among the fish species most responsive to EE2 treatment (Lange et al., 2012). It has been proved that even low concentrations (ng/L) are sufficient to be harmful to fish, especially during the early developmental periods, causing several endocrine disruptions: 0.1 ng/L EE2 increases vitellogenin (VTG) production (Purdom et al. 1994); 0.1–15 ng/L can disrupt normal sexual development and differentiation (Andersen et al. 2003; Metcalfe et al. 2001; van Aerle et al. 2002; Van den Belt et al. 2003; Weber et al. 2003); 2-10 ng/L reduces fecundity and concentrations ranging from 1 to 10 ng/L can diminished fertilization success and promote behavioural changes (Lange et al., 2008).

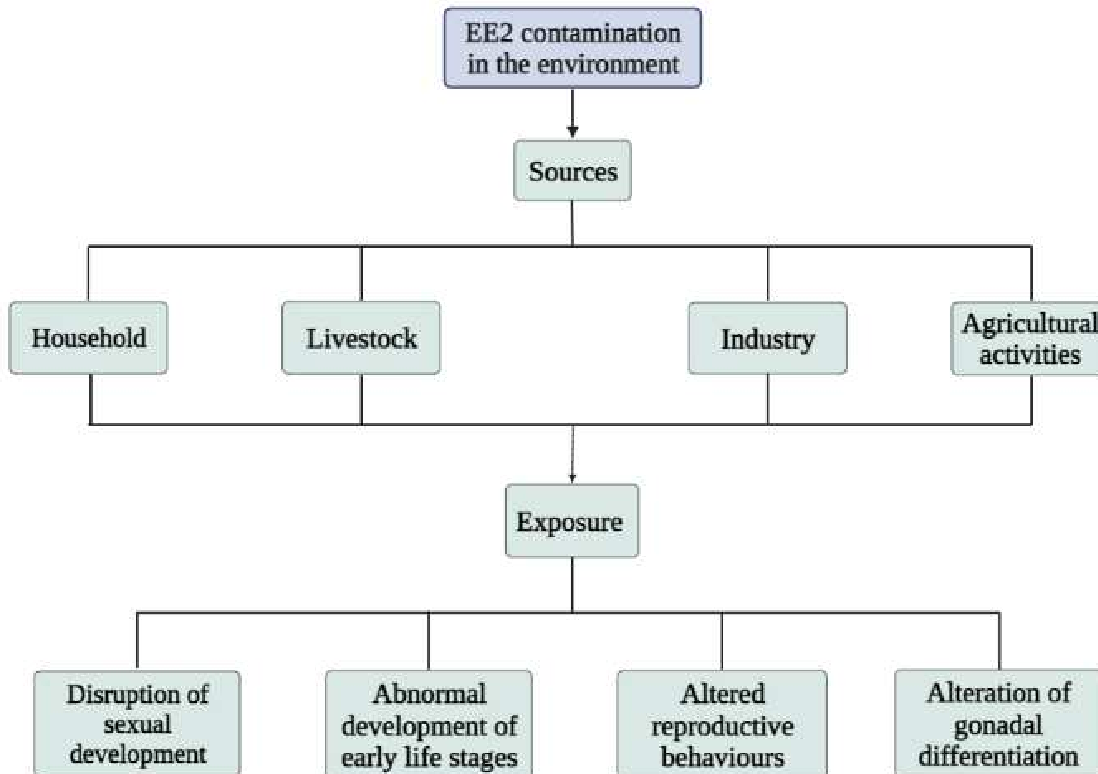


Figure 6. Conceptual diagram of the principal sources of contamination of EE2 and the effects of its exposure. Figure Created with BioRender.com and based on: (Aris et al., 2014)

To conclude, the persistent presence in the environment and the detected negative effects transmissible to the future generations – probably by epigenetic mechanisms -, make EE2 as one of the major compromisers of the aquatic population survival (Anway et al., 2012).

1.4.3. Mechanism of action

EE2 shows a high affinity for binding the estrogen receptors (ERs), inducing similar responses to endogenous estrogens. Its lipophilicity helps to be taken up by the gills of fish, as their membrane is mainly constituted by lipids, facilitating the diffusion (Blewett et al., 2014).
In teleost are present three different isotypes of the ER (ERα, ERβ, and ERγ), which differs in binding affinities to natural and synthetic estrogens, distributions in tissues and transactivation properties (Hawkins et al., 2000). EE2 tends to bind to the α and β isotypes, altering their mechanisms (Bertotto et al., 2020; Lange et al., 2012).
In absence of the natural ligand, estradiol (E2), the ER is organized in a heterodimeric complex with Hsp proteins (Hsp90, Hsp70, p23). This Hsp90-based chaperone complex stabilizes the

receptor in an inactive form. Furthermore, Hsp90 protects the ER from the ubiquitin/proteasome-mediated degradation, ensuring an adequate hormonal response. After crossing the membrane of cells, EE2 binds to the receptor ER, which dissociates from the chaperone complex, dimerizes and bind special DNA sequences in the nucleus, called estrogen-responsive elements (EREs). In this way it activates the transcription of specific genes through direct or indirect interactions with co-activators and other general transcription factors (M. Lee et al., 2002).
The conserved domains characterizing the vertebrate ER are six -from A to F- and each of them has a specific function. The N-terminal A/B domain and E domain include the transcriptional activation functions, ligand independent and ligand dependent, respectively. The DNA binding function is site in the C domain and the D domain seems to act as a hinge between DNA- and hormone-binding domains (Taylor et al., n.d.). After its dissociation from the chaperone complex, the ER is degraded by the ubiquitin-proteasome pathway.

1.4.4. Neurobehavioural effects

While the impact of EE2 on the reproductive behaviour of fish has already been extensively examined by many investigators, the effects on non-reproductive tracts are not still well established (Volkova et al., 2015).
Zebrafish is considered a consolidated vertebrate model in many research areas, such as neurobehavioral science, toxicology and genomic as several biochemical and physiological aspects resemble that of the mammals (Panula, 2010; Sárria et al., 2011). Additionally, the similarities of their genome with the human one permit to better understand the neurochemical mechanisms and to develop drugs screenings (Rico et al., 2011). Thanks to these features they represent an important tool to predict EE2 effects on human health (Fenske et al., 2020).
The impact of EE2 in social and reproductive behaviours of fish is strictly connected to the many endocrine system targets located in the brain, at both peripheral and central level (Reyhanian et al., 2011). Indeed, the disruption of hormone levels during the early stage of life induced by estradiol alters not only the gonad development but also the development of several brain regions, which are regulated by transcription factors and endogenous hormones, like gonadal steroid. Thus, the effects of EE2 could affect both the adult endocrine and behavioral responses (Fernandez-Galaz et al., 1997). It has been demonstrated that EDC exposure affects

risky behavior, schoaling behavior and bottom dwelling (Hebert et al., 2014). For what concerned EE2, it increases aggressive behaviour in different fish species (Colman et al., 2009). Short and long EE2-exposure induces anxiety and reduces shoaling behaviour in adult zebrafish (both males and females), compromising reproductive and anti-predator responses. Furthermore, the observed effects affect also the unexposed progeny (Sárria et al., 2011; Volkova et al., 2015).

One of the most important aspects to consider in the early development of fish is locomotor activity. Indeed, altered movement responses analysis could help to predict disruptions in both reproductive and non-reproductive behaviours which may impact fitness of fish population (Sárria et al., 2011). Dopaminergic and serotonergic system are strictly involved in this kind of alterations, as they control several brain functions. Indeed, dysregulations of their mechanism are related to a variety of neuropathologies, such as Parkinson's or schizophrenia, as both these neurotransmitters modulate the brain physiology and behaviours (anxiety, aggressiveness, pain) (Lucki, 1998). Through the investigation on gene transcription conducted by Nasri et al., 2021, larvae zebrafish exposed to EE2 showed a downregulation of th2, which encodes tyrosine hydroxylase, involved in the dopamine synthesis. Since dopamine regulates th2 expression, its downregulation is correlated to a negative feedback loop in the pathways of this neurohormone (Brand et al., 2002). Changes in swimming behavior are probably correlated to alterations of these biological pathways.

Although more evidence is needed to clearly establish the effects of this potent endocrine disruptor, the current results show that the survival of fish and other aquatic organisms could be compromised by relevant concentrations exposure.

2. Aim of the thesis

The main objectives of this study are:

1. Optimization of feedings protocols for zebrafish husbandry, focusing on the reduction of costs and working time.
2. Evaluation of the EE2 toxicity on zebrafish model.
3. Evaluation of the EE2 effects upon spontaneous locomotory behaviour and avoidance response in zebrafish larvae trough validated behavioural assays.
4. Examination of the reversibility of EE2 effects over time on the behaviour of zebrafish larvae.

3. Experimental Part

Material and methods

3.1 Zebrafish

3.1.1 Care and maintenance

The adult wild-type zebrafish supplied by CIIMAR were maintained in 70L aquarium with de-chlorinated tap water at 28 ± 1°C, under a 14h:10h light-dark cycle.
The fish were fed twice a day with the commercial fish food flakes TetraMin®, increasing to three times a day during breeding days.
Every day the water was siphoned to remove debris and replaced with the corresponding amount. Three times a week the walls of the aquarium were scrubbed and one time a week filters, aeration system and thermostat were rinsed. The pH (pH test, Sea test) and Ammonium level (ProdacTest NH_3/NH_4) of the water were measured every two weeks.
Zebrafish were treated according to European ethical guidelines on the care and use of animals in research.

3.1.2 Breeding protocol and eggs' collection

Adult couples were randomly placed into individual small transparent plastic tanks (one couple per tank) with aquarium water at 28 ± 1°C on 14:10 hr (light: dark) lightning conditions. The couples were left in the breeding tanks from the afternoon until the following morning.
Eggs were collected one hour after the start of the light period and cleaned through a decantation process to remove possible contaminants. They were moved through disposable plastic pipette into Petri dishes (Ø 90 mm) filled with water and methylene blue (1µM), an antifungal agent, until a maximum of 100-150 eggs per dish. The dishes with the eggs were stored in incubator (INCU-Line Digital Mini Incubator, VWR, Radnor, Pennsylvania) at 28°C on the same light conditions as the adult fish (14:10 light: dark).

After 6 hours post fertilization, the embryos were checked, and the dead or undeveloped ones were removed.

3.2 Husbandry of larvae

The eggs were obtained and collected using the protocols described in 4.1.2. Then, they were divided in 6 Petri dishes (55 Larvae each one) at the 2 dpf.

Two different conditions of feeding were established: continuous and discontinuous feeding; the larvae of the second condition were not fed on Sunday.

The feeding started at 6 dpf, two times per day with dry food (TetraMin®), following the food size showed in Table 2**.** At 9 dpf fish were moved to two different rigid plastic containers filled with 1 L chlorine-free tap water, equipped with aeration system and thermostat to maintain the temperature at 28 ± 1°C. After 18 dpf, larvae were fed with Artemia nauplii trough a disposable plastic pipette (2 mL) 2 times per day. After each feeding the remaining food was removed with a net (on the top) and with a little siphon (on the bottom). At 21 dpf the juvenile zebrafish were moved to bigger containers with a water capacity of 4 L.

Dpf	TetraMin® (µm)	Artemia (mL)
6-14	2x 100	
14-21	2x 180	
18-21	2x 180	2x 2
21-24	2x 250	2x 2

Table 2. Feeding protocol for each group of larvae from 6 dpf to 24 dpf.

The water was changed every day replacing the corresponding amount with chlorine-free tap water; dead or diseased larvae and any other debris was removed daily to not compromise the water quality. The Ammonia levels and pH were checked one time per week, trying to respect the following values: 0-0.2 mg/L (NH_4) and 7-8 (pH).

3.2.1 Artemia hatching system

A “V” shaped transparent container closed with a cap was filled with 1 L of tap water heated at 28°C by thermostat and held by a metal system to guarantee its stability. An aeration source was provided to keep cysts in suspension and to reach an adequate level of oxygen. To avoid the settling of unhatched eggs, the air tube was placed in the bottom part of the container. A light source was used to ensure a successful hatching, especially during the first hours of incubation. 32 grams of Artemia salt and a knife tip of eggs provided by Hobby (Dohse Aquaristik GmbH & Co. KG) were added to the water. 24-36 hours later, the air supply was turned off. While the eggshells floated to the surface because of their density, the unhatched eggshells tended to stay in the bottom of the hatchery. The newly hatched shrimps vibrated around the neck of the container (Figure 7).

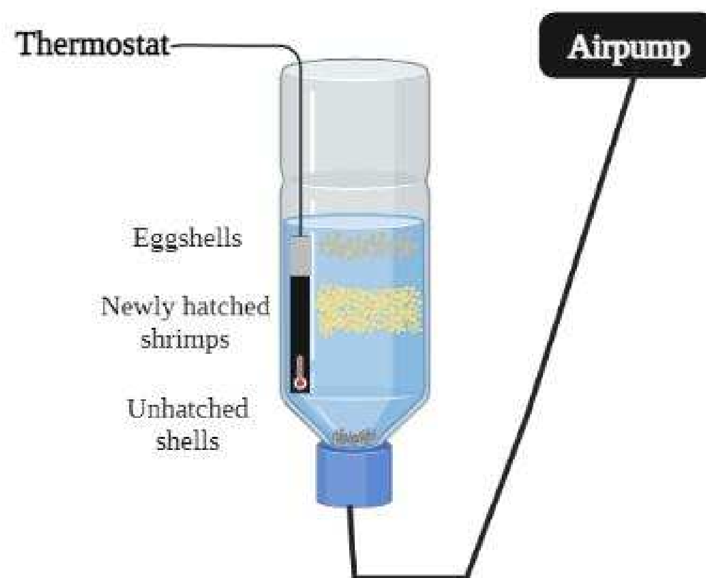


Figure 7. Representation of the Artemia hatching system. Created with BioRender.com

To separate the nauplii from the unhatched eggs, a light source was directed to the neck of the container to attract the nauplii. After that, the air tube was lowered below the bottle level and the water was drained through a small net into a container with fresh clean water to remove salt and bacteria that could contaminate the fish. Containers and hatching equipment were cleaned and sterilized daily.

3.3 Drugs, solvents and solutions

DMSO, Life technologies (California, USA)

Methylene blue, Sigma- Aldrich (St.Lois, MO, USA)

17-α-ethinylestradiol Sigma- Aldrich (St.Lois, MO, USA)

3.4 Range of concentrations

Drug solutions in 1% DMSO were: 0.5 ng/L, 5 ng/L, 50 ng/L 17-α-ethinylestradiol. The solvent control was 1% DMSO solution.

3.5 Equipment

24-wells plates

Plastic pipettes

Automatic pipettes with adjustable volume: 10μL, 20 μL, 100 μL, 200 μL, 1000 μL, 5mL (PIPETMAN, GilsonTM, Middleton, WI, USA)

Petri dishes

Water tanks

Thermostat

Mini incubator (INCU-Line Digital Mini Incubator, VWR, Radnor, Pennsylvania)

Stereo Zoom microscope (SZT 300, VWR, Radnor, Pennsylvania)

Inverted fluorescence microscope (Eclipse TE 300, Nikon, Tokyo, Japan)

HDWebcamC525 (Logitech, Lausanne, Switzerland)

DinoEye camera AM-423U (Dino-Lite Digital microscope)

Ipad Air APPLE 4[a] generation (10.9”- iPadOS14)

“V” Bottomed container

Aeration tubes

Brine Shrimp Eggs Hobby (Dohse Aquaristik GmbH & Co. KG)

Artemia Salt Hobby (Dohse Aquaristik GmbH & Co. KG)

3.6 Programs

Excel (Microsoft Office for Microsoft 365 MSO (16.0.14026.20304), 32 bit)

Fiji (Imagej version 1.8.0-64bit)

Graph Pad Prism 8.3.0. (San Diego, CA,USA)

Logitech Webcam Quick Capture

Dino Xcope (Version 1.7.3)

Power Point (Microsoft® PowerPoint® per Microsoft 365 MSO (16.0.14026.20304) a 32 bit)

3.7 Drug treatments

After the collection and incubation of eggs, the larvae (4 dpf) were checked under the microscope and distributed by plastic pipette into 24-well plates (5 larvae/well). They were treated with 17-α-ethinylestradiol (Sigma-Aldrich) solutions (250 µL/well) at different concentrations. The concentrations administered, using the solvent (1% DMSO) as a control, were: 0, 0.5, 5, 50 ng/L. 200 µL of the drug solution were removed daily and replaced by 250 µL of fresh solution -because of the evaporation – until the 7 dpf.

After the 7 dpf, the feeding with 100 µg of dry food started and larvae were moved into Petri dishes (1 concentration/ dish).

Two different drug treatments were conducted: a long exposure (LE), from 4 dpf to 14 dpf and a short exposure (SE), from 4 dpf to 7 dpf, followed by a recovery period in a 1% DMSO water solution (25 mL/Petri dish) until 14 dpf (Figure 8). Every day larvae were checked under the microscope to remove dead ones

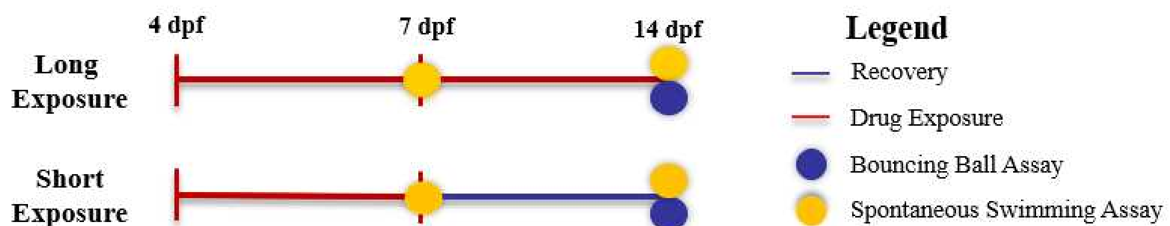


Figure 8. EE2 treatment's scheme on zebrafish larvae from 4 dpf to 14 dpf.

3.8 Behavioural assays

3.8.1 Spontaneous swimming assay

Zebrafish larvae (4 dpf) were treated with 17-α-ethinylestradiol at different concentrations (0ng/L, 0.5 ng/L, 5 ng/L, 50 ng/L) in 24-well plates. The spontaneous swimming assay was performed at 7 dpf and 14 dpf. For monitoring the movement, we used two 6-Well plates per condition, containing each one agarose 0.5% (NZYtech Lisbon, Portugal) rings. The rings were obtained pouring 2 mL of agarose solution into each well. After its solidification, a plastic vial was used to stamp the rings (Ø 25 mm). This step is necessary to avoid the swimming of the larvae in the edge of the well, where it is difficult to detect them because of the shadows generated by the wells' walls. The central part was filled with 3 mL of autoclaved water until the coverage of the ring. The plates were placed in the fridge overnight and put in the incubator at 28°C the next morning. One larva was added to each well of 6-well plates containing 500 µL of autoclaved water. Two plates per concentrations were used (1%, DMSO, 0.5 ng/L, 5 ng/L, 50 ng/L). Then, each larva was tail touched three times to evaluate the ability to move. If the larvae did not move, it was replaced by another one. Movement of larvae was recorded for 10 minutes using a HD Camera C525 connected to the software Logitech Webcam Quick Capture. Each 6-well plate containing larvae was placed in a water bath heated at 28°C through thermostat (Figure 9).

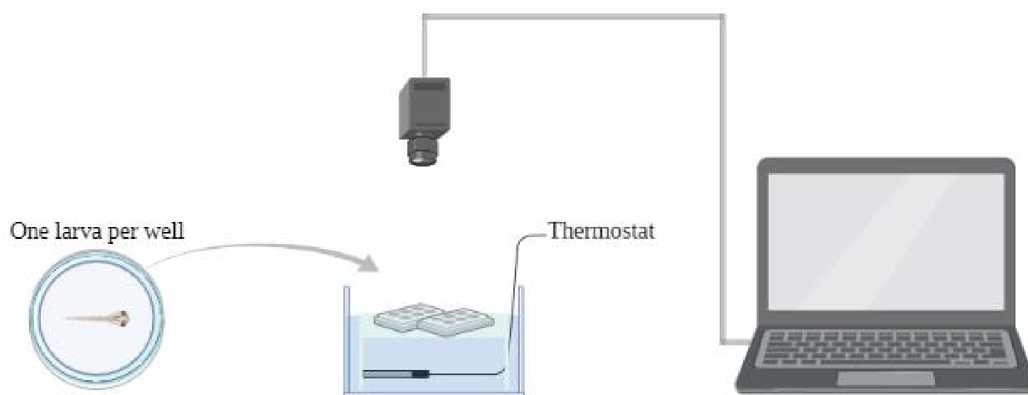


Figure 9. Representation of the spontaneous swimming assay. Created with BioRender.com

The larvae were left to acclimatize for 10 minutes and then the movement was recorded for 10 more minutes. At the end, the videos were converted in a sequence of 3000 images (5 frames

per second) using iWisoft Free Video Converter (http://iwisoft.com/videoconverter) and analysed with ImageJ particle tracker plugin. Then, Excel spreadsheets (Microsoft Corporation) were used to calculate several parameters such as distance, distance/initiation, initiation, time/initiation, velocity. The graphs were obtained and statistically analysed using GraphPad Prism Version 8.3.0. (San Diego, CA, USA).

3.8.2 Bouncing ball assay

Zebrafish larvae were treated with 17-α-ethinylestradiol at different concentrations (0 ng/L, 0.5 ng/L, 5 ng/L, 50 ng/L) in 24-well plates. The bouncing ball assay was performed at 14 dpf with different exposures to the drug as mentioned in 4.7. after 10 minutes of acclimatation period and the spontaneous movement assay. Two 6-well plates per concentrations were used for monitoring the movement of the fish. The process to obtain the agarose rings it is the same explained in 4.8.1 for the spontaneous swimming assay.
The bouncing red discs of 1.35 cm diameter were created on Microsoft PowerPoint following the method and the parameters described by Loscalzo, 2011 (supplement 1 and 2). They moved from left to the right in 2 seconds over a straight 2 cm trajectory only in the upper half of the wells. The multi-well plates containing zebrafish larvae were placed on an iPads' screen projecting the Power point with the visual stimuli (Figure 10).

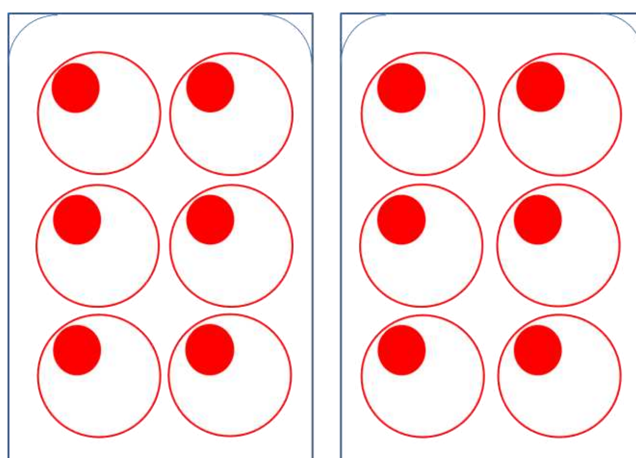


Figure 10. Power Point representation projecting the visual stimuli in the bouncing ball assay. Based on: Loscalzo, 2011.

The plates were imaged for 10 minutes at 5 frame per second (fps). Then the videos were converted in a sequence of 3000 images using iWisoft Free Video Converter and analysed with ImageJ. The parameter considered for the analysis was the standard deviation (SD) of the time that each larva spent in the lower half of every well. It was obtained cropping the well with the option *segmented line* in ImageJ as shown in Figure 11. The graphs were obtained and statistically analysed using GraphPad Prism Version 8.3.0. (San Diego, CA, USA).

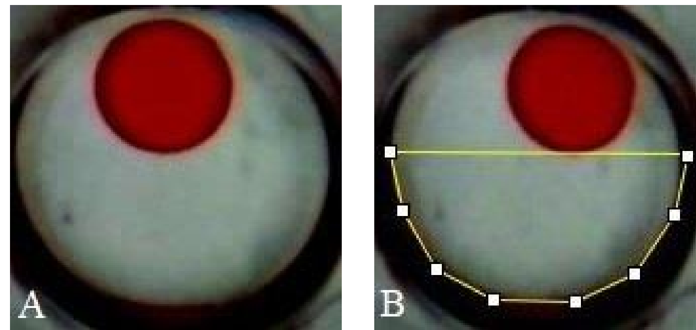


Figure 11. Representation of the wells in bouncing ball assay. A) Well with a visible larva; B) Method used to analyse the SD of the lower half of the well.

3.9 Statistical analysis

All the statistical analyses were performed with Graph Pad Prism 8.3.0 (San Diego, CA, USA). The graphs used to represent data of the behavioural analysis are "column bar type", showing the mean with SEM (Standard Error of Mean).

For analysing the survival data, it was used a "staircase" graph and the Kaplan-Meier estimator. To analyse the Spontaneous Swimming Assay, it was used two-way ANOVA followed by a Sidak's multiple comparison test.

The Bouncing Ball Assay was analysed considering the values of Standard Deviation obtained with ImageJ, using an ordinary one-way ANOVA followed by a Dunnett's multiple comparison test. Significant differences were considered for P<0.05 (*) vs the control and P<0.0.1 (**).

4. Results

Effects of EE2 on zebrafish model

4.1. Survival time

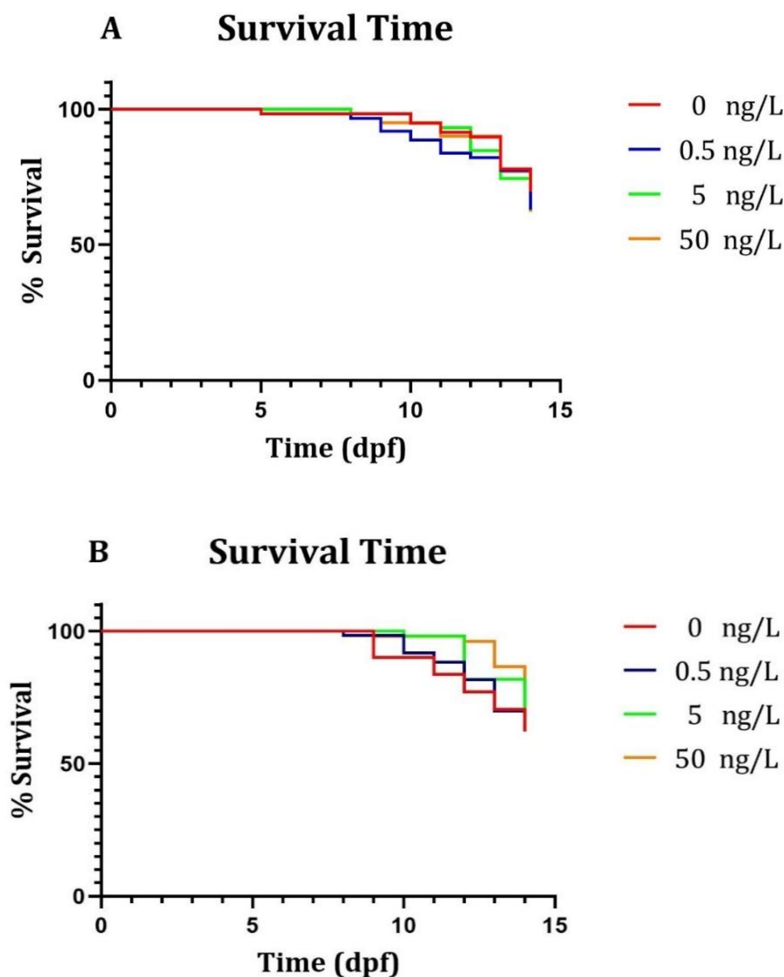


Figure 12. Kaplan-Meier response curves of survival.

Percent survival of zebrafish larvae treated with 1% DMSO as control (0 ng/L) and EE2 (0.5 ng/L, 5 ng/L, 50 ng/L). A) Long exposure, from 4 dpf to 14 dpf. B) Short exposure, from 4 dpf to 7 dpf followed by 7 days of depuration.

After 14 days, there was no difference in survival between larvae exposed to EE2 and control (Figure 12) as data were not statistically significant. The number of EE2 exposed dead larvae

in the long drug exposure treatment (Figure 12.A) was slightly higher than the ones exposed to the control, but still not statistically significant.

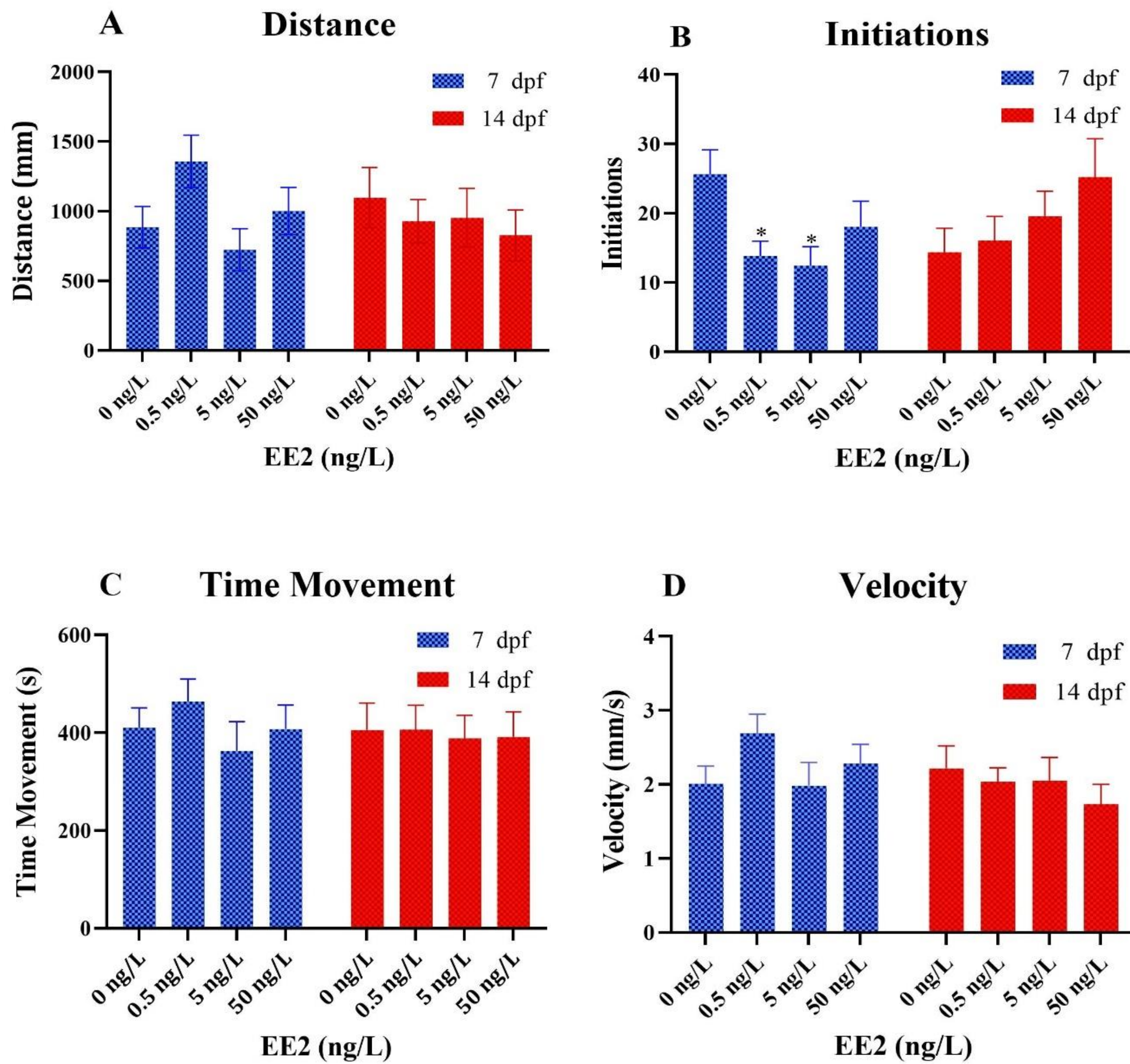
4.2 Behavioural analysis

Parameters considered to analyse larvae locomotion:

1. *Distance*: mm that larva swam in 10 minutes.
2. *Initiations*: number of times that the larva stopped to move and started to swim again.
3. *Time in movement*: time in seconds the larva spent in movement.
4. *Velocity*: ratio between distance and time in movement.
5. *Distance/Initiations*: ratio between the two parameters mentioned before.
6. *Time/Initiations*: this parameter is a correlation between the time in movement and the initiations explained before.
7. *Standard deviation*: indicate the standard deviation of the time that each larva spent in the lower half part of the well.

4.2.1 Spontaneous swimming assay

Long drug exposure



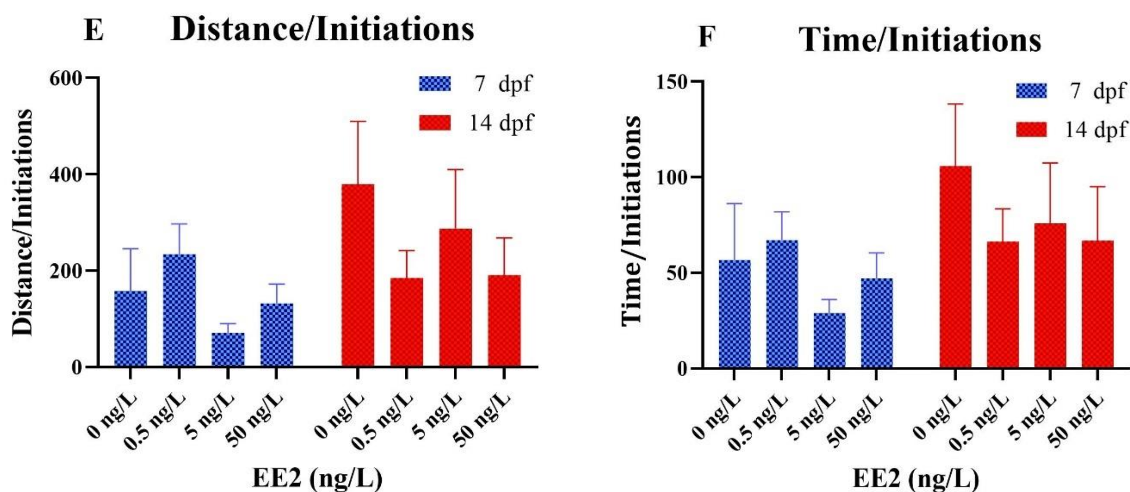
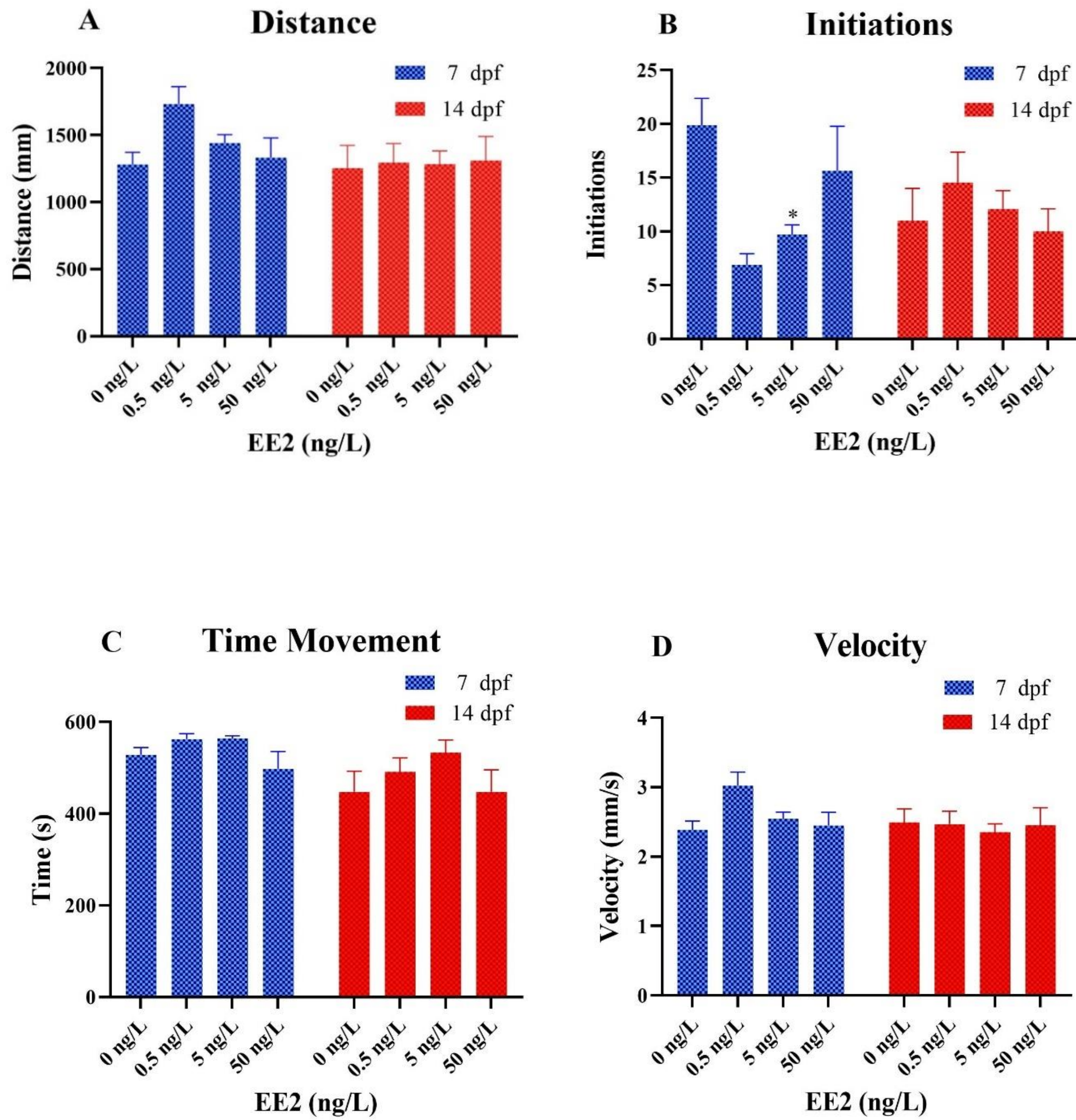


Figure 13. Spontaneous swimming assay parameters of long drug exposure.

Spontaneous swimming parameters in zebrafish larvae treated with different concentrations of EE2 (0.5 ng/L, 5 ng/L, 50 ng/L) and with DMSO 1% as control (0 ng/L) from 4 dpf to 14 dpf. The blue and red columns indicate respectively 7 dpf and 14 dpf, in which the behavioural assay was performed. Data were statistically analysed using two-way ANOVA, which showed a significant interaction. Data were then analysed with one-way ANOVA, followed by Sidak's multiple comparison test. *P<0.05 vs control. Data are from 4 independent experiments with a total of 20-24 larvae for each concentration.

Initiations have decreased significantly by the treatment with 0.5 ng/L and 5 ng/L of EE2 at 7 dpf; initiations at 14 dpf seems to be concentration dependent but data are not statistically significant (Figure 12. B). The other parameters did not show any significant result.

Short drug exposure



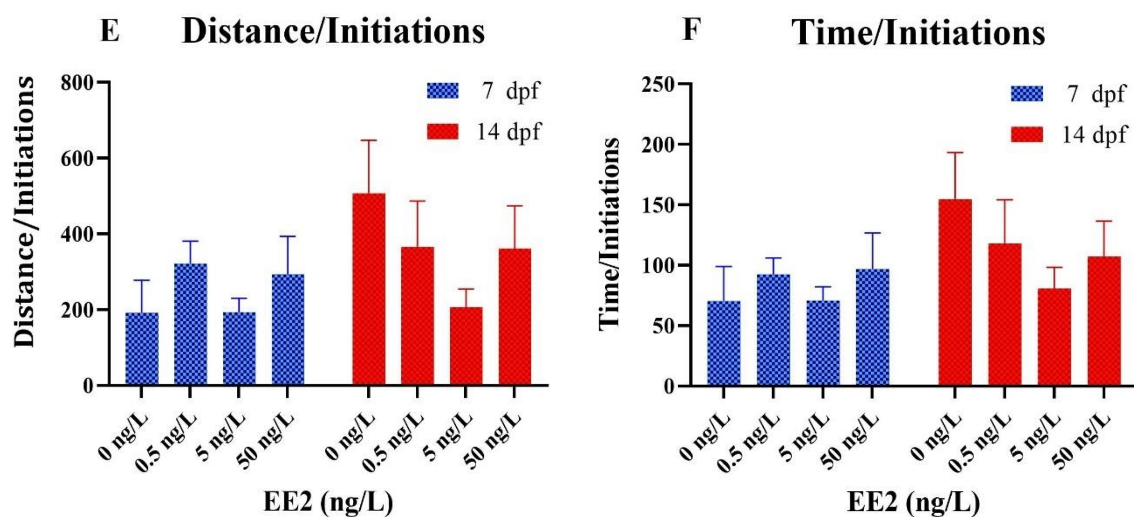


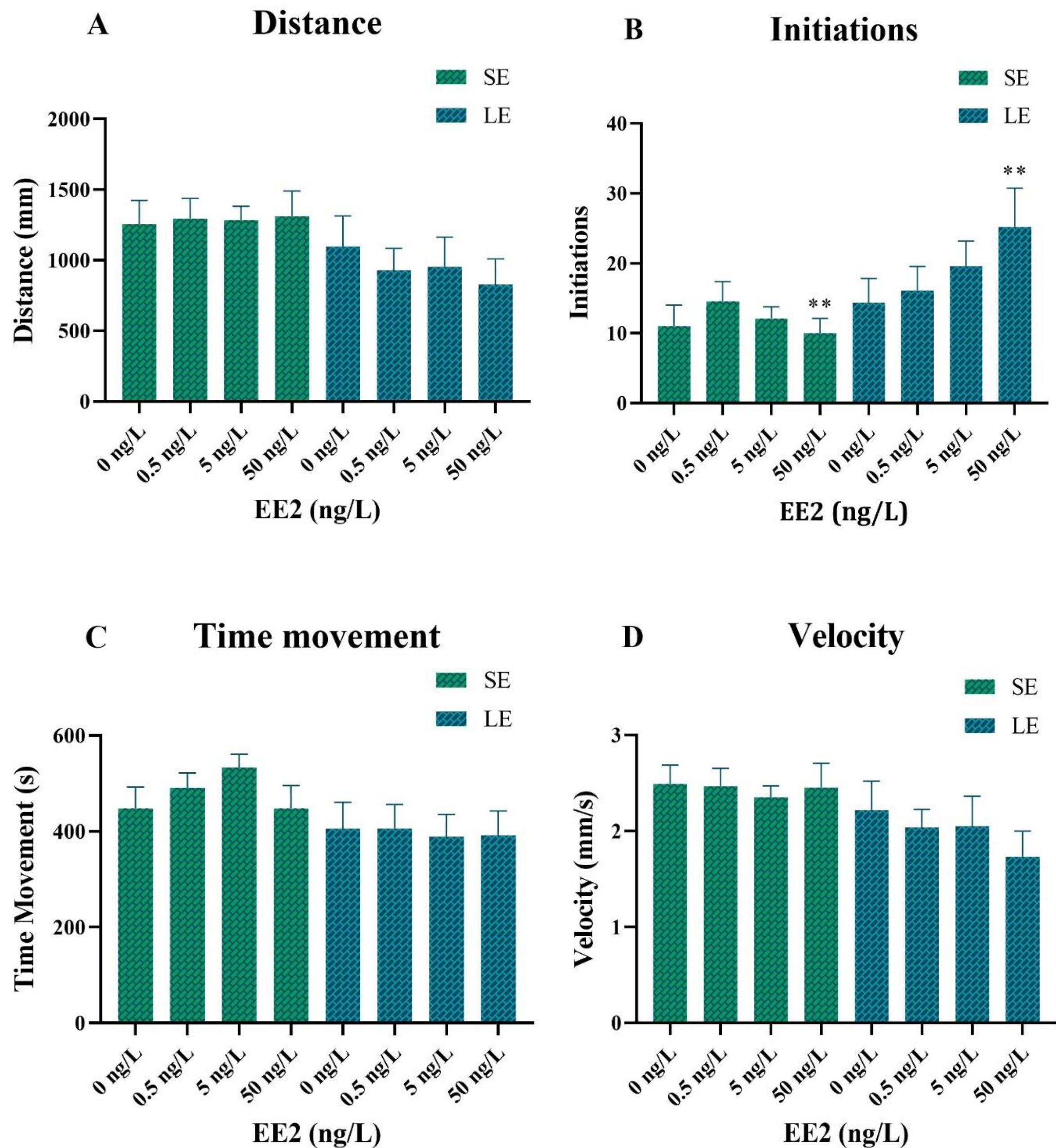
Figure 14. Spontaneous swimming assay parameters of short drug exposure.

Spontaneous swimming assay parameters in zebrafish larvae treated with different concentrations of EE2 (0.5, 5, 50 ng/L) and with 1% DMSO as control from 4 dpf to 7 dpf followed by a recovery period of 7 days. The blue and red columns indicate respectively 7 dpf and 14 dpf, in which the behavioural assay was performed. Data were statistically analysed using two-way ANOVA, which showed a significant interaction. Data were then analysed with one-way ANOVA, followed by Sidak's multiple comparison test. *P <0.05 vs control. Data are from 4 independent experiments with a total of 20-24 larvae for each concentration.

Initiations have decreased significantly by the treatment with 0.5 ng/L and 5 ng/L of EE2 at 7 dpf, as for the long exposure treatment (Figure 14 B.)

Comparison between long and short drug exposure

In this section, the comparison between the short and long drug exposure results at 14 dpf will be shown.



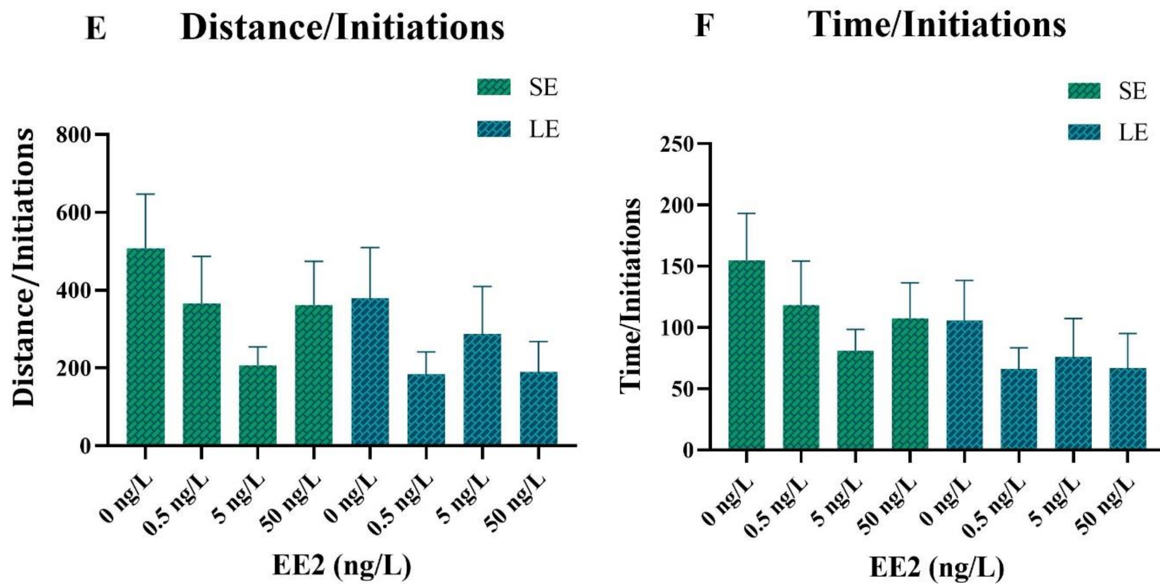


Figure 15. Long and short exposure comparison of swimming assay at 14 dpf.

Spontaneous swimming assay parameters in zebrafish larvae treated with EE2 (0.5 ng/L, 5 ng/L, 50 ng/L) and 1% DMSO as control at 14 dpf. The green and blue columns indicate respectively short exposure (SE) and long exposure (LE). Data were statistically analysed using ordinary one-way ANOVA, followed by Sidak's multiple comparison test. (** $P<0.001$).

The only parameter that showed significant result was the "initiations". From the comparison of every concentration between the short and the long exposure, only one concentration (50 ng/L) showed an increment (Figure 15 B.) This could mean that a longer treatment with highest concentrations could impair the locomotor behaviour.

All the other parameters did not show statistically significant differences, meaning that there is not a relevant change in the swimming behaviour with these concentrations and timepoints.

4.2.2 Bouncing ball assay

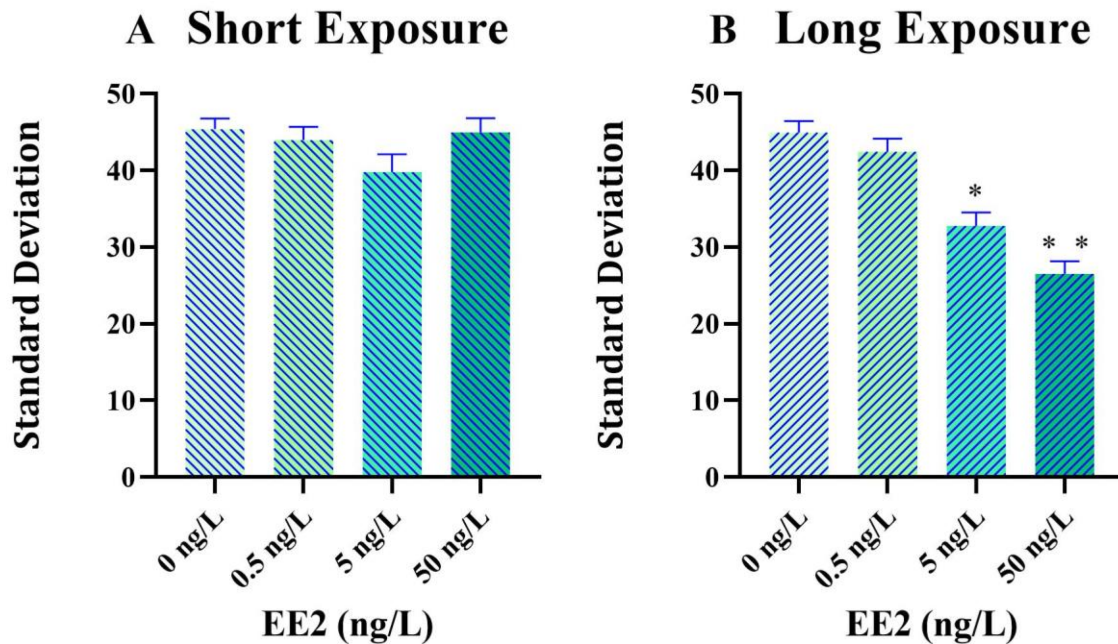


Figure 16. Standard deviation of bouncing ball assay at 14 dpf.

SD of the lower half of the well in zebrafish larvae treated with 1% DMSO as control and EE2 (0.5 ng/L, 5 ng/L, 50 ng/L). A) SD of short drug exposure; B) SD of long drug exposure. Data were statistically analysed using ordinary one-way ANOVA, followed by Dunnet's multiple comparison test. *P<0.05 vs control, **<0.01. Data are from 4 independent experiments with a total of 24 larvae for each concentration.

Concerning the results obtained with the bouncing ball assay at 14 dpf, only larvae treated with 5 ng/L and 50 ng/L of EE2 after a long drug exposure (Figure 16. B) showed a significant decrease in the SD, meaning that they spent more time in the lower half the well, avoiding the ball. Larvae under a shorter EE2 exposure treatment (Figure 16. A) did not display any statistically relevant effect on their behaviour, meaning that there is not any prolongation of the effects of EE2.

Husbandry of larvae: effects of different feedings

4.3 Survival time of husbandry experiment

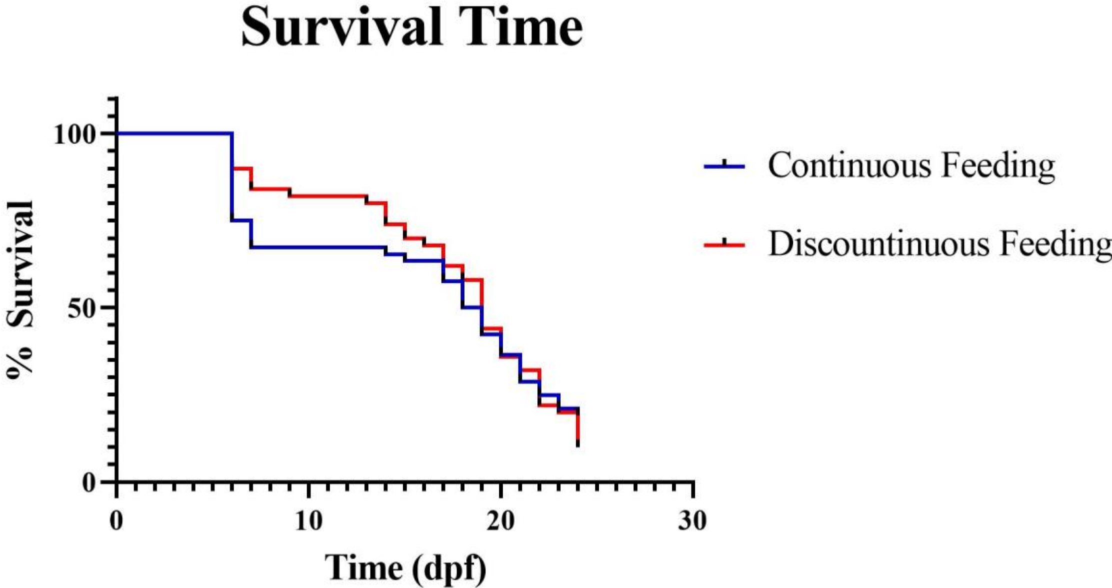


Figure 17. Kaplan-Meier response curves of survival.

Percentage of survived zebrafish larvae from 0 dpf to 24 dpf following two different feedings protocols, continuous and discontinuous as described in 4.2.

After 24 days, there was no significant difference in survival between larvae under continuous feeding and larvae under the discontinuous one since data were not statistically significant. The possible cause of the decrement of survival in both conditions could be a not optimal quality of the water or an inappropriate feeding.

5. Discussion

The synthetic estrogen, 17-α-ethinylestradiol (EE2), widely used in many contraceptive and hormonal therapies, is one of the most bioactive agonists of the nuclear ER among the EDCs.
It has been proved that low concentrations of EE2 (ng/L) in the aquatic system promote disruptions in the eggs production, reproductive behaviour and sex organs development of fish (Sárria et al., 2011). In this study it has been examined the locomotory and avoidance behaviour, two behavioural endpoints that are often overlooked in the risk assessment of EE2 in fish populations. Considering that the concentrations of EE2 are not stable in the aquatic environment, we examined the EE2 effects for brief periods of exposure and the possible recovery from them in zebrafish larvae. The choice of testing this compound on zebrafish during early stage of life it is due to the high susceptibility to xenoestrogen during this “critical window” of their development (Van Leeuwen et al., 1985).
Furthermore, the survival percentage of larvae under EE2 treatment has been considered.

5.1 EE2 exposure did not affect larvae survival

The graphs showed in Figure 12 demonstrated that EE2 did not have any impact on larval survival with the concentrations and timepoints considered in this work. Our results agree with those obtained from other studies in which the survival was not affected by the considered concentrations of EE2, even with longer treatments and/or longer depuration periods (Versonnen & Janssen, 2004; Foster & Brown, 2018). According to Foster & Brown, 2018, higher concentrations of EE2 (100 ng/L) decreased the survival of zebrafish under 14 days of exposure. Andersen et al.,2000 recorded an increased mortality in fish exposed to 100 and 200 ng/L for 30 days. The prolonged exposure and concentrations used by these investigators, could have induced the mortality rates.

5.2 17-α-Ethinylestradiol effects on spontaneous swimming behaviour

While reproductive behaviours have been widely investigated in fish, effects on non-reproductive behaviours after EDCs exposure are still understudied. Short exposure to EE2 demonstrated to increase anxiety and shoaling behaviour in adult zebrafish (Bell, 2004, Xia et al., 2010). Furthermore, several fish species displayed an enhanced aggressive behaviour after EE2 exposure (Reyhanian et al., 2011).

Behaviour is strongly controlled by neurotransmitter mechanisms, such as the dopaminergic one. Indeed, DA regulates emotions, locomotion, perceptions, and reward-motivated behaviour (Rico et al., 2011). Several studies asserted that endocrine disruptors like EE2 can interfere with the dopaminergic pathway, leading to alterations of the nervous system and changes in swimming behaviour (Nasri et al., 2021). These kinds of alterations, especially during the early period of zebrafish development, can compromise their survival. Moreover, it is not still clear if these kind of effects are reversible (Legradi et al., 2018, Liu et al., 2016).

Considering that few studies have focused on the effects of EE2 during early stage of life of fish, in this research we decided to test spontaneous swimming changes in larvae zebrafish after 17-α-ethinylestradiol exposure with different timepoints at environmentally relevant concentrations. According to Nasri et al., 2021, the swimming behaviour of zebrafish larvae is affected by short period of EE2 exposure (8 days). They demonstrated that with a precise concentration of the drug (1000pM) there were longer periods of inactivity compared to control and to lower concentrations. In our results, the parameter "initiations" that indicate how many times the larva started and stopped the swimming activity, was significantly influenced by EE2 exposure. Indeed, it decreased with 0.5 and 5 ng/L compared to the control at 7 dpf, reflecting an alteration in the movement. As reported in the same study (Nasri et al., 2021), these changes in swimming behaviour could be correlated to the role that EE2 plays in the dopaminergic mechanism. Indeed, EE2 seems to downregulate the *th2* transcription levels, that encodes for the tyrosine hydroxylase, catalyst of the DA synthesis, causing a negative feedback loop in the dopaminergic system. On the other hand, the behavioural assay performed at 14 dpf after long exposure treatment (Figure 13. B) shows a concentration-dependent tendency of the initiations, but data are not statistically significant. More investigations are needed to establish if a longer exposure to EE2 could lead to an initial reduction of the swimming activity followed by its increment. For what concerned the short exposure treatment, data did not show significant

results after the recovery time (7 days) from the drug exposure. Even if fish are more sensitive to endocrine disruptors during the early stage of life than the adult phase, it is possible that relevant effects will be manifested later, in the adulthood (Gore et al., 2006). In this case, it could be necessary evaluate the behaviour two-three months after the exposure.

In the comparison between the behavioural assays conducted at 14 dpf in both treatments, the parameter "initiations" still display relevant differences. Indeed, the number of initiations with 50 ng/L of EE2 resulted higher in the long exposure compared to the short one (Figure 15.B). This result may indicate the need to use higher concentrations or longer exposures to obtain notable variations in terms of behaviour between the two treatments.

To conclude, it is important to point out that experiments performed by adding compounds in water make difficult to predict the amount of absorbed substance as it depends on several individual physical factors (gill activity, skin surface area) (Rubinstein, 2006). Thus, it could result complex to correlate the concentration of compound in solution that caused the toxic effect. A possible solution to this issue could be the detection of the amount of compound that reach target tissues with chemo-analytical methods, such as mass-spectroscopy or high-performance liquid chromatography (Fontana et al., 2018).

5.3 Effects of EE2 on avoidance behaviour

In this work, the term 'avoidance' is referred to the locomotory activity of larva to maintain a certain distance from a threat.

Usually, zebrafish larvae begin to develop swimming capacity after 72 hpf, moving fins, flaps, and eyes. All these changes lead to a progressive acquisition of avoidance behaviours, escape response and hunting (Kimmel et al., 1995). Escape and avoidance behaviours are correlated to the feeling of anxiety and fear, which are important to evade predators or any other danger (Colwill & Creton, 2011). Recent studies demonstrated how EE2 exposure during the development of zebrafish could increase anxiety, influencing feeding and reproductive performance and compromising fish survival. Considering that, it is important predict the risks faced by aquatic populations exposed to chemical pollutants, even for short periods of life.

In our work, larvae treated for shorter time with EE2 did not show any avoidance response to the visual stimuli of the ball, meaning that after the recovery time from the treatment there is not any visible effect caused by the compound. Thus, larvae exposed for short periods of their

lives to EE2 may not have any negative effects or, as already explained in 5.1, visible changes may occur over time. Larvae under long exposure treatment displayed an avoidance reaction to the visual simuli with 5 ng/L and 50 ng/L of EE2, that can be interpreted as an anxiety-like behaviour as demonstrated by other researchers (Reyhanian et al., 2011). Probably the 0.5 ng/L was too low to induce a significant effect.
However, the life stage, duration of exposure and concentrations of EE2 could affect differently the behavioural responses, meaning that further investigations are needed to establish the real role of this powerful estrogen.

5.4 Different feeding protocols did not affect larvae survival

Optimized feeding protocols are crucial for zebrafish husbandry. Indeed, several factors should be considered: frequency of feedings per day, type of food (live or dry food), quality and quantity of food etc.; the lack of attention towards them could compromise the survival, growth rate and reproductive success of fish. Nowadays, these parameters have not been defined yet for zebrafish culture. Thus, the aim of our experiment was to improve the existing dietary protocols, reducing costs and times of maintenance which often represent the main problems in fish cultures. Considering the small size of zebrafish larvae, we reasonably decided to feed them with small amounts of food per feeding (two times per day during the first days post fertilization), increasing them over time, as shown in Table 2; the size of food has been carefully chosen according to fish age. Additionally, the number of feedings per day was not higher to not compromise the water quality, as it could easily decreased with debris and leftovers of food. It has been demonstrated that zebrafish could been reared successfully without any live feed (Siccardi et al., 2009), but not all investigators agree on the suitability of these diets, at least during the early stage of life (C. Certal, 2016). Despite the several advantageous that characterized live foods, the nutritional quality varies according to the geographical origin and rearing conditions. Furthermore, as discussed in 1.2 they could work as vectors for bacteria, causing developmental malformations or death. Taking into consideration all these factors, we concluded that a combination of dry and live food could be the most suitable approach for fish husbandry. The choice of using Artemia as live food instead of Paramecium depended on many factors, including the easier culturing protocols and richer nutritional profile.
From the results obtained (Figure 17), it emerged that there is not a significant difference on

the survival of fish between the two feeding protocols tested. The possible causes of the increased mortality may be various. The most plausible causes are an inadequate water quality or the use of a live food with an incomplete nutritional profile. Keeping water in good conditions has always represented on of the major problems in zebrafish husbandry as its irregularly cleaning could intoxicate fish with their own excretions and other debris. According to Brand et al., 2002, the easiest way to avoid low water quality levels is replace it frequently and to maintain less fish in one system. The error we probably came across was an excessive fish density into every single water system, causing elevate levels of ammonia which resulted toxic for fish.

6. Conclusion

It has been widely demonstrated in literature the role EDCs in the dysregulation of endocrine system, metabolism, reproduction, and homeostasis of animals and humans. In particular, the aquatic population resulted to be most at risk since most of these compounds can easily reach the water systems, compromising its survival. While many researchers based their studies on the reproductive disruptions, few investigations have been carried out on the neurobehavioural changes caused by EDCs. Behaviour is considered one of the most relevant parameters for assessing dysfunctions of several districts in the organism and it is strictly correlated to the neuroendocrine mechanisms. In our study we evaluated the neurobehavioural effects of 17-α-ethinylestradiol (EE2), a synthetic estrogen used for birth control pills, in zebrafish model, performing two validated behavioural assays: the spontaneous swimming assay and the bouncing ball assay. From the results obtained we established that EE2 has effects on the swimming locomotion of zebrafish larvae after few days of exposure, without any significant prolongation of effects. The bouncing ball assay, used to assess the avoidance response of zebrafish larvae after EE2 treatment, showed an increased anxiety-like behaviour with larvae treated with 5 and 50 ng/L, whose spent more time far from the virtual stimulus. Further experiments with higher concentrations and longer periods of exposure are required to establish the effects of EE2 on these two important behavioural parameters. All these data contribute to enrich the knowledge about endocrine disruptors consequence on aquatic wildlife and to highlight the correlation between the endocrine system and neurobehavioral activity.

In addition, the second aim of our study was to propose an optimized feeding protocol that can reduce costs and working time without affecting the survival rate of zebrafish larvae. The lack of relevant differences between the effects of the proposed feeding protocols permits to consider them as a first approach to raise zebrafish with easier conditions. Future modifications are needed.

7.Abbreviations

Dpf days post fertilization

ECs estrogenic chemicals

EDCs endocrine disrupting chemicals

EE2 17-α-ethinylestradiol

E1 estrone

E2 estradiol

ER estrogen receptor

Hpf hours post fertilization

LE long exposure

SE short exposure

8.List of figures

Figure 1. Adult female and male zebrafish adapted 1
Figure 2. Advantages of using zebrafish as an animal model in biomedical research 3
Figure 3. Newly hatched artemia nauplii.. 6
Figure 4. Paramecium under the microscope 7
Figure 5. Chemical structure of 17-α-ethinylestradiol.. 9
Figure 6. Conceptual diagram of the principal sources of contamination of EE2 and the effects of its exposure 12
Figure 7. Representation of the Artemia hatching system.. 19
Figure 8. EE2 treatment's scheme on zebrafish larvae from 4 dpf to 14 dpf 21
Figure 9. Representation of the spontaneous swimming assay 22
Figure 10. Power Point representation projecting the visual stimuli in the bouncing ball assay. Based on: Loscalzo, 2011 23
Figure 11. Representation of the wells in bouncing ball assay... 24
Figure 12. Kaplan-Meier response curves of survival.. 25
Figure 13. Spontaneous swimming assay parameters of long drug exposure. 28
Figure 14. Spontaneous swimming assay parameters of short drug exposure. 30
Figure 15. Long and short exposure comparison of swimming assay at 14 dpf................................. 32
Figure 16. Standard deviation of bouncing ball assay at 14 dpf... 33
Figure 17. Kaplan-Meier response curves of survival.. 34

9.List of Tables

Table 1. Physico-chemical proprerties of estrogen hormones 11

Table 2. Feeding protocol for each group of larvae from 6 dpf to 24 dpf. 18

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