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Embryonic exposures to chemicals acting on brain aromatase lead to different locomotor effects in zebrafish larvae

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Abstract :

Pathways underlying neurodevelopmental effects of endocrine disruptors (EDs) remain poorly known. Expression of brain aromatase (aroB), responsible for estrogen production in the brain of teleosts, is regulated by estrogenic EDs and could play a role in their behavioral effects. We exposed zebrafish eleutheroembryos (0-120 hours post-fertilization) to various concentrations of 16 estrogenic chemicals (incl. bisphenols and contraceptives), and of 2 aroB inhibitors. Behavior was monitored using a photomotor response test procedure. Both aroB inhibitors (clotrimazole and prochloraz) and a total of 6 estrogenic EDs induced significant behavioral alterations, including DM-BPA, BPC and BPS-MPE, three bisphenol substitutes which behavioral effects were, to our knowledge, previously unknown. However, no consensus was reported on the effects among tested substances. It appears that behavioral changes could not be linked to groups of substances defined by their specificity or potency to modulate aroB expression, or by their structure. Altogether, behavioral effects of estrogenic EDs in 120 hours post-fertilization larvae appear unrelated to aroB but are nonetheless not to be neglected in the context of environmental safety.

Highlights

► Zebrafish eleutheroembryos were exposed to 18 (anti-)estrogenic chemicals (EDs) ► Concentrations were based on the regulation of brain aromatase (aroB) expression ► Behavioral effects were measured and compared in 5 day larvae ► Results show limited link between aroB dysregulation and locomotor effects ► Substitute chemicals such as BPC exhibit neurobehavioral effects in this model

Keywords : Bisphenol, cyp19a1b, neurotoxicity, endocrine disruption, larval photomotor response

Abbreviations

ED: Endocrine Disrupter

aroB: Brain aromatase

hpf: hours-post-fertilization

DMSO: Dimethylsulfoxide

EC50: Concentration leading to 50% of the maximum induction

TCBPA: Tetrachlorobisphenol A

BPA: Bisphenol A

BPB: Bisphenol B

BPC: Bisphenol C

DM-BPA : Dimethyl-Bisphenol A

BPF: Bisphenol F

BPS: Bisphenol S

BPAF: Bisphenol AF

BPS-MPE: Bisphenol S-MPE

BPS-MAE: Bisphenol S-MAE

4,4'-ODP: 4,4'-oxydiphenol

4-tOP: 4-tert-octylphenol

E2: estradiol

EE2: Ethinylestradiol

LNG: Levonorgestrel

NET: norethindrone

CLO: Clotrimazole

PCZ: Prochloraz

LOEC: Lowest Observed Effect Concentration

LON1: first period of light in the photomotor response test procedure

LOFF: period of darkness in the photomotor response test procedure

LON2: second period of light in the photomotor response test procedure

OECD: Organization for Economic Co-operation and Development

1. Introduction

Endocrine disrupters (EDs) are substances capable of interfering with the endocrine system *via* different mechanisms such as synthesis, release, receptor binding or biodegradation of endogenous hormones, and resulting in an adverse effect in an intact organism (Bergman et al., 2013; Mihaich et al., 2017). Compounds showing endocrine disrupting properties are structurally diverse and have been identified among natural substances (e.g. estradiol), industrial chemicals (e.g. bisphenol A) and pharmaceuticals (e.g. 17α-ethinylestradiol, levonorgestrel) (Bergman et al., 2013). The use of many endocrine-disrupting chemicals is under restrictions, or even forbidden around the world. However, there is an increasing number of substitute chemicals that in fact exhibit similar properties than the compound they are supposed to replace. This is exemplified by the number of bisphenol A alternatives (bisphenols

B, S, F, AF) that are known or suspected EDs (European Commission Endocrine Disruptor list, April 2022; UN list of identified endocrine disrupting chemicals, July 2017).

Endocrine disrupters were primarily widely studied because of their deleterious effects on reproductive function (Brion et al., 2004; Cheshenko et al., 2008; Hayes et al., 2011; Holley et al., 2020; Rempel and Schlenk, 2008). Nonetheless, there is accumulating evidence showing that exposure to EDs can interfere with brain circuits and functions which lead to neurological or neurodevelopmental defects (Lupu et al., 2020; Saili et al., 2012; Schantz and Widholm, 2001; Seralini and Jungers, 2021; Street et al., 2021). So far, behavior of zebrafish was used to explore effects of exposure to a limited set of EDs, i.e. mainly 17β-estradiol, 17α-ethinylestradiol and bisphenol A, revealing disruption of various neurobehavioral traits such as aggressivity (Colman et al., 2009), sociability (Moraes et al., 2021; Rosenfeld, 2015), anxiety (Porseryd et al., 2017; Volkova et al., 2015), locomotion (Kinch et al., 2015; Nasri et al., 2021), learning (Naderi et al., 2020; Saili et al., 2012). Besides, zebrafish has also been useful to the study of ED targets and underlying mechanisms of action (Segner, 2009).

It is estimated that 20 % of neurodevelopmental effects of EDs go through dysregulation of thyroid signaling, the rest acting *via* other -yet mostly unidentified pathways (Colborn, 2004; Seralini and Jungers, 2021; Takesono et al., 2022). A feature common to many ED substances relies on their capacity to target the developing brain to alter the estrogen signaling pathway and brain aromatase expression (Brion et al., 2012; Vosges et al., 2010). In teleosts, brain aromatase (aroB) is responsible for neuroestrogen synthesis and it is therefore a key-player in neurosteroid homeostasis (Diotel et al., 2018; Pellegrini et al., 2013). In zebrafish, expression of aroB is under strict control of estrogen receptor signaling through a positive feedback-loop mediated by interaction between estrogen receptor and estrogen response elements located in the promoter region of aroB (Diotel et al., 2010; Menuet et al., 2005; Mouriec et al., 2009). The high responsiveness of aroB transcription to EDs promoted the development and the validation

of the EASZY assay at the OECD level (OECD 250) to reliably quantify estrogenic activities of substances acting in the developing brain of zebrafish embryos (Brion et al., 2012).

Previous studies have shown that modulation of brain aromatase expression or activity (up-regulation or inhibition) led to significant changes in neurogenic activity (Diotel et al., 2018; Ogawa et al., 2020; Pellegrini et al., 2016; Vaillant, 2020). Further, the role of brain aromatization in fish sexual, social and aggressive behavior as well as in cognition has been documented in various fish species (Garcia-Segura, 2008; Huffman et al., 2013; Ogawa et al., 2020; Ramallo et al., 2017). Mechanisms underlying effects of estrogenic chemicals on behavior are, however, poorly understood (Takesono et al., 2022).

In the present study, we aimed at evaluating locomotor defects in zebrafish larvae following exposure to chemicals interfering with aroB expression as measured in the EASZY assay. Behavioral alterations in larvae are often highlighted using the larval photomotor response test (Ali et al., 2012; Jarema et al., 2015; Legradi et al., 2015; MacPhail et al., 2009; Noyes et al., 2015; Shen et al., 2020). It relies on that a sudden switch to darkness represents an anxiogenic stimulus for larvae that triggers an escape response, which can be altered by exposure to exogenous substances (MacPhail et al., 2009). It is often used for evaluating the neurotoxic potential of chemicals or for detection of neuroactive pharmaceuticals (Ali et al., 2012; Basnet et al., 2019; Kokel et al., 2010). Two objectives of this study were 1) to assess behavioral alterations following exposure to a set of established and suspected EDs in a standard test procedure using 120 hours post-fertilization (hpf) zebrafish larvae and 2) to evaluate whether locomotor defects could be directly linked to aroB induction.

2. Material and Methods

2.1.Chemicals

All chemicals in this study were purchased from Sigma-Aldrich (France) except for tetrachlorobisphenol A (abcr), BPS-MPE (Santa Cruz Biotechnology), 4-4'oxydiphenol (TCI Europe n.v.) and BPS-MAE (Ark Pharm. Inc.). Tested chemicals are listed in Figure 1 and Table 1. Stock solutions of all chemicals were prepared as 10,000X concentrates in dimethyl sulfoxide (DMSO, Sigma-Aldrich) to keep a final solvent concentration of 0.01% v/v in all test vessels, including a solvent control. Test concentrations were based on the induction of brain aromatase expression in *cyp19a1b*-GFP zebrafish embryos obtained following EASZY guideline (OECD, 2021). For each substance, three concentrations were used: the concentration leading to 50% of their respective maximum induction level (EC_{50}) (Brion et al., 2012; Christophe et al., 2023), as well as to the $EC_{50}/2$ and $EC_{50}/4$. Three exceptions were TCBPA, CLO and PCZ which did not induce *cyp19a1b* expression. For TCBPA, half the lowest observed effect concentration (LOEC/2) in the Fish Embryo Toxicity Test (OECD, 2013) was chosen as the highest tested concentration (Christophe et al., 2023). CLO and PCZ were tested as they inhibit aromatase activity. The highest tested concentrations refer to the inhibition of approx. 50 % of *cyp19a1b* expression obtained following the EASZY guideline (Christophe et al., 2023).

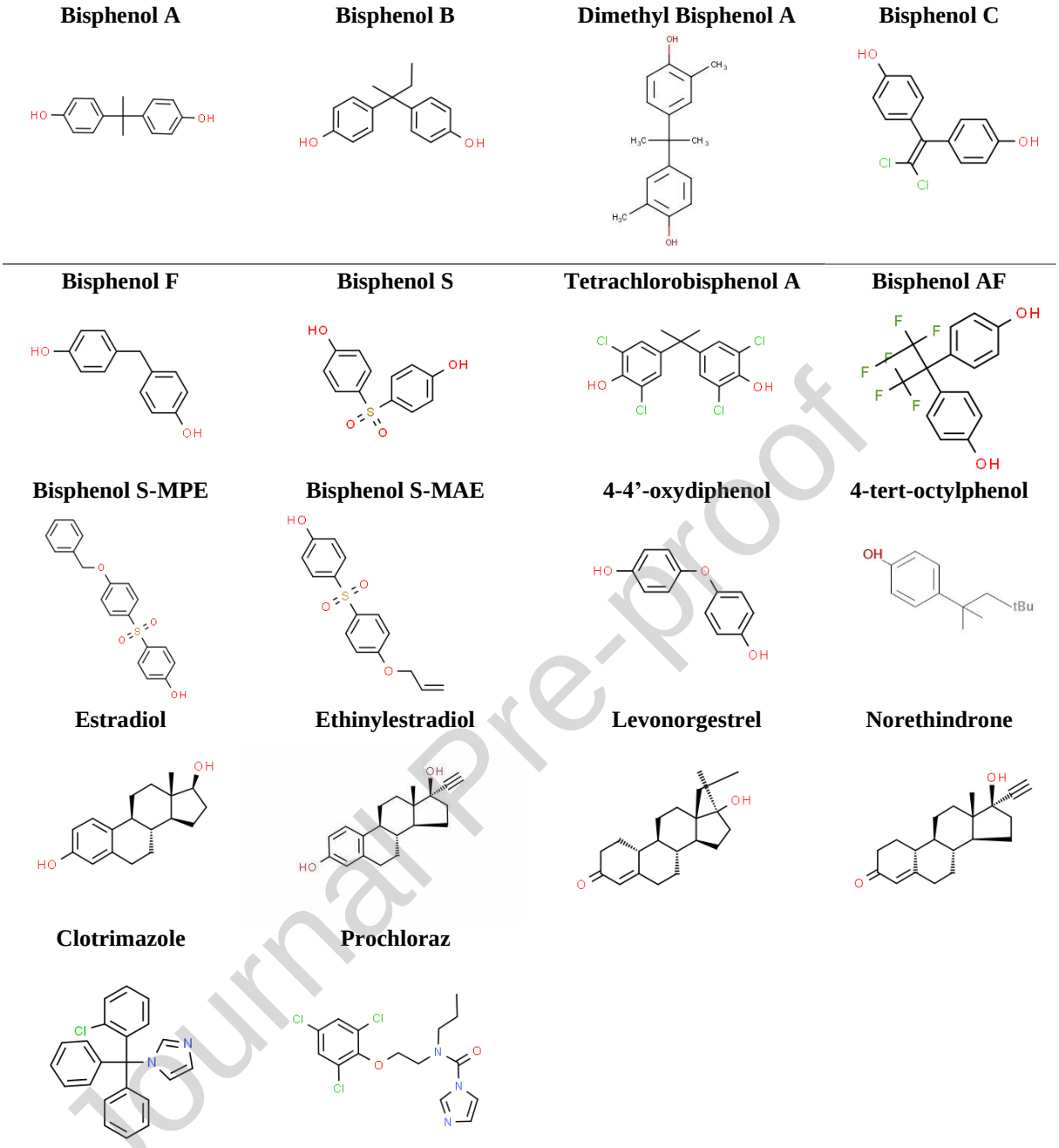

Figure 1: Structure of chemicals tested in the study.

2.2.Fish husbandry

The study was conducted using the *cyp19a1b*-GFP zebrafish line (Tong et al., 2009). Fish were reared in a recirculating water system at 27 ± 1 °C under a 14 h/10 h light/dark cycle with light onset at 8:45 a.m. Physical water conditions were maintained constant during the

experiment and were within recommended ranges (Lawrence, 2007). Fish were fed in the morning and evening with dry food (SDS of appropriate size according to age (Dietex International, England and InicioPlus 500 µm, Biomar SAS, France) and with freshly hatched artemia (Ocean Nutrition®, Belgium) at midday. In the evening before eggs were needed, random pairs were created and transferred to 1 L spawning boxes containing fresh water. Eggs were collected the next morning within 3 hpf.

2.3.Embryo exposure

Embryos were exposed to the test solutions from 0 to 120 hpf. Fertilized eggs were sorted and transferred in reconstituted ISO water for embryo rearing, prepared according to ISO 7346-2 (1996) (294 mg/L CaCl2·2 H2O, 123.3 mg/L MgSO4·7 H2O, 63 mg/L NaHCO3, 5.5 mg/L KCl, pH 7.8). Per condition, an even mixture of 25 eggs issued from 3-5 different spawns was quickly transferred to a glass crystallizer containing 25 mL of pre-warmed exposure solution. Experiments were performed in triplicate, for a total of n=61±6 embryos per condition. Dishes were incubated at 27 ± 1 °C. The medium was exchanged every 24 h to maintain stable concentrations over the course of the experiment, and embryo development was monitored daily by microscopic observation until behavioral testing at 120 hpf.

2.4.Locomotor behavior

The swimming activity of eleutheroembryos was evaluated at 120 hpf using Daniovision (Noldus, The Netherlands) between 1:00 and 6:00 p.m. (MacPhail et al., 2009). In the morning before testing, zebrafish embryos were transferred in 24-well plates (one embryo per well) (Clearline, Dutscher, France) and the plates moved for acclimation to a lightened thermostatic incubator at 28 °C in the room where the Daniovision is located. They were left there for at least two hours to recover after transfer. The behavioral protocol was as follow: one plate was gently introduced in the Daniovision for 10 minutes of acclimation in darkness, free swimming

was then recorded during 5 min of light (LON1), 5 min of darkness (LOFF) and 5 min of light (LON2), as described in (Alfonso et al., 2019). At the end of the experiment, larvae were euthanized by immersion in ice-cold water.

2.5.Data analysis

EthoVision (Noldus) was used for automatic recording and analysis of total distance travelled, meander of movement, and time spent in well center (thigmotaxis). Because of deviation from normality, the effects of exposure to each substance on behavioral endpoints were analyzed using Kruskal-Wallis tests on individual light periods (LON1, LOFF, LON2), and followed by Dunn's post-hoc multiple comparison test to identify concentration-specific differences using R v.4.1.0 (packages stats and FSA 0.8.32). If not representative of other larvae from the same condition, larvae that did not move at all during the entire experiment were removed before analysis (less than 1% of total population). An adjusted p-value ≤ 0.05 (Holm method) was considered to show a significant difference between exposed and control larvae. Graphics represent mean ± SEM with a total number of n=61±6 larvae per condition.

Table 1: Substances tested in the present study.

Substance	**Acronym**	**Chemical Abstract Service (CAS) number**	**Final tested concentrations (µg/L)**
Bisphenol A	BPA	80-05-7	1000-500-250
Bisphenol B	BPB	77-40-7	600-300-150
Dimethyl Bisphenol A	DM-BPA	79-97-0	540-270-135
Bisphenol C	BPC	14868-03-2	17-8,5-4,25
Bisphenol F	BPF	620-92-8	400-200-100

Bisphenol S	BPS	80-09-1	63000-31500-15750
Tetrachlorobisphenol A	TCBPA	79-95-8	250-125-62,5
Bisphenol AF	BPAF	1478-61-1	270-135-67,5
Bisphenol S-MPE	BPS-MPE	63134-33-8	1140-570-285
Bisphenol S-MAE	BPS-MAE	97042-18-7	350-175-87,5
4-4'-oxydiphenol	4,4'-ODP	1965-09-9	400-200-100
4-tert-octylphenol	4-tOP	140-66-9	76,4-38,2-19,1
Estradiol	E2	50-28-2	0,444-0,222-0,111
Ethinylestradiol	EE2	57-63-6	0,004-0,002-0,001
Levonorgestrel	LNG	797-63-7	50-25-12,5
Norethindrone	NET	68-22-4	2,5-1,25-0,625
Clotrimazole	CLO	23593-75-1	500-250-125
Prochloraz	PCZ	67747-09-5	3700-1850-925

3. Results

Under our experimental conditions, no significant effect on mortality or development was reported following exposure to the test substances, except for the highest concentration of PCZ for which a high proportion of unhatched embryos was observed at 120 hpf, and therefore not further analyzed (data not shown). No significant effect was observed following exposure to the 10 following substances at tested concentrations: EE2, BPA, BPB, BPF, BPS, TCBPA, BPAF, BPS-MAE, 4,4'-ODP and NET (Figure S1-S2). For other substances, modifications of larval behavior varied according to substance identity and/or concentration used (Table 2).

Table 2: Summary of significant behavioral modifications obtained in 120 hpf zebrafish larvae following exposure to effective substances. Effects were not necessarily observed during all light periods but for sake of clarity no distinction was made in this summary table. For details, please refer to text and figures.

Acronym	Distance travelled (cm/5 min)			Total meander (deg/cm/5 min)			Time spent in well center (%/5 min)		
	EC_{50}	$EC_{50}/2$	$EC_{50}/4$	EC_{50}	$EC_{50}/2$	$EC_{50}/4$	EC_{50}	$EC_{50}/2$	$EC_{50}/4$
E2	↑		↑						
BPC	↑	↑	↑			↑		↑	
4-tOP	↓	↓	↓	↓					
DM-BPA	↓			↓					
LNG	↓	↓		↓	↓	↓		↑	
BPS-MPE				↑	↑		↓	↓	
CLO	↓			↓					
PCZ		↓	↓		↓	↓		↓	↓

Exposure to E2 induced a significant increase in the distance travelled by larvae during LOFF observable at the EC_{50} and $EC_{50}/4$, the intermediate concentration leading to no effect (Figure 2). No change in path meander or thigmotaxis was reported (Figure 3, 4). Exposure to BPC showed similar effects, with an increase in distance travelled by larvae at all tested concentrations during LOFF, which was also observed during LON2, significant only after exposure to $EC_{50}/4$ (Figure 2). This was accompanied by a significant increase in path meander during LOFF and LON2 (Figure 3). Interestingly, results also showed a significant increase in

the time spent in the well center following exposure to $EC_{50}/2$ and the same tendency at the other concentrations during LON1 (Figure 4).

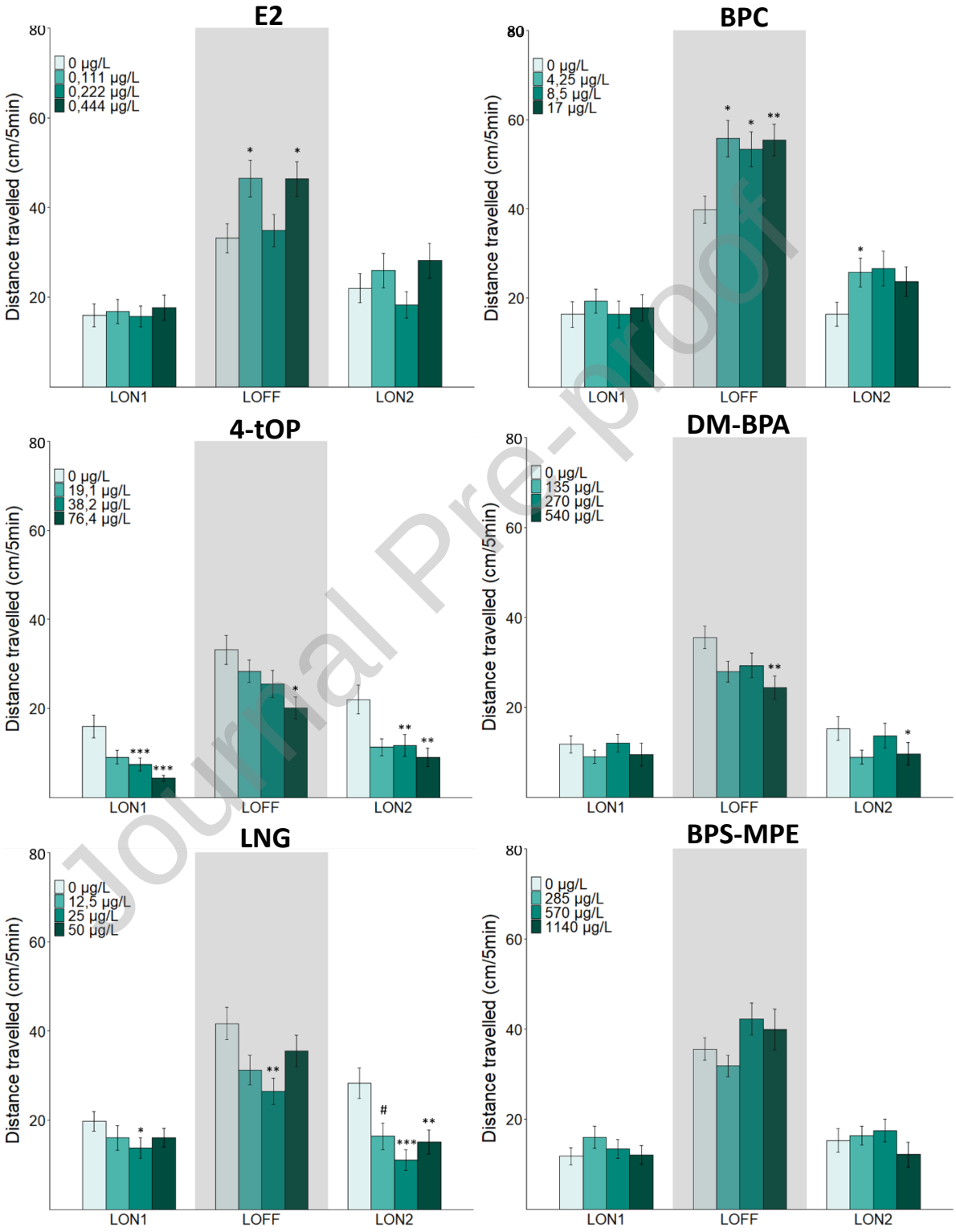

Figure 2: Modification of distance travelled in 120 hpf zebrafish larvae after exposure to the estrogenic substances E2, BPC, 4-tOP, DM-BPA, LNG and BPS-MPE. #: p≤0.1; *: p≤0.05; **: ≤0.001; ***: p≤0.0001.

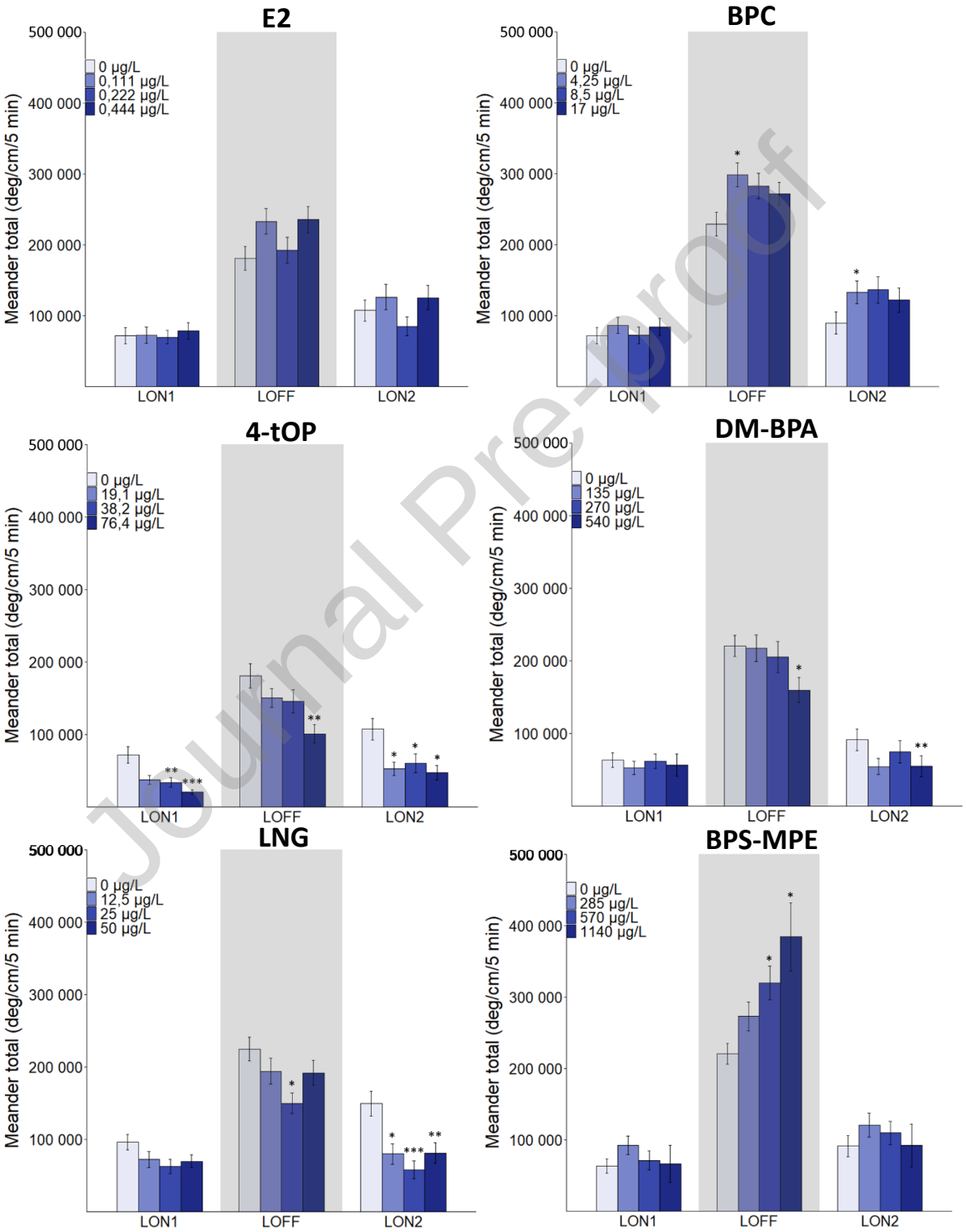

Figure 3: Modification of path sinuosity (as total meander) in 120 hpf zebrafish larvae after exposure to the estrogenic substances E2, BPC, 4-tOP, DM-BPA, LNG and BPS-MPE. *: $p \leq 0.05$; **: ≤ 0.001; ***: $p \leq 0.0001$.

In contrast, exposure to 4-tOP led to a concentration-dependent decrease in distance travelled (significant for the EC_{50} whatever the light period considered and $EC_{50}/2$ during LON1 and LON2) (Figure 2). This was further accompanied by significant decrease in path meander during LON1 (EC_{50}, $EC_{50}/2$), LOFF (EC_{50}) and LON2 (EC_{50}, $EC_{50}/2$ and $EC_{50}/4$) (Figure 3). No significant effect on thigmotaxis was observed (Figure 4). Exposure to DM-BPA led to a significant decrease in distance travelled by the larvae during LOFF and LON2 at the EC_{50} level (Figure 2). This was accompanied by a significant diminution in path meander, but no change in thigmotaxis (Figure 3, 4). Exposure to LNG led to similar defects, with a decrease in distance travelled during LON1 ($EC_{50}/2$), LOFF ($EC_{50}/2$) and LON2 (EC_{50}, $EC_{50}/2$), and a significant diminution of path meander during LOFF ($EC_{50}/2$) and LON2 (all concentrations) (Figure 2, 3). A significant increase in time spent in well center was observed during LON2 ($EC_{50}/2$) (Figure 4).

Exposure to BPS-MPE induced no effect on distance travelled; nonetheless, strong alterations of path meander and thigmotaxis behavior were reported (Figure 2-4). BPS-MPE induced a dose-dependent increase in path sinuosity during LOFF and a significant reduction in the time spent in well center after exposure to the EC_{50} (LON1, LOFF, LON2) and the $EC_{50}/2$ (LON1, LOFF) (Figure 3, 4).

Exposure to both substances known to act as inhibitors of aroB, CLO and PCZ, induced the most dramatic effects. Exposure to PCZ led to a dose-dependent decrease in all measured variables (Figure 5). Exposure to CLO led to a decrease in distance travelled during LON1 (highest and lowest concentrations), LOFF (highest concentration) and LON2 (highest

concentration) (Figure 5). The highest concentration also induced a decrease in path meander at all light periods (Figure 5); however, no effect on thigmotaxis was reported (Figure 5).

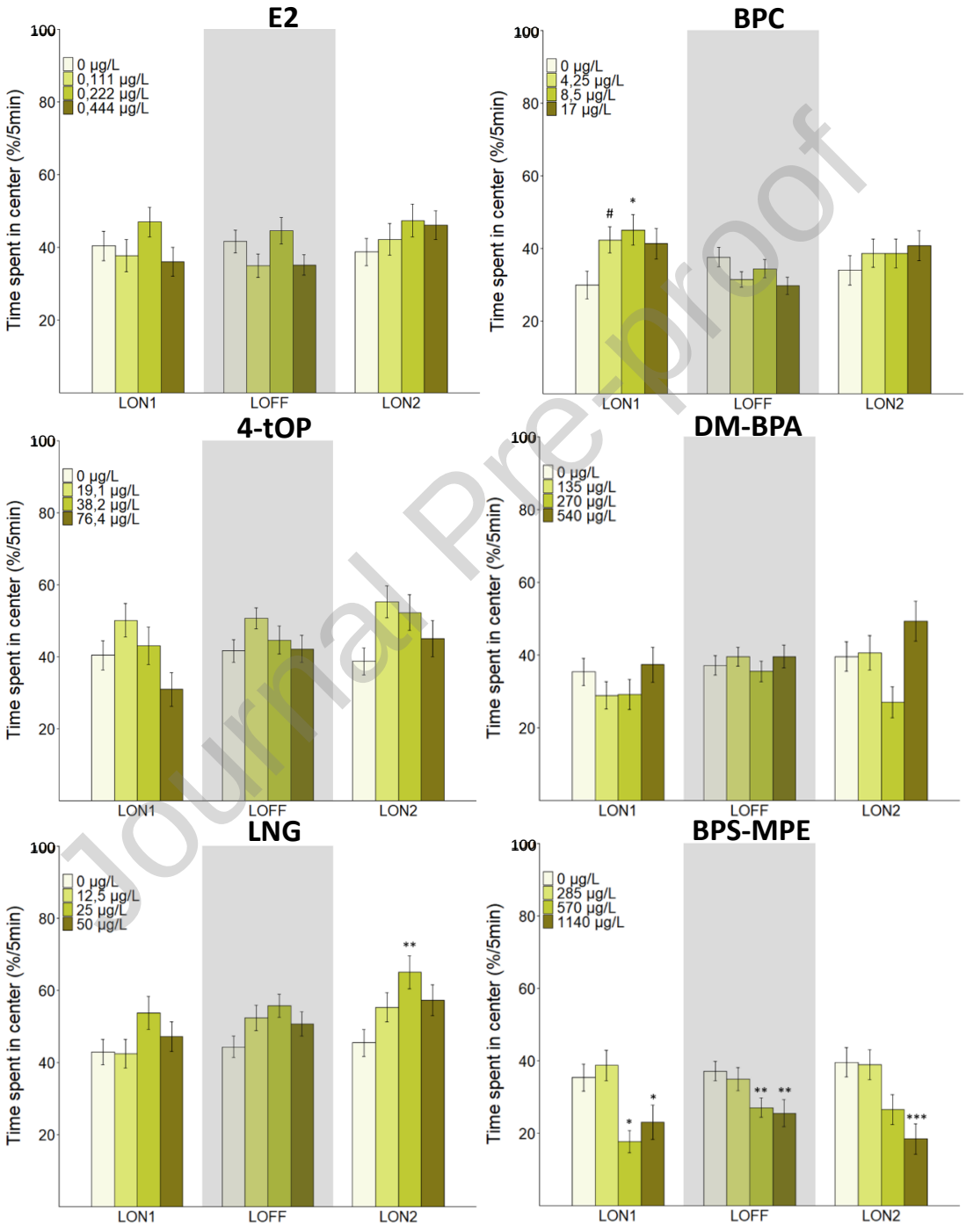

Figure 4: Time spent in well center (% of total period time) by 120 hpf zebrafish larvae after exposure to E2, BPC, 4-tOP, DM-BPA, LNG and BPS-MPE. #: p≤0.1; *: p≤0.05; ***: p≤0.0001.

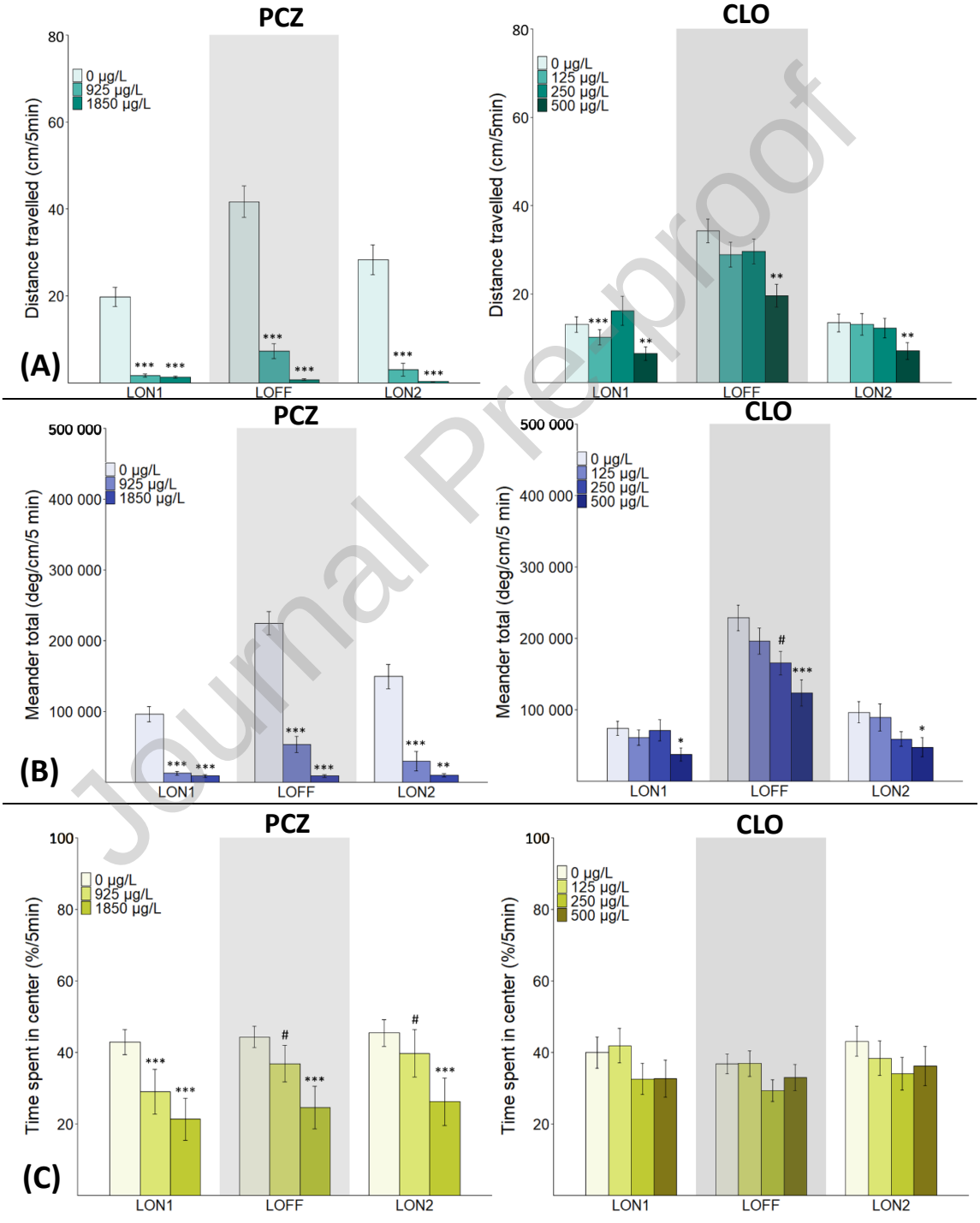

Figure 5: Modification of locomotor traits induced by embryo-larval exposure to aroB inhibitors PCZ and CLO in 120 hpf zebrafish larvae. A) Distance travelled (cm); B) Total meander (path sinuosity) (degrees/cm); C) Time spent in well center. #: $p \leq 0.1$; *: $p \leq 0.05$; **: ≤ 0.001; ***: $p \leq 0.0001$.

4. Discussion

The purpose of this work was to evaluate disruption of locomotor activity in 120 hpf zebrafish larvae resulting from exposure to a set of EDs chosen according to their ability to disrupt the expression of brain aromatase using the EASZY assay (Christophe et al., 2023). In parallel, we also sought to evaluate the possibility to include larval behavior monitoring in a regulatory framework as this would provide with a high-throughput assay for assessing neurobehavioral toxicity. For this reason, exposure procedure followed regulatory guidelines (TG236 and TG250) (OECD, 2021, 2013) and behavioral monitoring was performed according to a routinely used procedure based on larvae photomotor response (Alfonso et al., 2019). Consequently, concentrations and exposure protocol used here may differ from that applied in other studies and further lead to different results.

In order to evaluate the relationship between estrogenic activity of substances and behavioral disruption, we exposed zebrafish to 16 substances able to trigger aroB expression in zebrafish larvae. In order to allow comparison between EDs, the concentration inducing a response of 50% of the respective maximum (EC_{50} EASZY) was chosen as the highest tested concentration. Our data show that only 6 estrogenic substances significantly affected larvae behavior at the tested concentrations. As previously mentioned, the choice of concentrations can explain why we did not observe any behavioral alteration for some compounds that have previously been identified as neurotoxic, such as BPA. In fact, chemicals can act at different levels of a pathway and interfere with different pathways simultaneously (Grimaldi et al., 2019; Spaan et al., 2019),

and the full extent of these chemical-specific differences could not be compared in this study as test concentrations were based on aroB induction in EASZY. For BPA, it is however important to mention that previous results are controversial regarding its behavioral effects in zebrafish larvae. In fact, hyperactivity, hypoactivity or null effects were reported in the literature over a wide range of tested concentrations (~1-14500 µg/L) (Alla et al., 2021; Fraser et al., 2017b; Gyimah et al., 2021; Kinch et al., 2015; Qiu, 2021; Saili et al., 2012; Selderslaghs et al., 2013). In addition, it was shown by Saili et al. (2012) that behavioral effects of BPA followed an inverted U-shape curve between 0.2 and 2000 µg/L. They showed that behavior was unaltered for concentration in the 1 to 10 µM (250 to 2500 µg/L) range, which is in agreement with our results. Concerning BPAF and BPB, we could not reproduce the reduction in larval activity previously observed in a different study, albeit using similar concentrations (Qiu et al., 2021). The same authors reported the absence of effects of exposure to 100 µg/L of BPF (Qiu et al., 2021) which is in agreement with our results. On the contrary, two recent reports highlighted a reduction in 6-day larval swimming activity after exposure to BPF (Gu et al., 2020; Yuan et al., 2019). Effects of BPS on locomotor behavior were also observed in older larvae and/or at lower concentrations than that tested in our study (Gu et al., 2019; Gyimah et al., 2021; Kinch et al., 2015). These differences may directly arise from methodological variations between studies as emphasized in a report (Fraser et al., 2017a), such as differences in larval age, length of exposure, water temperature, photoperiods, strain, exposure regimen, and absence or presence -and number, of dark-light cycles.

To our knowledge, no previous study showed behavioral alterations following exposure to the BPA-alternatives DM-BPA, BPC and BPS-MPE. Our results showed that the effects were strongly dependent on the tested substance. The effects of BPS-MPE were rather specific as they showed no alteration of the average distance travelled, but significant changes in path sinuosity during LOFF and time spent in the center of the well (thigmotaxis). In zebrafish, a

few studies demonstrated that the thigmotactic response was altered following chemical exposure (e.g. Richendrfer et al., 2012; Schnörr et al., 2012), while most experiments investigating locomotor defects in zebrafish larvae do not include thigmotaxis (Champagne et al., 2010). Since in at least one case upon eight (BPS-MPE), modification of meander and thigmotaxis was observed without modification of distance travelled, our results advocate for the monitoring of more variables than only the latter. In general, comparison of the modifications in different locomotor traits can be useful for effect characterization. For example, the simultaneous decrease in distance travelled and meander after exposure to DM-BPA suggests a global activity reduction. In contrast, the increase in distance travelled and sinuosity after exposure to BPC suggests induction of erratic and rather uncoordinated movement, which is supported by the increase in time spent in the center of the well. Overall, this study strongly supports that 120 h zebrafish larvae are a sensitive tool to highlight behavioral alterations following chemical exposure. This is further exemplified by a study on a different set of chemicals which results showed - albeit applying a different protocol, that effects in the photomotor response in embryos were observable at lower doses than morphological malformations (Ortmann et al., 2022). In the present work, effects of EDs could also be observed at levels that did not induce any acute toxicity. In particular, the magnitude of the defects induced by BPC at low concentration (down to 4 µg/L) raises concern about its toxic potential. While previous investigations showed that disruption of brain aromatase expression affects neurogenic activity (Diotel et al., 2018; Ogawa et al., 2020; Pellegrini et al., 2016; Vaillant, 2020) and support a neurotoxic mode of action from estrogenic EDs interfering with aroB, no functional analysis was performed in the present study, thus, we cannot ascertain that observed effects are related to neurotoxicity. Altogether, and whatever the underlying mechanism, this advocates for the introduction of a behavior-based test guideline to increase the sensitivity of regulatory testing framework.

In this study, we first hypothesized that if aroB plays a role in the behavioral effects reported after exposure to estrogenic substances, similar alterations would be highlighted across the range of tested chemicals at a fixed EC_{50} (EASZY) level. We did however not report consensus on the behavioral effects (or lack thereof) among tested substances. References compounds (EE2, E2) induced no to limited effects on zebrafish larvae behavior. Out of the 16 other substances, only BPC exerted similar effects than E2. Other inducers, DM-BPA, 4-tOP and LNG, showed similar patterns to the aroB antagonists, PCZ and CLO (i.e., reduction in locomotor activity independent from the light period, sometimes accompanied by changes in path sinuosity and time spent in well center). Because EC_{50} values vary a lot in magnitude among chemicals, we sought to investigate whether we could establish some links between behavioral effects and specificity (low EC_{50}) or potency (maximum GFP induction in EASZY tests) toward ER-mediated aroB expression. However, no specific link between behavioral outcomes and these reference values could be observed (Figure 6 and Figure S3). We also evaluated if acute toxicity potential -which may include neurotoxicity that could translate into altered behavior, may explain behavioral responses. Again, no correlation could be established (Figure S3). Finally, chemicals used in this study may be compared according to their structural (or functional) family. Our chemicals set included estrogens (E2, EE2), analogues of progesterone (LNG, NET), alkyl phenols (4-4'-ODP, 4-tOP) and bisphenols (BPA, BPB, DM-BPA, BPC, BPF, BPS, BPAF, BPS-MPE, BPS-MAE and TCBPA) which all bind to estrogen receptors while showing sometimes very distinct structures. These chemicals have different modes of action (Combarnous and Nguyen, 2019; Fraser et al., 2017b; Frye et al., 2012). Some studies have compared effects of these substances. In particular, several papers evaluated specificity of activation of the ER pathway by different bisphenol substitutes and revealed distinct mechanisms and/or toxic potential (Chen et al., 2016; Christophe et al., 2023; Gu et al., 2022; Liu et al., 2021). As an example, Liu et al. (2021) observed that chloride halogenation of

BPC triggers intensified interaction with estrogen receptors, resulting in both ERα agonist and ERβ antagonist properties, which may explain differences in locomotor effects between different bisphenols. Overall, no consistency can be observed between chemical groups and behavioral effects.

Taken together, no specific behavioral pattern was observed in response to aroB modulation. These results strongly suggest that behavioral effects of tested substances in zebrafish larvae are not solely related to their property to interfere with aroB expression. Interestingly, this is in agreement with the fact that co-exposure of EE2 or BPA with fulvestrant, an ER inhibitor, does not inhibit the behavioral alterations induced by EE2/BPA alone (Fraser et al., 2017b; Kinch et al., 2015). It is however in partial disagreement with the findings that co-exposure with fadrozole, an inhibitor of aroB activity, abolishes behavioral effects induced by selected bisphenols (Kinch et al., 2015). It is noteworthy that tested concentrations were very different, as were the reported behavioral defects; suggesting that the role of brain aromatase may be depending on the tested dose and/or the evaluated endpoint. It is also important to mention that many EDs have multiple modes of action on the endocrine system as shown by their capacity to bind and activate steroidal nuclear receptors and to alter endocrine pathways (Grimaldi et al., 2019; Spaan et al., 2019; Suresh et al., 2022). The behavioral defects reported here may therefore result from different modes of action in the developing brain and not solely on the modulation of the ER-signaling pathway. In this regard, both measurements of brain aromatase disruption using EASZY, and assessment of larval behavior represent complementary approaches to assess the hazard posed by chemicals.

Finally, brain aromatase has been previously implicated in nervous system development and in various behavioral alterations such as aggressivity, social behavior and cognition (Garcia-Segura, 2008; Huffman et al., 2013; Rosenfeld et al., 2018), although underlying mechanisms of action are still poorly understood. Therefore, one additional explanation to the absence of a

link between aroB induction and behavioral disruption may be that 120 hpf larvae do not display enough complexity in their behavioral repertoire to reveal the extent of neurotoxic effects of brain aromatase disruption that may manifest in adults.

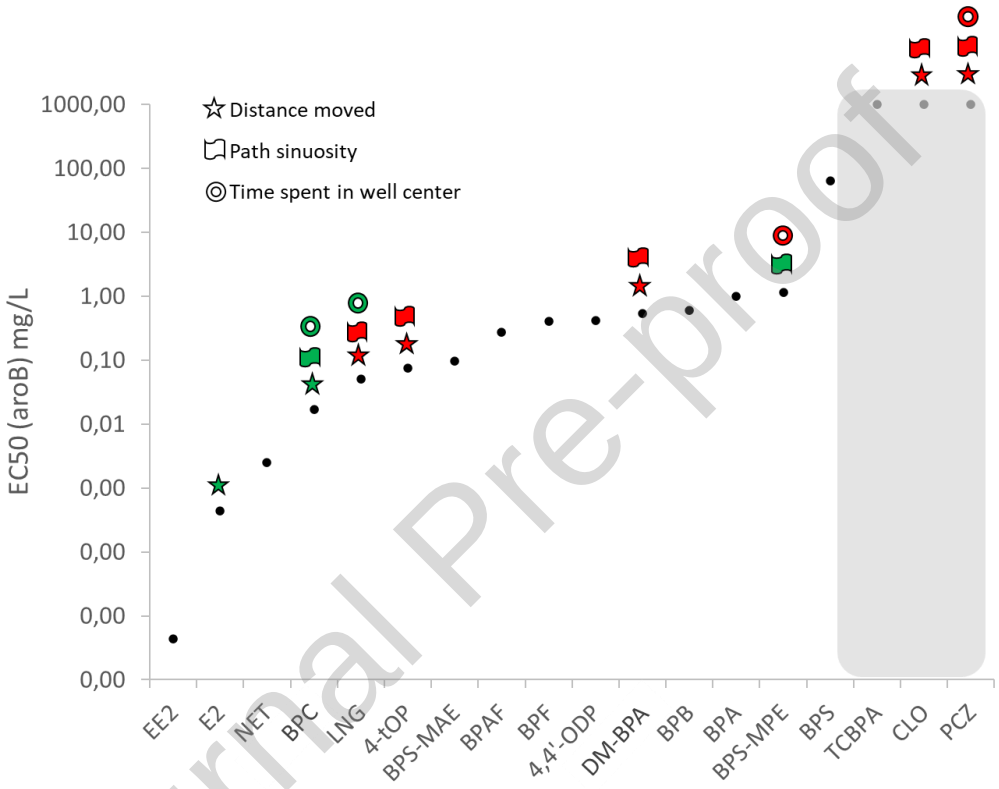

Figure 6: Summary of behavioral effects following developmental exposure to a set of EDs in zebrafish larvae. Green: Upregulated; Red: Downregulated. Chemicals are ranked according to their EC_{50} (aroB) as measured in the EASZY test. nb: TCBPA, CLO and PCZ have been assigned an arbitrary high EC50 for visualization however they do not induce aroB expression.

Conclusion

Taken together, the present data suggest no key-role of the expression or modulation of aroB in the disruption of photomotor response of 120 hpf zebrafish larvae induced by EDs interfering with the estrogenic pathway. The absence of common response based on either structure,

chemical properties suggests that different mechanisms may be involved in the onset of behavioral responses. Additional experiments are required to understand the relationship between brain aromatase and behavioral disruption by analyzing more complex behavioral changes in adults and the use of genetic models to manipulate brain aromatase.

5. Ethical statement

This work has been performed using non-protected stages according to Directive 2010/63/EU. It has however been completed under the umbrella of a larger project which has received the approval of the Ministry of Research under the project authorization number APAFIS #32568-2021072622109117 v3 and followed the recommendations of Directive 2010/63/EU. All experiments were performed at MARBEC Palavas Experimental Marine Platform which has agreement D34121926 for animal experiments.

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8. Author contributions

Funding acquisition: XC, MLB and FB; Conceptualization/Methodology: XC, MLB and MB; Investigation: SS, MB, XC and AC. Data curation/Formal analysis: SS, MB. Writing – original draft: MB; Writing – review and editing: XC, FB, MLB.

9. Declaration of competing interest

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

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CRediT authorship contribution statement

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Declaration of interests

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

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

Highlights:

- Zebrafish eleutheroembryos were exposed to 18 (anti-)estrogenic chemicals (EDs)
- Concentrations were based on the regulation of brain aromatase (aroB) expression

- Behavioral effects were measured and compared in 5 day larvae
- Results show limited link between aroB dysregulation and locomotor effects
- Substitute chemicals such as BPC exhibit neurobehavioral effects in this model