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Exploring estrogen antagonism using CRISPR/Cas9 to generate specific mutants for each of the receptors

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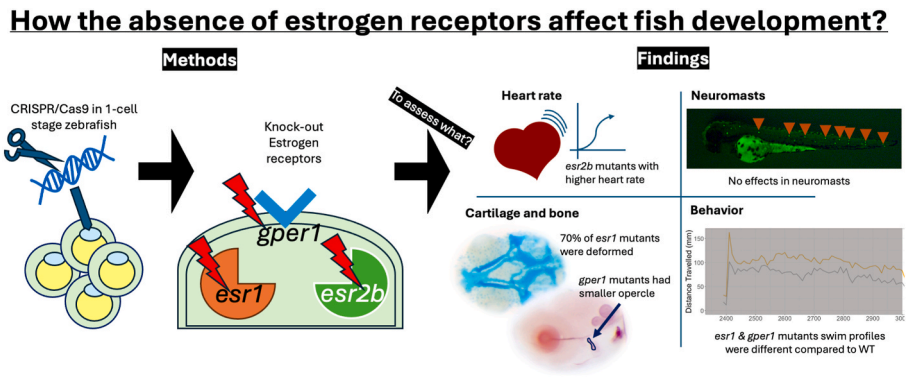
HIGHLIGHTS

- Genetic tools to study endocrine antagonism.
- Individual estrogen receptors invalidated in zebrafish.
- Receptor-specific estrogen antagonism.
- Effects on growth, behavior, bone development, and heart beat.

GRAPHICAL ABSTRACT



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ABSTRACT

Endocrine disruptors are chemicals that have been in the spotlight for some time now. Their modulating action on endocrine signaling pathways made them a particularly interesting topic of research within the field of ecotoxicology. Traditionally, endocrine disrupting properties are studied using exposure to suspected chemicals. In recent years, a major breakthrough in biology has been the advent of targeted gene editing tools to directly assess the function of specific genes. Among these, the CRISPR/Cas9 method has accelerated progress across many disciplines in biology. This versatile tool allows to address antagonism differently, by directly inactivating the receptors targeted by endocrine disruptors. Here, we used the CRISPR/Cas9 method to knock out the different estrogen receptors in zebrafish and we assessed the potential effects this generates during development. We used a panel of biological tests generally used in zebrafish larvae to investigate exposure to compounds deemed as endocrine disrupting chemicals. We demonstrate that the absence of individual functional estrogen receptors (Esr1, Esr2b, or Gper1) does affect behavior, heart rate and overall development. Each mutant line was viable and could be grown to adulthood, the larvae tended to be morphologically grossly normal. A substantial fraction (70%) of the *esr1* mutants presented severe craniofacial deformations, while the remaining 30% of *esr1* mutants also had changes in behavior. *esr2b* mutants had significantly increased heart rate and significant impacts on craniofacial morphometrics. Finally, mutation of *gper1* affected behavior, decreased standard length, and decreased bone mineralization as assessed in the opercle. Although the exact molecular mechanisms underlying these effects will require further investigations in the future, we added a new concept and new tools to

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explore and better understand the actions of the large group of endocrine disrupting chemicals found in our environment.

1. Introduction

In the past decade, one of the major breakthroughs within the field of biology is undoubtedly the discovery, description, and application of the CRISPR/Cas9 system to perform gene editing. Although other gene editing tools already existed, such as chemical mutagenesis coupled to "targeting induced local lesions in genomes" (TILLING) (Wienholds et al., 2003), "transcription activator-like effector nucleases" (TALENs) (Bedell et al., 2012) or morpholino antisense RNAs (Nasevicius and Ekker, 2000), the advent of the "clustered, regulatory interspaced, short palindromic repeats" CRISPR/Cas9 (Gagnon et al., 2014) system has greatly improved speed and accessibility of such tools (Housden et al., 2017).

In the field of ecotoxicology, gene editing tools have been used to generate transgenic or mutant lines to study the underlying molecular mechanisms of chemical compounds and their toxic effects (Zhao et al., 2023). Some examples across species have been useful to determine how certain compounds could be more toxic in the presence or absence of specific genes/receptors. This is the case of the *Daphnia magna* CYP360A8 mutant, where the absence of a functional cytochrome P450 (CYP) clan 3 gene enhanced the observed toxic effects from exposure to paraquat in comparison to their wildtype counterparts (Religia et al., 2021). Similarly, generation of a mutant in the aryl-hydrocarbon receptor gene *arh2* in zebrafish has revealed its involvement in 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD)-induced developmental defects (Garcia et al., 2018).

The zebrafish has been used as a model organism due to its many perks. It is a model organism sharing around 84% of gene orthologues known to be related to human diseases. They are cost-effective, easy to handle, they have high reproductive rates and a rather easy to manipulate genome (Segner, 2009; Howe et al., 2013). Historically, fish have been used as sentinel species to indirectly assess the health of the environment and to model effects on humans. Their use has yielded good, reliable, and precise data, while they are cost-effective approaches compared to their mammalian counterparts.

Estrogen receptors (ERs) are targets for many chemical compounds, they trigger responses such as activation or inhibition of gene expression or phosphorylation of signaling molecules. Four genes code for ERs in zebrafish: three corresponding to the classical nuclear receptors acting on transcription of their target genes depending on the presence of their ligand (Menuet et al., 2002), which are *esr1* (ortholog to mammalian ERα) and *esr2a* and *esr2b* (closer to mammalian ERβ). Moreover, the *gper1* gene encodes a membrane receptor acting on the MAPKinase pathway (Liu et al., 2009; Pang and Thomas, 2010). Exposure of zebrafish larvae to estrogens leads to feminization of their gonads, with males being sterile (Van den Belt et al., 2002; Fenske et al., 2005). Transcriptomic analysis of whole larvae mRNA has revealed regulation of target genes such as *vtg1* et *cyp19a1b* at these early stages (Hao et al., 2013; Schiller et al., 2013b) upon β-estradiol exposure. Estrogens affect cartilage, variations in their concentration may lead to cartilage disruptions when zebrafish is exposed during early development (He et al., 2018). Besides their involvement in female sexual maturation, little is known about the function of individual ERs. Their expression pattern during development is known in the liver, brain, lateral line, pituitary, and other tissues (Bardet et al., 2002; Lassiter et al., 2002; Menuet et al., 2002; Froehlicher et al., 2009; Liu et al., 2009; Pang and Thomas, 2009; Hao et al., 2013). Recently, effects of estrogen exposure on early development of zebrafish larvae have been shown (Soares et al., 2009; Schiller et al., 2013a). Gper1 has been shown to be involved in brain development (Shi et al., 2013), liver growth (Chaturantabut et al., 2019), and oocyte maturation (Pang and Thomas, 2009), while Esr1 and Esr2b seem to be required for lateral line development (Froehlicher et al., 2009; Gamba et al., 2010).

In our study, we focused on the developmental effects caused by antagonizing estrogenic signaling, either by antagonistic chemical compounds, or using a novel approach by knocking out genes expressing three of the four known estrogen receptors by means of CRISPR/Cas9. We knocked out the *esr1*, *esr2b* and *gper* genes in zebrafish and performed a panel of biological tests on zebrafish larvae to determine the effects such a selective antagonism may cause.

2. Materials and methods

2.1. Zebrafish husbandry and ethical considerations

Fish maintenance, breeding conditions, and egg production were described in detail (Westerfield, 2007; Lammer et al., 2009) and are in accordance with internationally accepted standards. Adult wild-type zebrafish of the AB strain were obtained from breeding facilities at the GIGA-Institute, Liege, Belgium. Animal care and all experimentation were conducted in compliance with Belgian and European laws (Authorization: LA1610002 Ethical commission protocol uLg19-2134 and Ulg19-2135).

2.2. Generation of mutant zebrafish lines, DNA extraction and genotyping

To generate mutant lines for *esr1*, *esr2b* and *gper1* genes, we used the CRISPR/Cas9 method (Hwang et al., 2013; Doudna and Charpentier, 2014). Guide RNA (gRNA) sequences were introduced into the Alt-R™ Cas9 system from Integrated DNA Technologies (IDT, Leuven, Belgium). Their sequences were, for *esr1* (exon 2): 5′-TGCTTCAGTGGGATACTACC-3'; for *esr2b* (exon 2): 5′-GGGCAGTGCAGAGAGTGAGG-3'; and for *gper1* (exon 3): 5′-GGAGGTTGACCACCAAAATG-3'. The solutions were made following protocols from the manufacturer (IDT, Leuven, Belgium). The Alt-R crRNA (gRNA for the gene of interest) and tracrRNA were resuspended in Nuclease-Free IDTE Buffer to reach a final concentration of 100 μM each (Raman et al., 2023). Prior to injection, we also added to the final mix 0.5 μL tracer dye (0.5 mg/mL, rhodamine dextran (RD), Molecular Probes, Carlsbad, California USA). Microinjection was carried out into one- and two-cell stage zebrafish embryos using a pneumatic PicoPump PV 820 assembled on an Olympus dissecting microscope. Every injected droplet was calibrated to contain around 2 nL. After 24 h, the DNA from single embryos was extracted and the genomic region surrounding the target sequences for the genes of interest was amplified using PCR. Primer sequences used for PCR were, for *esr1*: forward 5′-GAGTACCCGAACCCCACAC-3′, reverse 5′-TGGACGAGGAATCCAGGTAG-3'; *esr2b*: forward 5′-GATTACTCTCCACCGCATGG-3′, reverse 5′-TGAACGTGTTTCGCTGTAGG-3'; *gper1*: forward 5′-AGTTTTACATCATCGGCCTGTT-3′, reverse 5′-CACCAGAATAAGATCTGCCACA-3'.

2.3. Search of mutants and streamlining genotyping – DNA extraction, qPCR, and sequencing

DNA was isolated from whole larvae or from finclips of adults at various stages of development in 50 mM NaOH by heating at 95 °C water bath for 20 min. The solution was cooled down on ice for 10 min and the DNA extraction was stopped by adding Tris-HCl 1 M, pH = 8.0, 1/5th the volume of NaOH and spun down using a desktop centrifuge for 2 min to recover the supernatant. The extracted DNA was stored at 4 °C until further processing. Genomic fragments covering the targeted region were obtained by PCR using the above primers. The mutants were

identified using Heteroduplex Mobility shift Analysis (HMA) by polyacrylamide gel electrophoresis, selected DNAs were further processed for Sanger sequencing to identify the exact position and extent of the mutation (Raman et al., 2024).

Homozygous mutants were obtained by crossing heterozygous parents carrying the desired mutation and then further tested in each of the assessed endpoints. After each experiment, genotyping of each individual was performed in either 5 days post fertilization (dpf) or 10 dpf fish. qPCR was performed to identify and sort the mutants into 96 well-plates, one fish in each well to be easily located for further analysis. DNA extraction was performed as described above by adapting the volume to fit in the 96-well-plates. Only homozygous WT or mutants were then assigned to their location for phenotype determination.

2.4. Alizarin red (AR) staining

Larvae were euthanized at 10 dpf with an overdose of MS-222 (400 mg/L) (Ethyl 3-aminobenzoate methane sulfonate; Merck, Overijse, Belgium), fixed in PFA 4% overnight at 4 °C and thereafter rinsed three times with PBST for 10 min each time. They were then bleached using H_2O_2 3%/KOH 0.5% during 30 min, then washed thrice for 10 min with 25% glycerol/0.1% KOH to remove the bleaching solution and subsequently stained with alizarin red-S (Merck, Overijse, Belgium) at 0.05% in the dark for 30 min. The pictures were obtained on a dissecting microscope (Olympus and camera SZX10 using cell B software), from at least 96 individuals in three or more independent experiments for each condition.

2.5. Alcian blue (AB) staining

Larvae were euthanized at 5 dpf with an overdose of MS-222 (400 mg/L) (Ethyl 3-aminobenzoate methane sulfonate; Merck, Overijse, Belgium), fixed in PFA 4% for 16 h at 4 °C and thereafter rinsed three times with PSBT for 10 min each time. The larvae were then stained with 1 ml of alcian blue (0.04% Alcian blue/10 mM $MgCl_2$/80% EtOH pH 7.5) for 16 h on low agitation. The pictures were obtained on a dissecting microscope (Olympus and camera SZX10 using cell B software), coming from at least 96 individuals in three or more independent experiments for each condition.

2.6. Estrogen receptor antagonist exposure tests

We have selected three antagonist compounds, one for each of the estrogen receptors. The chosen chemicals were MPP, PHTPP and G15 antagonists for ERα, ERβ and GPER1, respectively (https://www.tocris.com/products/). Stock solutions were prepared in Dimethyl sulfoxide (DMSO, >99.9%, CAS number 67-68-5; Merck kGaA, Darmstadt, Germany). Every exposure test was performed at least three times independently using three final concentrations (10, 100 and 1000 nM) of each compound. 6-well plates were used and in each well 25 fertilized eggs were placed in E3 medium mixed with one of the exposure treatments. The DMSO concentration was corrected to achieve a total concentration of 0.1%, including in the control groups. To keep stable chemical concentrations, we used a static-renewal approach where at least 90% of the media was refreshed every 24 h. Exposure started between 0 and 6 h post fertilization (hpf); the larvae were treated for at least 96 h.

2.7. Morphological observations, heart rate, and behavior

A set of morphological features was recorded, including presence of edemas, inflation of the swim bladder, eye malformations, etc. Pictures of treated and untreated larvae at different stages were taken. All observations were made with a stereomicroscope Leica M165 FC (Leica Microsystems©, Leica, Wetzlar, Germany). Standard length was estimated in fish at 5- and 10- dpf using the FIJI line tool for measurement (ImageJ2, v. 2.3.0/1.53f).

Heartbeats were counted manually using an inverted Nikon Eclipse TS100 microscope and a hand counter for 15 s. To obtain the beats per minute (BPM), measurements were multiplied by 4. The heart rate was estimated on 96 hpf larvae that were acclimated to the lighting conditions for no less than 5 min prior to counting; the larvae were not immobilized by anesthetics or other means. Each larva was observed sequentially at least three times. Ten larvae were observed per mutation and each experiment was performed at least in triplicate.

Neuromasts were counted in each fish at 4 dpf. The neuromasts of the posterior lateral line (PLL) were stained using Di-Asp (4-(4-diethylaminostyryl)-*N*-methylpyridinium iodide) (Di-Asp; Sigma D3418, USA) (Harris et al., 2003). Live staining was achieved by incubating larvae in 5 mM Di-Asp for 5 min and then rinsing three times with normal E3 rearing medium. Stained larvae were euthanized with an overdose of MS-222 (400 mg/L), then mounted in Petri dishes with 3% methylcellulose, placed on their lateral side, and visualized under fluorescent light on a stereomicroscope Leica M165 FC (Leica Microsystems©, Leica, Wetzlar, Germany). 24 larvae were used for each mutant and each experiment was performed at least in triplicate.

Behavioral tests were conducted on zebrafish larvae at 120–125 hpf as previously described (Guerrero-Limón et al., 2023); every test was performed between 10:00 and 13:00 to maintain a constant position in the circadian cycle. Special care was taken during the entire rising period to avoid interference of environmental factors. Exposed larvae were shielded from loud noises, changes in the temperature of the incubator (27–28 °C) and the raising medium (~26° at the time of testing), changing light conditions and activities in the room by putting them in a specific enclosure harboring its own, constant dark/light regime. Prior to each behavioral test, the zebrafish larvae were inspected under a stereomicroscope to select and transfer to the testing plates only individuals devoid of any malformation that might interfere with mobility outcome (e.g., yolk sac or pericardial oedemas, spinal aberrations, aberrations in pigmentation, and/or loss of equilibrium, etc.). The larvae were placed individually in a well of a 96-well plate and observed using a ViewPoint® Zebrabox system and its tracking software (ViewPoint Life Sciences, Lyon, France). The light level was set to 20% on the ViewPoint software (7.45 klux, TES 1337 light meter), while infrared light (850 nm) was used to track larval activity. We applied a light–dark cycle that lasted for a total of 1 h and consisted of 20 min of light, allowing for the larvae to acclimate to the situation and discarded from the analysis, followed by 10 min of darkness, 10 min of light, 10 min of darkness and 10 min of light. The video and tracking software were used to screen larval locomotion behavior in10 s intervals; the distance travelled (DT) and the time spent active (TSA) were determined and, from these parameters, the mean swimming speed when active (SS) was calculated by dividing the cumulated distance travelled by the total time spent active. For each condition, at least 3 batches of 24 individuals each were observed in independent experiments, taking care that always one control (WT or untreated) batch was present on the same 96-well plate.

2.8. Data and statistical analysis

Morphological data were transferred to Prism 9.0.0 (v86) (Graphpad, San Diego, CA, USA). Each data set was tested for normality (e.g., using a visual cue (QQ plot), D'Agostino–Pearson omnibus normality and Shapiro–Wilk tests) and equal variances (Bartlett's test). Therefore, parametric or non-parametric tests were performed depending on the case, as indicated in each figure.

Raw behavioral data sets consisted of tables holding the positions of

each larva in each video frame (30 FPS). This table was first trimmed to eliminate very short, oscillating, and likely artefactual movements, and then aggregated into 10-s periods for further analysis. These data were transferred to R version 4.0.2 to analyze motility during the dark and light phases. To assess behavior, we used linear mixed effect (LME) models within the "nlme" package (Pinheiro and Bates, 2000). Three dependent variables were used, either the "mean time spent active" (seconds), the "mean distance travelled" (mm), or the "mean swimming speed" (calculated as the mean distance travelled/mean time spent active) within each 10 s period, with "compound" and "time" as the categorical and continuous independent variables, and "batch" as a random effect. The "Anova" command within the "car" library (Fox and Weisberg, 2019) was used to extract the results for the main effects whereas the "lsmeans" command (Lenth et al., 2022) within the "emmeans" library was used as a post-hoc test to compare groups against one another while adjusting for the means of other factors within the model (Lenth, 2016). Type II sum of squares was used for the model. We performed our analyses using the 600 s (10 min) of every alternating phase (dark-light). Confidence was assigned at $\alpha = 95\%$ and a p-value of ≤ 0.05 was considered as significant, $p \leq 0.05$ (*), ≤ 0.01 (**), ≤ 0.001 (***), ≤ 0.0001 (****).

3. Results

3.1. Generation of estrogen receptor mutants

To elucidate the role of the estrogen receptors in development, we generated mutants for each of the *esr1*, *esr2b*, and *gper1* genes using the CRISPR/Cas9 method. We focused our studies on three specific lines: line ulg078 disrupting the *esr1* coding region with a 43-nucleotide deletion in exon 2, -line ulg079 disrupting the *esr2b* coding region with a 31-nucleotide deletion in exon 2, -and ulg080 disrupting the *gper1* coding region with a 22-nucleotide deletion in exon 3. Heterozygous mutant populations were established for each line, the mutations and the resulting disruption of the coding sequence was thoroughly checked in homozygous mutant offspring before further characterization. Detailed description of the mutants can be found on http://zfin.org.

3.2. Streamlining genotyping for large number of samples was successful using qPCR

Genotyping individual fish can be time and resource-consuming using traditional means such as the Heteroduplex Mobility Assay, where a polyacrylamide gel is used to tell apart individuals that have a mutation from those being wildtype. Here we streamlined a process where we would identify mutants, heterozygotes and wildtype fish using the differences in the melting curves of the fragment amplified from the genomic target region for each individual. Briefly, depending on the type of mutation, melting curves would appear at a lower or a higher temperature, if the mutation is large enough, the peaks are easily discriminated both in heterozygotes and homozygotes (Fig. S1).

3.3. $esr1^{-/-}$ mutants present malformations in craniofacial cartilage

Initial analysis of larval growth at 5 dpf did not reveal any obvious morphological malformations, nor did we observe any significant differences in standard length between WT and their mutant siblings for any of the estrogen receptors (Fig. 1A).

Skeletal development in zebrafish starts with the formation of cranial cartilage that can be easily observed at 4 dpf by alcian blue staining. We thus compared WT larvae to homozygous mutants for each of the three receptor genes *esr1*, *esr2b*, and *gper1*.

Mutation of the *esr2b* or *gper*1 genes did not cause any defects in craniofacial development. In contrast, mutation of the *esr1* gene led to 70% of the $esr1^{-/-}$ mutant fish presenting malformations in the cranial cartilage compared to WT (Fig. 1B), ranging from mild (31%), severe (29%) to very severe (10%) (respectively Fig. 1C and D) and consisted in significant reduction or total absence of structures such as Meckel's cartilage, ceratohyals, palatoquadrates, and ceratobranchials. The ethmoid plate, although present, was clearly deformed and/or reduced

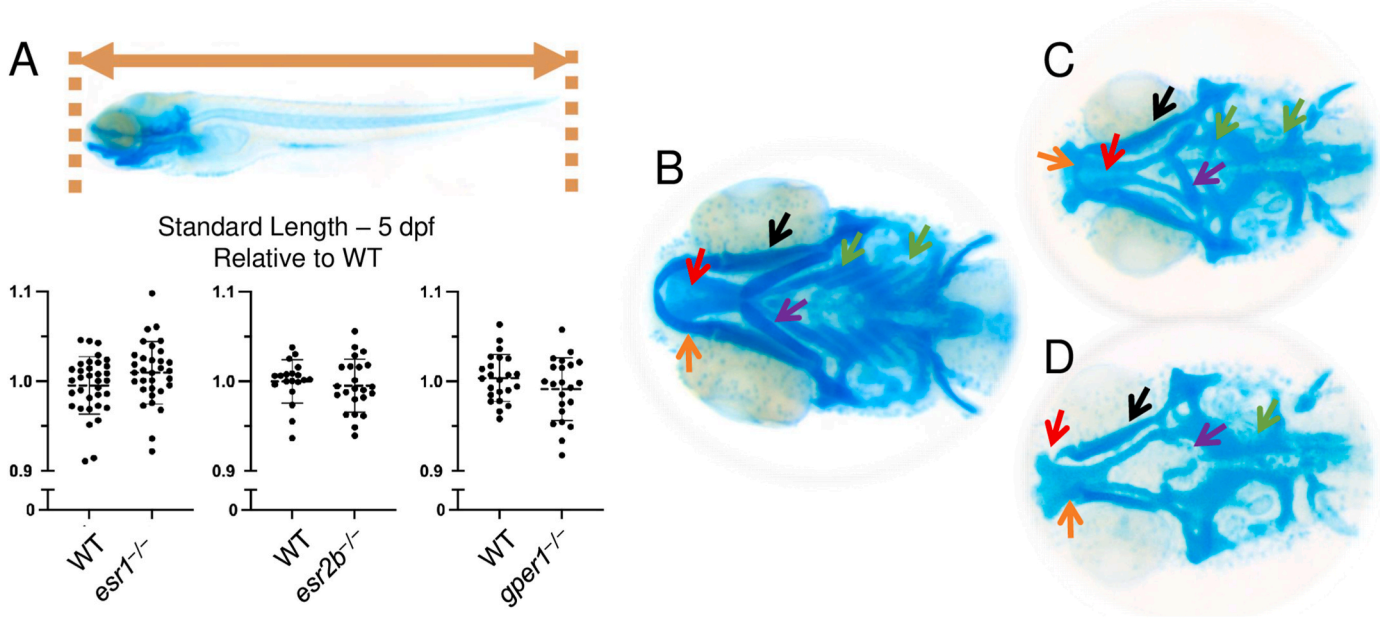

Fig. 1. Phenotypes found in *esr1* mutants. A) Lateral view of a WT sibling, illustrating the measure of the standard length (orange arrow) and graphs representing the measures of standard lengths (relative to the mean of the WT larvae) of each mutant compared to their WT siblings; B) Dorsal view of the stained head cartilage of a 5 dpf WT larva; C) Observed chondrocranium malformations in *esr1* mutant fish: total absence of ceratobranchial 1–5 (green arrows), reduced Meckel's cartilage (orange arrow), thinner palatoquadrate (black arrow) and deformed ceratohyals (violet arrow; D) another example of observed malformations in 5 dpf zebrafish larvae: heavily reduced or total absence of Meckel's cartilage (orange arrow), deformed ethmoid plate (red arrow), thinner palatoquadrate (black arrow), heavily deformed ceratohyal (violet arrow) and absence of ceratobranchials 1–5 (green arrow). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

in size.

To obtain a more precise and quantified analysis of craniofacial features, morphometric analysis was performed on individual images (Aceto et al., 2015; Guerrero-Limón et al., 2023). The assessed parameters were "Angle between ceratohyals" (ACT), "Distance between the frontal end of the ceratohyals and the line connecting the posterior ends of the hyosymplectics" (DCH), "Length of ceratohyals" (LC), "Distance covering the entire Meckel's, palatoquadrate, and hyosymplectics" (MPH), and the "Distance between the left and right Meckel's cartilages/Palatoquadrates" (MPQ) (see also Fig. 2).

These measures could not be performed on the severely affected *esr1*$^{-/-}$ mutant fish, therefore we only analyzed the 30 % (10 individuals) that were less affected, which were indeed not significantly different from their wildtype counterparts (Fig. 2). Although all assessed parameters were slightly different in *esr2b*$^{-/-}$ mutants compared to their WT siblings, only LC was significantly lower (Fig. 2A). Analysis of the *gper1*$^{-/-}$ mutants revealed that the DCH and LC were significantly different from their wildtype siblings (Fig. 2A and B), all other parameters were unchanged.

3.4. The transmembrane receptor Gper1 plays a role in overall growth and bone mineralization

To assess bone formation in the estrogen receptor mutants, we decided to measure the size of the opercle in 10 dpf larvae. Measuring the standard length of the 10 dpf larvae revealed that all mutants were slightly smaller compared to their wildtype siblings, however only the *gper1*$^{-/-}$ mutants were significantly smaller than WT (Fig. 3A). In addition, after performing alizarin red staining for the calcified tissues, we measured the area of the mineralized opercle in each larva. This measure revealed that only the *gper1*$^{-/-}$ mutant displayed a significantly smaller opercle area compared to its WT siblings (Fig. 3B–D).

3.5. Swimming behavior is differently affected by mutation of the estrogen receptors

Next, we tested the swimming behavior at 5 dpf of the different mutants in comparison to their WT siblings. Tracking the fish movements could provide important insights that could be linked to the effects caused by the studied factor, positional data is collected in a semi high-throughput fashion, then actograms of 10 s intervals are plotted to find changes in patterns (Fig. 4 and Fig. S2). We used the previously described alternating 10 min dark/light cycling protocol to measure the "swimming speed when active", the "time spent active", and the "distance travelled" respectively during the dark and the light phases (Guerrero-Limón et al., 2023). As expected, the larvae were significantly more active during the dark phases (Fig. 4).

The *esr1*$^{-/-}$ mutants showed a significantly reduced time active and distance covered in both the dark and the light phase, while swimming speed was significantly reduced only during the light phase. No change in swimming behavior was observed for the *esr2b*$^{-/-}$ mutants (Fig. 5B). In contrast, the *gper1*$^{-/-}$ mutants were significantly less active and covered a significantly smaller distance only during the light phase (Fig. 5F and I, respectively).

3.6. The heart rate was significantly increased in *esr2b*$^{-/-}$ mutants

The heart rate was measured in mutants and their WT siblings at 4 dpf. Both *esr1*$^{-/-}$ and *gper1*$^{-/-}$ mutants presented a slightly lower heart rate, but only *esr2b*$^{-/-}$ zebrafish mutants revealed a significantly increased heart rate compared to their WT siblings (Fig. S3).

3.7. Neuromasts were unaffected by mutations

Previously, a defect in neuromast development was reported upon morpholino knock-down of the *esr2b* gene in zebrafish. Using Di-Asp staining, we thus compared neuromast development in each of the estrogen receptor mutant lines relative to their WT siblings. No significant difference was observed in the number of neuromasts for any of these mutants (Fig. S4).

3.8. Exposure to chemical estrogen receptor antagonists affects zebrafish larvae behavior and heart rate

To gain further insight into the specific effects caused by inhibition of each of the estrogen receptors, chemicals with known antagonistic properties on specific human estrogen receptors were tested in zebrafish to determine whether they would mimic the effects on some of the endpoints observed in mutants.

First, the ERα -specific antagonist MPP was shown to have no effect on zebrafish development at any of the concentrations tested (10–1000 nM) and, importantly, had no effect on cranial cartilage development in 4 dpf larvae. In contrast, several effects on swimming behavior were observed upon treatment with ER antagonists. The ERα-specific antagonist MPP caused a significant increase in distance travelled at 100 nM, and in swimming speed at 100 and 1000 nM in the dark phase, while we found a surprising inverted "U"-shaped pattern in the light phase, with only the 10 nM dose causing a significant increase in all measures compared to untreated or higher doses (Fig. 6A). A similar inverted "U"-shaped pattern was observed using the inhibitor of the ERβ (PHTPP) in the light phase, where the values for all parameters were the highest at the 100 nM dose (Fig. 6B). In the dark phase, the same inhibitor caused a significant decrease in all parameters at the lowest, 10 nM dose, followed by an increase at 100 nM, and a dramatic decrease at the 1000 nM dose (Fig. 6B).

Finally, the inhibitor of GPER1 (G15) had significant effects only at the highest concentration, with an increase of the Swimming Speed in the light phase and a decrease on the Time Spent Active in the dark phase (Fig. 6C).

The exposure tests using estrogen receptor antagonists revealed a decreased heart rate at 1000 nM PHTPP relative to control, while the GPER1 antagonist G15 increased the heart rate at all concentrations tested (Fig. 7).

4. Discussion

4.1. Disrupting estrogen signaling in zebrafish

Estrogen receptors play important roles within the bodily functions of an organism, substantial effort has been put into elucidating the function of these receptors in fish using different approaches. Total antagonism was previously tested using aromatase inhibitors such as fadrozole that blocks the synthesis of endogenous estrogens and thus would affect all receptors. The effects observed were impairment of gonad histology and severe pathological changes (Luzio et al., 2016), gonadal sex differentiation (Fenske and Segner, 2004), altered gene expression in brain and ovaries (Villeneuve et al., 2009), developmental toxicity, delayed hatching and decreased heart rate (Santos et al., 2014), while reduced uptake of vitellogenin into developing oocytes (Ankley et al., 2002) and inhibition of both brain and ovarian aromatase activity was observed in fathead minnow (*Pimephales promelas*) (Villeneuve et al., 2006). However, this approach is limited when investigating the effects on early development, as it would not affect the maternal hormone stock provided by the mother within the egg. Known anti-estrogenic compounds, such as tamoxifen or ICI 182,780 are also difficult to use, due to their complex actions on the different receptors (e.g. partial agonism of tamoxifen, or agonism of ICI 182,780 on GPER1). Therefore, we decided to investigate the function of each receptor separately by knocking out each one separately to assess their effects individually. Individual receptors have been previously targeted using morpholino antisense RNAs to specifically block transcription, or by targeted mutation of the individual genes. The focus of these studies was

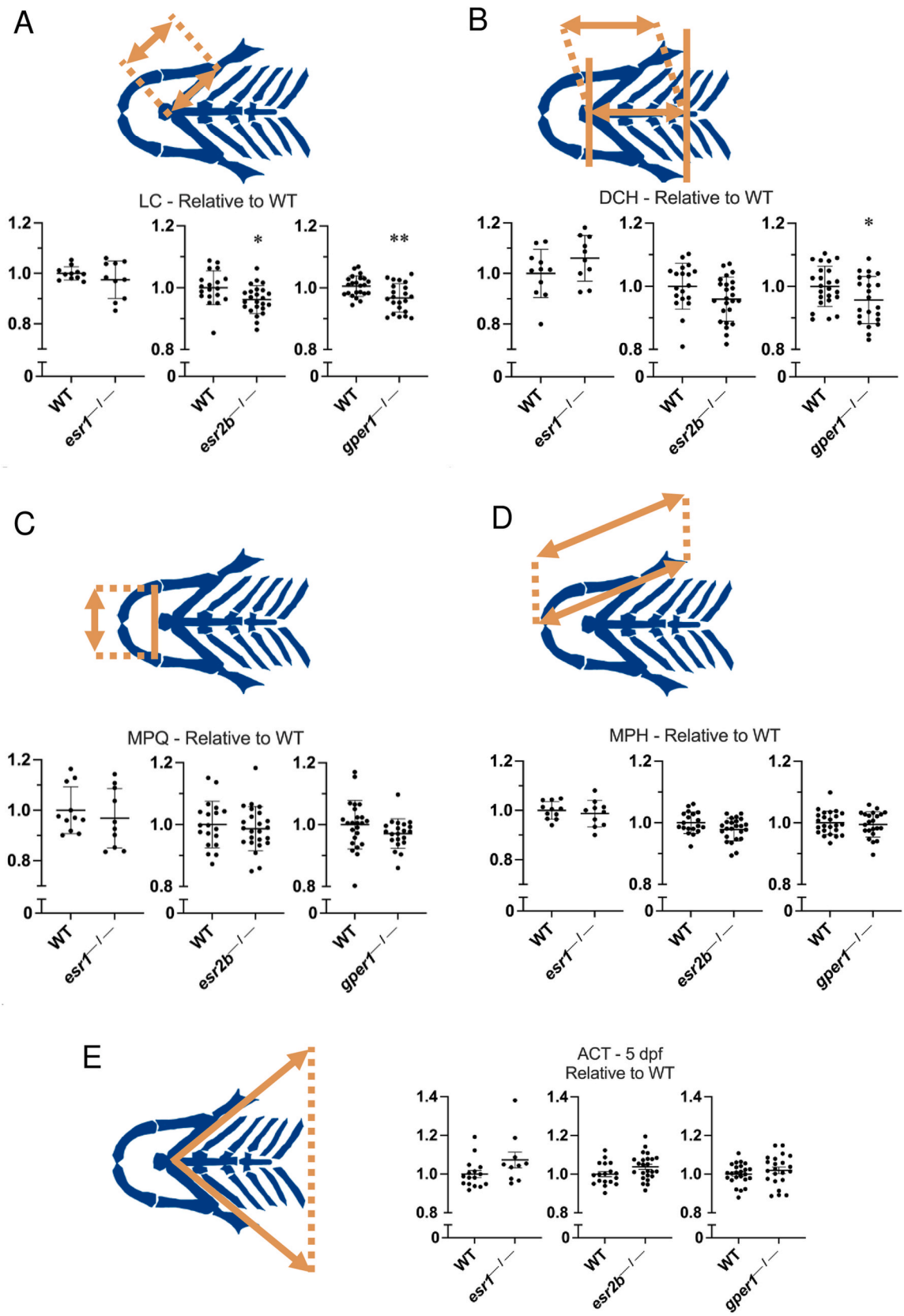

Fig. 2. Morphometrics of *esr1*$^{-/-}$, *esr2b*$^{-/-}$, and *gper1*$^{-/-}$ mutants compared to WT. Illustrations above each plot, or on the left for figure E, are a representation of how the different measurements were performed. A) Length of ceratohyals (LC); B) Distance between the frontal end of the ceratohyals and the line connecting the posterior ends of the hyosymplectics (DCH); C) Distance between the left and right Meckel's cartilage/Palatoquadrates (MPQ); D) Distance covering the entire Meckel's cartilage, palatoquadrate, and the hyosymplectics (MPH); E) Angle between ceratohyals (ACT). An Unpaired Mann-Whitney *t*-test was performed on 24 fish per group (either Mutants or WT) per assessed parameter. Asterisks (*) indicate when significant differences were found for a mutant strain compared to its WT siblings, $p < 0.05$ (*), $p < 0.01$ (**). All values were normalized relative to the mean value of the corresponding WT siblings for each mutant strain.

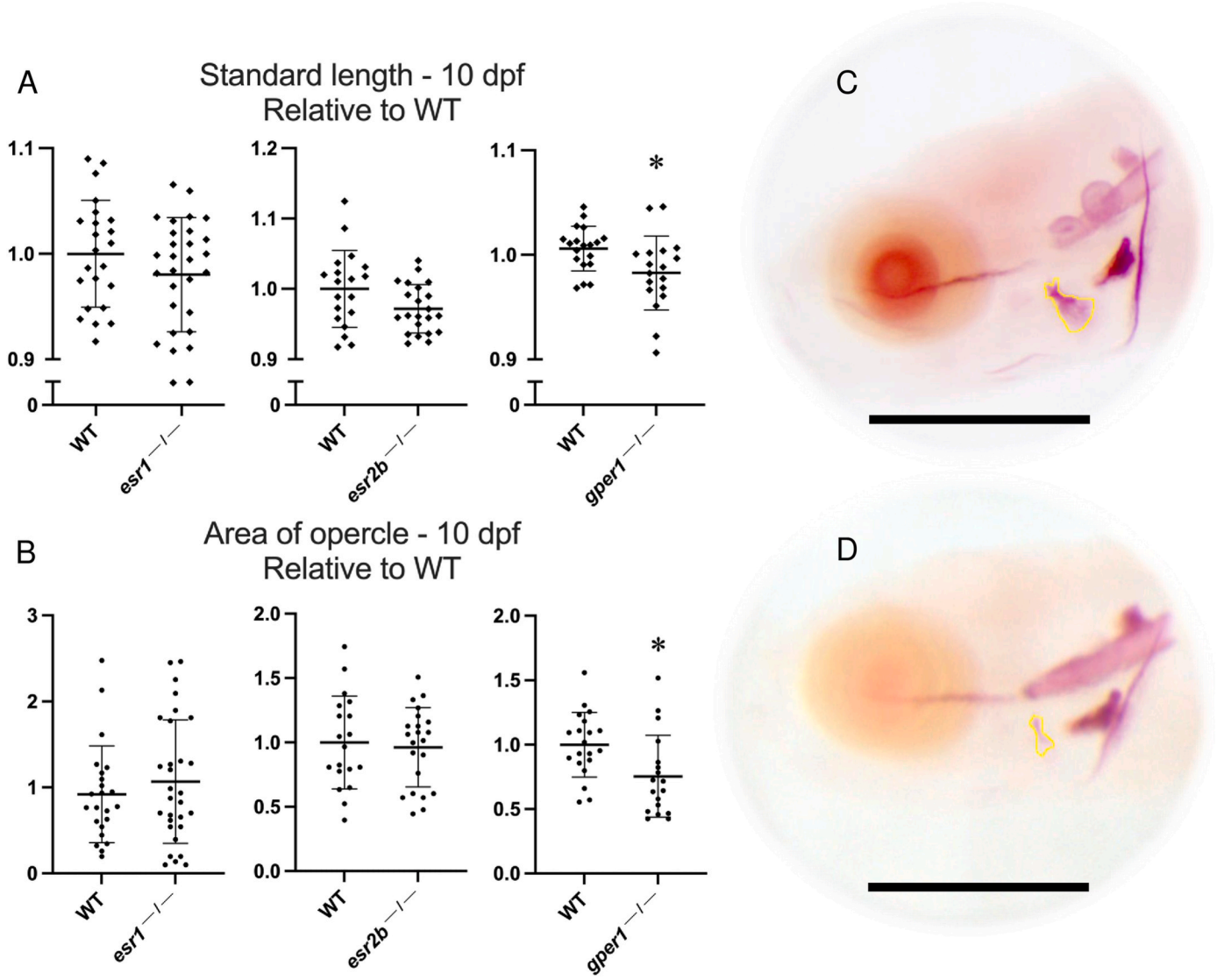

Fig. 3. A) Standard length of mutant larvae at 10 dpf relative to their WT siblings; B) opercle area measured for all mutant larvae relative to their WT siblings; C) Example of alizarin red stained WT larva and D) a *gper1*$^{-/-}$ mutant, the opercle area measured using FIJI is outlined in green, the size bar represents 500 μm. Unpaired Mann-Whitney *t*-test. Asterisks (*) indicate when significant differences were found for each mutant strain compared to its WT siblings, $p < 0.05$ (*). All values were normalized relative to the mean value of the corresponding WT siblings for each mutant strain. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

on sexual maturation, sex reversal and fertility or function of reproductive organs in older animals (about 3 months old) (Pang and Thomas, 2010; Lopez-Munoz et al., 2015; Lu et al., 2017; Chen et al., 2018, 2019; Bertotto et al., 2019; Wu et al., 2021). Impairment of the antiviral response in adults was observed in *esr2b* mutants (Lopez-Munoz et al., 2015), while defects in early development were observed in the lateral line in *esr2b* and in *esr1* morphants (Froehlicher et al., 2009; Gamba et al., 2010) and dopaminergic signaling was affected in *esr1* morphants (Bertotto et al., 2019). Growth retardation and brain defects were detected in *gper1* morphants (Shi et al., 2013), as well as impaired liver growth (Chaturantabut et al., 2019) and an increased embryonic heart rate (Romano et al., 2017).

4.2. Generation of mutants and streamlined genotyping

The generation of mutant zebrafish lines for any gene is greatly facilitated by using the CRISPR/Cas9 technology, which often results in obtaining several deficient lines, however working with mutant zebrafish can still be painstakingly labor-intensive. As a rule of thumb, the larger the number of individuals, the more reliable the statistical tests and their results will be. In situations like those described here, the absence of a clearly observable phenotype at early stages means that each larva obtained from heterozygous parents has to be individually genotyped before analyzing the results of a test. Here we used a previously reported method (D'Agostino et al., 2016) that allowed us to screen a large number of samples at once. Using the cost-effective and rapid screening technique of Melting Curve Analysis, which requires a real-time PCR instrument now widely available, we were able to run several batches of zebrafish larvae in skeletal staining and behavioral tests. We therefore chose, after initial selection and validation by sequencing, the mutant lines with the largest deletions/insertions to increase the difference in size relative to WT of the amplified genomic fragment, and to simplify the detection of mutant fish. Thanks to this, the fish were easily and efficiently genotyped so that we were able to obtain results faster than using more traditional methods such as Heteroduplex Mobility Shift assays or sequencing. Thus, we decided to evaluate different developmental endpoints in the different estrogen receptor mutants that we obtained. Interestingly, none of the mutants

Fig. 4. Actograms of the recorded parameters in 5 dpf zebrafish larvae of WT and *esr1*$^{-/-}$ fish. The graphs represent the mean Swimming Speed when active, Time Spent Active, or Distance Travelled of 72 individuals observed in10 s intervals during 20 min, with a switch from dark to light that occurred at Time 3000 s.

displayed an increased mortality or extensive larval deformities, except 70% of the population of *esr1* mutants, consistent with the previously published results where a grossly normal development was reported. How far this resistance to lethality is due to mutual compensation of the different homologous receptors remains to be seen, however we also did observe specific developmental defects in the mutants for individual receptors. All the results of this study are summarized in Table 1.

4.3. Neuromasts

The neuromasts are a sensory organ that is part of the lateral line system in teleost fish and is composed of sensory hair cells surrounded by supporting cells (Nicolson, 2005). It has been described previously that neuromast development and function can be disrupted upon exposure to some toxicants such as heavy metals (Hernandez et al., 2006), organophosphate flame retardants such as TCPP (Xia et al., 2021), bisphenol A (Lam et al., 2011), various pharmaceuticals (Ton and Parng, 2005) and even complex matrices such as stormwater runoff (Young et al., 2018).

Although these treatments do not specifically affect estrogen signaling, it was also shown that knock-down of the *esr2b* gene using antisense morpholino oligonucleotides reduced the number of neuromasts at 72 hpf in the posterior lateral line in the morphants (Froehlicher et al., 2009), while knock-down of *esr1* caused a delay in the migration of the posterior lateral line primordium between 35 and 41 hpf (Gamba et al., 2010). Here, none of the mutants in estrogen receptor genes were affected in the number or positioning of the neuromasts at 96 hpf. Discrepancies between phenotypes observed in morphants and in mutants targeting the same gene have been previously observed (Schulte-Merker and Stainier, 2014). Two opposing explanations have since been formulated: on one hand, morpholino antisense oligonucleotides are able to target mRNAs with a similar, but not identical sequence, leading to off-target inhibition of a different gene, which would not be phenocopied by the mutant (Joris et al., 2017); on the other hand, deficient mRNA produced in a mutant is able to trigger a compensatory mechanism, not induced by antisense morpholinos, leading to higher or ectopic expression of a closely related gene (Rossi et al., 2015; El-Brolosy et al., 2019). The existence of a family of estrogen receptors makes the second explanation likely, however a compensation would not be complete as we still observe some significant phenotypes in the mutants. Further experiments will be required to determine the precise role of ERs in neuromast development (if any).

4.4. Effects on skeletal development

Estrogen receptors have important roles in bone and cartilage development, for instance, chondrogenesis may be disrupted in the presence of certain estrogenic compounds such as 17β-estradiol (E2). The latter is a pleiotropic factor for chondrocytes and osteocytes, it plays an important role in regulating the proliferation, differentiation, and the synthesis of the cartilage and bone extracellular matrix (Fushimi et al., 2009; Lara-Castillo, 2021). Its presence in high concentrations causes

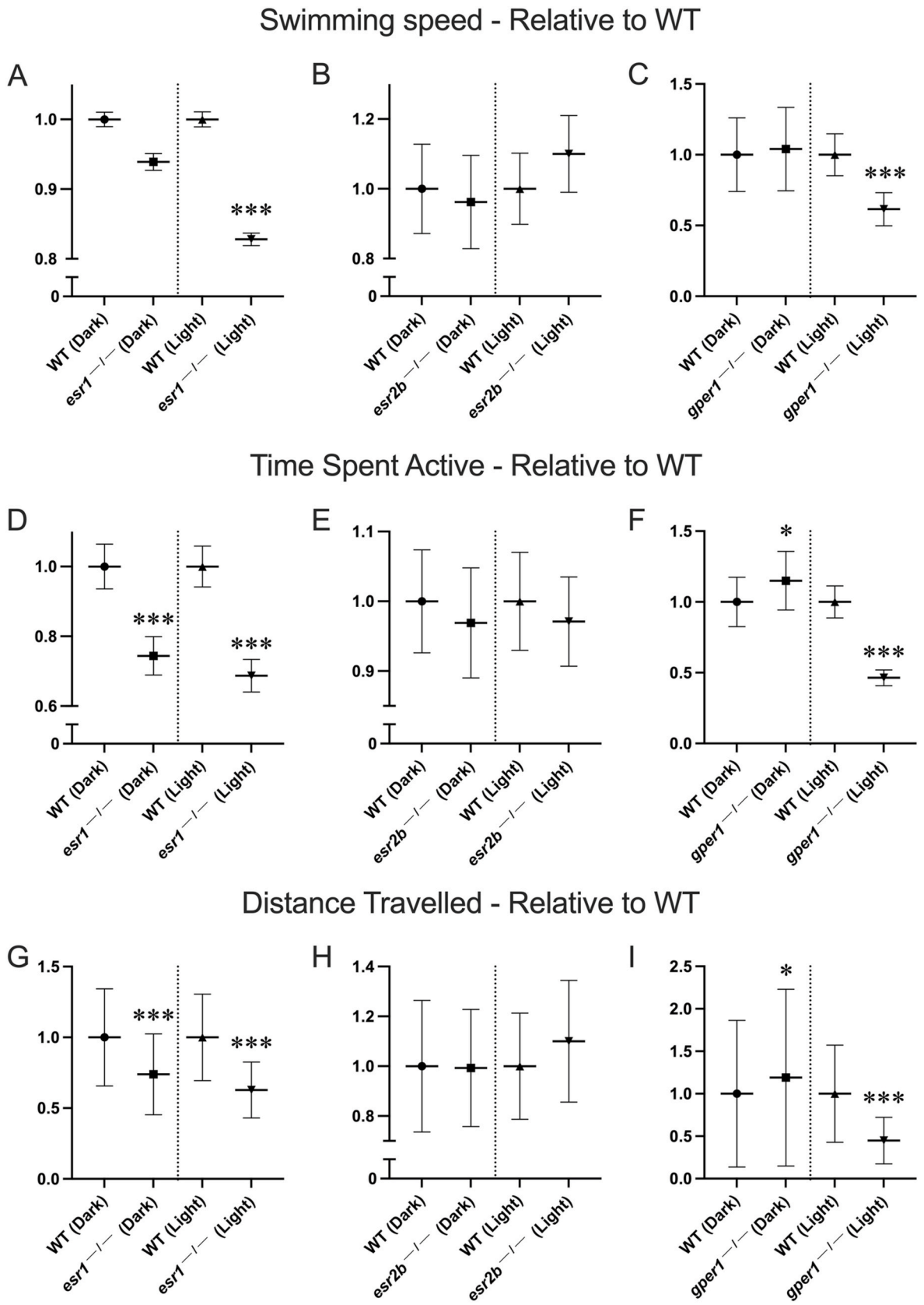

Fig. 5. Relative mean values of the three assessed parameters in each mutant strain during the motility test: Swimming speed (A–C), Time Spent Active (D–F) and Distance travelled (G–I). Asterisks (*) indicate when significant differences were found compared to WT of each mutant strain, $p < 0.05$ (*), <0.001 (***). All values were normalized relative to the mean value of the corresponding WT for each mutant strain.

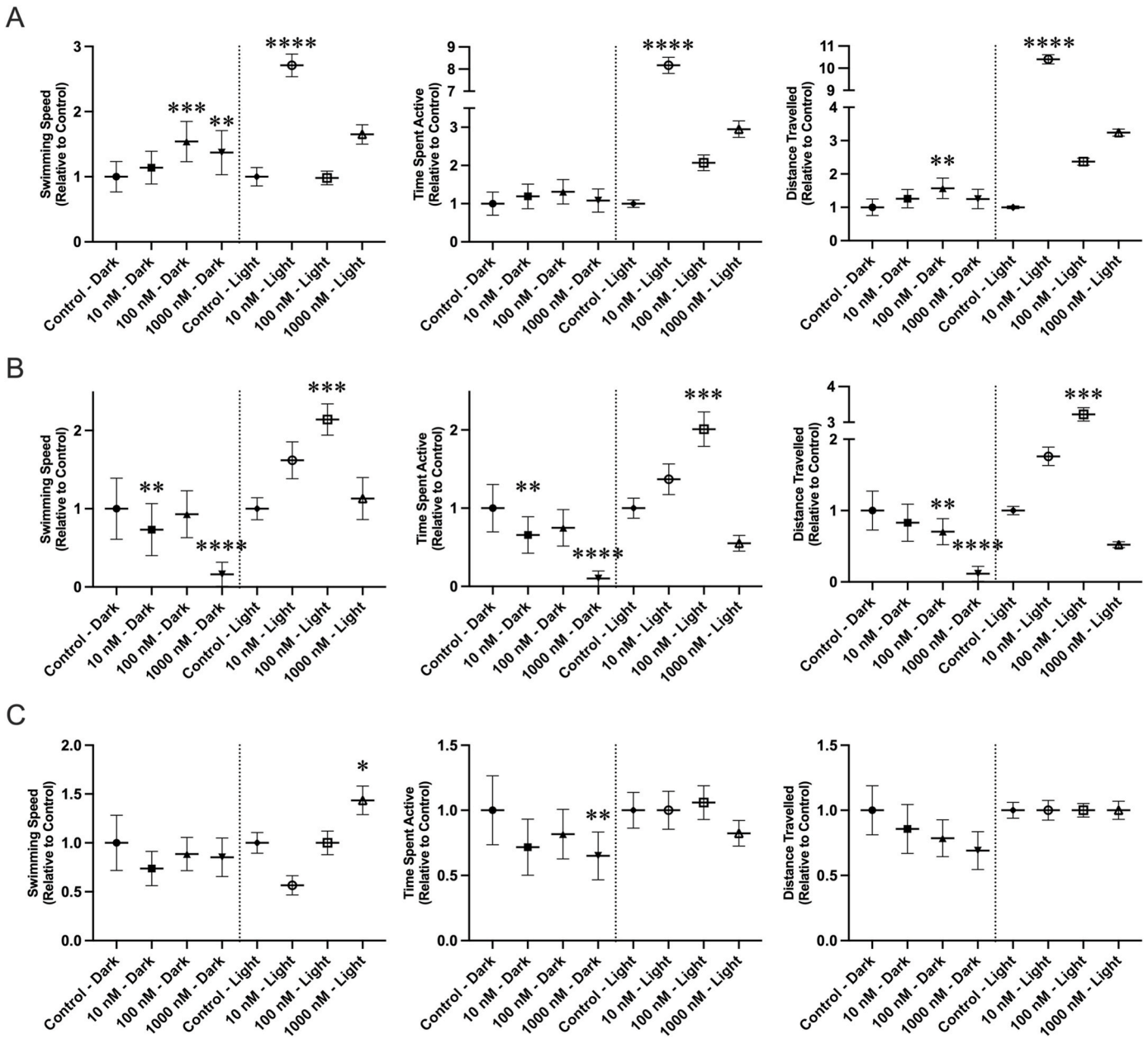

Fig. 6. Zebrafish larvae behavioral parameters recorded following 96 hpf chronic exposure to (by row) A) MPP (ERα antagonist); B) PHTPP (antagonist of ERβ) C) G15 (antagonist of GPER1). Asterisks (*) indicate when significant differences were found compared to control, $p < 0.05$ (*), <0.01(**), <0.001 (***), <0.0001 (****). All values were normalized relative to their respective control group.

craniofacial abnormalities in zebrafish larvae at ages 6 dpf to 40 dpf (Zare Mirakabad et al., 2019; Stewart et al., 2023), indicating that estrogenic signaling is involved in normal cartilage development. The underlying mechanism has been studied by whole transcriptome sequencing (He et al., 2018), revealing that genes coding for ECM proteins, such as collagens, were downregulated, while MAPK, TGF-β, and cell cycle signaling was upregulated following exposure to 17β-estradiol. Fish in those experiments had craniofacial deformities at 7 dpf. Other endocrine disruptors, such as glyphosate, were shown to affect craniofacial skeleton, bone, and behavior (Staal et al., 2018; Díaz-Martín et al., 2021).

We observed a dramatic effect of *esr1* mutation on craniofacial development, while significant effects were also observed in *gper1* and *esr2b* mutants by morphometric analyses at 5 dpf. These results clearly indicate that ERs are involved in regulation of the fine-tuned morphogenetic processes leading to an intact chondrocranium during these early stages, and that Esr1 is the main player. Interestingly, at later stages, we observe that only mutation of the *gper1* gene decreases mineralization of the opercle, an intramembranous bone that forms without a previous cartilage matrix. Taken together, these results indicate that different ERs play distinct roles in regulating the various pathways required for building an intact skeleton in zebrafish larvae.

4.5. Behavior of mutants

Previous studies revealed that exposure to E2 or 17α-ethinylestradiol (EE2) resulted in decreased "time spent active" and "total distance moved" in a dark-light behavioral test similar to the one used here (Fraser et al., 2017) or in longer durations of inactivity, exposing themselves in the center areas (Nasri et al., 2021). Here, we observed a decrease of activity in both phases in the *esr1* mutants, while the *gper1* mutant was less active in the light phase, but slightly more active in the

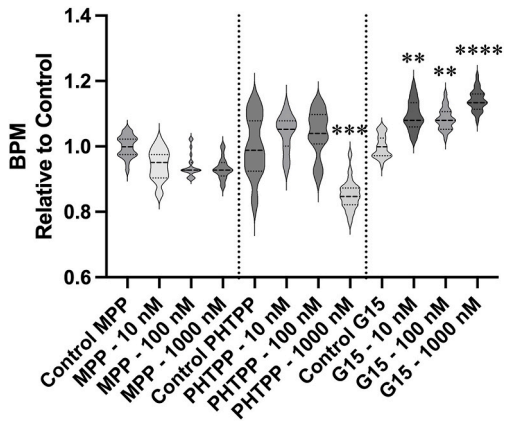

Fig. 7. Heart rate of zebrafish larvae measured at 4 dpf. Asterisks (*) indicate when significant differences were found compared to control, $p < 0.01$(**), <0.001 (***), <0.0001 (****). All values were normalized relative to control of each treatment.

Table 1
Recapitulation of the effects observed in ER mutant larvae and upon exposure to specific antagonists. The arrows indicate an up-(↑) or down-(↓)-regulation of the considered endpoint, (/) indicates no observed effect, n.d. means "not determined". The concentrations of antagonists resulting in a maximal effect are also given.

	esr1$^{-/-}$	*esr2b*$^{-/-}$	*gper1*$^{-/-}$	MPP	PHTPP	G15
Cartilage	↓↓↓↓	↓	↓↓	/	/	/
Bone	/	/	↓↓	n.d.	n.d.	n.d.
Behavior D	↓↓	/	↑	↑	↓↓	↓
Behavior L	↓↓↓	/	↓↓	↑↑↑ (10 nM)	↑↑ (100 nM)	↑
Heart rate	/	↑	/	/	↓	↑↑↑

dark phase (Table 1). Behavior is a complex endpoint that may respond to many factors; however, brain development may be an important one. EE2 was shown to decrease neuron proliferation and expression of tyrosine hydroxylase, a marker for dopaminergic neurons (Nasri et al., 2021), while *esr1* morphants have decreased dopamine levels and metabolism (Bertotto et al., 2019) and *gper1* morphants display decreased proliferation of brain cells and impaired development of peripheral neurons (Shi et al., 2013).

4.6. Heart rate in mutants

Previous studies showed that acute exposure for 1 h of zebrafish larvae to E2 led to an increased heart rate at 48 hpf, an effect that was attributed to the Gper1 receptor because ICI 182,780 (an antagonist of nuclear receptors but agonist for Gper1) was able to mimic this effect, acting most probably by increasing circulating levels of thyroid hormone T3 (Romano et al., 2017). The authors also show that larvae derived from a homozygous *gper1*$^{-/-}$ mother present lower maternal E2 levels, leading to decreased heart rates. The experiments reported here, using two heterozygous parents to generate *gper1* mutants, reveal that, at presumably normal levels of endogenous E2 in the larvae and presumably also some maternally deposited receptor, the absence of a functional *gper1* gene caused a trend to decreased heart rate, but did not lead to a significant effect.

4.7. Effects following antagonist exposure tests

In an attempt to correlate the results obtained with specific ER mutants with those observed using specific inhibitors, we chose to test three estrogen antagonists based on their alleged specificity against the human receptors. As the specificity and the affinities of these compounds for the zebrafish receptors are very controversial (Menuet et al., 2002; Notch and Mayer, 2011; Gorelick et al., 2014), we decided to use up to 1000 nM concentrations for these tests. Consistent with these previous observations, we did not find any correlation between the effects observed in the specific mutants and the supposed specificity of the antagonists. Taken together, our results show (confirm) that these antagonists against mammalian ERs are not effective in zebrafish, however we did observe different developmental defects caused by these chemicals that are presumably not endocrine mediated. Model endocrine modulators such as estradiol has, for instance, toxic effects that are not ER mediated, this was demonstrated using MCF7 breast cancer cell lines where high doses (above 10^{-6} M) of estradiol produced cytotoxic effects, but low doses (10^{-12} and 10^{-11} M) promoted cell proliferation (Welshons et al., 2003). Indeed, although orthologs are present across species, binding enzymes tend to react differently to the same compounds. That is the case of the aromatase inhibitor fadrozole, different fish species are indeed more or less sensitive (Doering et al., 2019).

An evaluation of selectivity for each specific receptor would need to use first cellular systems, as used for agonistic studies (Cosnefroy et al., 2012) where a zebrafish liver cell line was used to generate cells harboring only one of each of the receptors to test various chemicals for their ability to induce reporter gene transcription in this setting.

Another aspect that became apparent during these antagonist tests was the non-monotonic effects that we observed in the behavioral tests: e.g. the significantly increased movement parameters at 10 nM MPP or 100 nM PHTPP during the light phase were not observed at lower or higher concentrations (Fig. 6A and B). Such non-monotonic dose-response releationships have been previously described for EDC compounds (Vandenberg et al., 2012). For instance, using Bisphenol A (BPA) at environmentally relevant concentrations of 0.1 μM (low dose) BPA in male zebrafish would exert higher effects on the time spent active in comparison to a higher dose of 1 μM BPA (Weber et al., 2015). Mechanisms such as receptor selectivity, receptor down regulation, desensitization, and competition have been described to explain non-monotonic dose-responses. Using mutants deficient in potential compensatory genes could provide important insights into the processes involved.

5. Conclusions

Estrogen receptors play an important role in the overall development of organisms. Best known for their action in sexual maturation, our results emphasize their importance in early developmental stages. We present a reliable and cost-effective method, using qPCR, to tell apart mutants from WT fish, thus speeding up the findings and enabling medium throughput experiments, thus increasing statistical power of the results and their interpretation in a wider perspective. We have observed how the absence or deficiency of each one of the estrogen receptors (either nuclear or transmembrane) has consequences that can be measured using several biological tests. Although none of the individual ER mutations was lethal, we observed several effects at various frequencies and severity, however the underlying mechanisms remain at present unclear. Finally, we find that additional efforts are required to define receptor-specific antagonists for studying the effects of individual zebrafish estrogen receptors.

CRediT authorship contribution statement

Gustavo Guerrero-Limón: Conceptualization, Methodology, Software, Validation, Formal Analysis, Investigation, Data curation, Writing – Original Draft, Writing – Review & Editing, Visualization. **Marc Muller:** Conceptualization, Methodology, Validation, Formal Analysis, Investigation, Resources, Data curation, Writing – Review & Editing, Visualization, Supervision, Funding acquisition, Project administration.

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.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.chemosphere.2024.143100.

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