Title

The impact on an early exposure to 17α-ethynylestradiol on three-spined stickleback (*Gasterosteus aculeatus*) physiology in current and future climatic scenario

Authors:

Jimmy Devergne[1*], Arianna Servili[1], Sylvain Jodet[1], Titouan Brandicourt[1], Christophe Lebigre[2], Sophie Collet[1], Olivier Mouchel[1], Marie Lou Fleury[1], Sabine Roussel[1], Véronique Loizeau[1]

[1]Univ Brest, CNRS, IRD, Ifremer, UMR 6539, LEMAR, Plouzane, France

[2]UMR DECOD (Ecosystem Dynamics and Sustainability), IFREMER, INRAE, Institut Agro, F-29280, Plouzané, France

*Corresponding author

Abstract

Ocean warming and acidification are climate change related drivers that impact the physiology of marine organisms, affecting their coping ability to future environments. In addition, marine ecosystems are also facing pollution from an ever-growing diversity of chemical contaminants, including endocrine disruptors. A common example is the 17α-ethynylestradiol (EE2), which can affect the endocrine regulation of fish and hence potentially impacting their fitness. Thus, fish have to cope to multiple climatic and chemical stresses that can interact, influencing the overall impact on fish physiology. In this study, we investigated whether the impacts of early exposure to EE2 (15 ng.L^{-1}; one month during embryo-larval development) on survival, growth and reproductive axis of prepubertal sticklebacks is modulated by the RCP8.5 scenario (+3°C; -0.4 pH unit) after five months post-contamination. Fish used in this study were offspring of parents that had previously acclimated to identical climatic scenario, providing a baseline for understanding the potential interplay between endocrine disruptor exposure and projected climate change scenario. Our findings revealed that the survival of juveniles, when exposed to EE2 during early development, is reduced under Current but not RCP8.5 scenario. Furthermore, under RCP8.5-EE2, a significantly lower body length was observed. The study highlighted sex and tissue specific responses, regarding expression profiles of genes related with development and sexual maturation. Interestingly, the expression of ovarian aromatase (*cyp19a1a*) showed a significant interaction between RCP8.5 and EE2, suggesting a long-lasting estrogenic effect under RCP8.5 scenario. Additionally, skewed sex ratios and the presence of intersex individuals in both scenarios suggested feminization due to EE2, potentially disrupting sexual maturation and future reproduction. Hence, the early EE2 exposure had long-term physiological effects on sticklebacks, and these effects can be modulated by the climate scenario. This underscores the importance undertaking multiparametric and long-term studies to comprehensively understand the vulnerability on fish population in future environments.

"This work is licensed under a Creative Commons Attribution 4.0 International License"

Preprint not peer reviewed

This preprint research paper has not been peer reviewed. Electronic copy available at: https://ssrn.com/abstract=4795483

Key words: global change, xenoestrogens, multi-stress, survival, growth, sexual maturation.

Preprint not peer reviewed

"This work is licensed under a Creative Commons Attribution 4.0 International License"

This preprint research paper has not been peer reviewed. Electronic copy available at: https://ssrn.com/abstract=4795483

1. Introduction

The global change forcing associated with greenhouse gas emissions is altering the environmental conditions in which marine organisms live. The ocean has played a crucial role in regulating the Earth's climate by absorbing 93 % of the excess of heat, generated by the greenhouse gas since 1970s, but also by storing 28 % of the CO_2 emissions since the beginning of the industrial era in 1750 (Gattuso et al., 2015). However, these regulating role of the oceans could not prevent their overall warming and their increasing concentration of CO_2 has led to acidification (Gattuso et al., 2015). More specifically, sea surface ,temperature has risen by 0.7 °C over the last century, while ocean pH has fallen by 0.1 (Pörtner et al., 2014). Model projections from IPCC indicate that by 2100 the oceans will warm by 1.4 to 3.4 °C and experience additional acidification (Δ pH -0.2 to -0.4), depending on the region, habitat, and emission scenario (Kwiatkowski et al., 2020; Meinshausen et al., 2020). For instance, the RCP8.5 scenario, corresponding to the SSP3-7.0 and SSP5-8.5 more recent models, predict an average rise of temperature of 2.9 and 3.5 °C, respectively, while a decrease of pH of 0.3 and 0.4 unit, respectively, on the surface ocean water by 2100 (Kwiatkowski et al., 2020).

Such major changes in oceans' characteristics questions the ability of species that inhabit them to acclimate to future conditions. As these alterations could affect survival, growth rates, reproductive success, and ultimately impact fish recruitment dynamics (Baag and Mandal, 2022). For many marine species, physiological responses differ significantly from one life stage to another, with the larval stage being the most sensitive (Baag and Mandal, 2022; Pörtner et al., 2014; Przeslawski et al., 2015). To accurately quantify the impact of climate change, it is crucial to develop realistic approaches for assessing the impact environmental stressors on different life stages of an organism.

In addition to warming and acidification, marine biota is also affected by the chronic and widespread pollution of the marine environment by multiple contaminants resulting from human activities. Indeed, aquatic organisms are exposed to a growing number of chemical contaminants, including endocrine disruptors, which are likely to have an impact on their fitness (Alava et al., 2017). These include a group of molecules with estrogenic activity: the xenoestrogens. The 17α-ethynylestradiol (EE2), used in most human contraceptive pills, is one of the most widely reported xenoestrogenic endocrine disruptors in surface waters worldwide, with concentration varying between 0.2 and 73 $ng.L^{-1}$ (Bhandari et al., 2015). In France, in the Seine and its estuary, the concentrations in water range from 2 to 17 $ng.L^{-1}$ (Cargouet et al., 2004). Despite its short half-life in rivers (up to 6 days), the consequent and continuous discharge in the wastewater leads to a continuous presence of this contaminants in surface waters (Adeel et al., 2017). The EE2 is known to induce tissue damage, feminisation and reproductive dysfunction (Jackson et al., 2019), behavioural changes (Saaristo et al., 2019), disrupted steroidogenesis (Sridevi et al., 2015), and poorer spawning quality (Cosme et al., 2015) in teleost at environmental concentrations.

"This work is licensed under a Creative Commons Attribution 4.0 International License"

This preprint research paper has not been peer reviewed. Electronic copy available at: https://ssrn.com/abstract=4795483

Despite the recommendations to evaluate the impact of multiple environmental stressors on the whole life cycle of fish (Alava et al., 2017; Kibria et al., 2021), few studies have evaluated these effects. In this context, the general objective of this study was to contribute to filling these gaps, by focusing on the three-spined stickleback (*Gasterosteous aculeatus*), as model species. This species is an amphihaline teleost originating from marine environment that has been widely spread over the northern hemisphere. Its small size and short life cycle (sexual maturity at one year) combined with its ease of rearing in lab facilities has led an important use by the scientific community, with extensive knowledge on its physiology and ecology (Blaker et al., 2022). The Organisation for Economic Co-operation and Development (OECD) had also recognized this species as a good model for endocrine disrupting studies (OECD, 2011). Moreover, the full sequencing of its genome gives a variety of tools to explore the physiological responses at a molecular level (Jones et al., 2012). All these characteristics have made stickleback a good model for long-term studies on the impact of anthropogenic environmental variations on teleost physiology.

Several studies have reported the effect of increase temperature on key physiological functions of the stickleback: under warmer condition growth in size and mass is lower (Hani et al., 2018), as well as fecundity and egg diameter, while egg development is accelerated (Kim et al., 2017b; Shama, 2015, 2017). Moreover, higher temperatures are also shown to up-regulate metabolic and redox processes as well as the expression of several genes involved in growth and reproduction (Kim et al., 2017a). Studies on the effects of acidification on this species are still scarce and a few managed to revealed an impact of acidification on the reproductive axis, resulting in higher fecundity under acidification (Schade et al., 2014), but no effects on the egg survival and size (Glippa et al., 2017). As a sentinel species for endocrine disruption, the stickleback has been extensively studied facing estrogenic contamination, particularly EE2. EE2 contamination at environmental concentration increased this species' growth at early life stages as well as activity level (Bell, 2004), but did not impact the growth in adults (Björkblom et al., 2009). Moreover, a feminisation was observed through the testis when fish were exposed to EE2 during the two first weeks of life (Hahlbeck et al., 2004). As presented above, warming, acidification and EE2 contamination at environmental concentrations are all factors that, taken individually, are likely to impact the physiological traits of the stickleback and thus increase the vulnerability of its population. However, to our knowledge, the combination of these stress factors remains unexplored.

In this context, we investigated whether the effects of an early exposure to EE2 on the survival, the growth and the reproductive axis of stickleback are modulated by the RCP8.5 climatic scenario (Δ pH -0.4; Δ T°C = 3°C during six months).Individuals were issued of parents that had been acclimated to the same climatic conditions (Current and RCP8.5) since their juvenile stage (Devergne et al., 2023). The EE2 concentration applied at embryo-larval stage (15 $ng.L^{-1}$ during 1 month) corresponded to estrogenic environmental contamination observed in France (Cargouet et al., 2004).

"This work is licensed under a Creative Commons Attribution 4.0 International License"

This preprint research paper has not been peer reviewed. Electronic copy available at: https://ssrn.com/abstract=4795483

Using an integrative approach based on physiological, biochemical, and molecular analysis, this study aimed to assess the effects of this multi-stress on the growth and the development of the reproductive axis, to improve our knowledge of the vulnerability of fish species to predicted future environments. We hypothesised that the interaction between simulated climate change (RCP8.5) and exposure to EE2 at environmental concentrations would lead to more pronounced physiological alterations than those observed with each stressor taken individually.

Preprint not peer reviewed

"This work is licensed under a Creative Commons Attribution 4.0 International License"

This preprint research paper has not been peer reviewed. Electronic copy available at: https://ssrn.com/abstract=4795483

2. Materiel and methods

This experimental study adhered to the regulations outlined in French national laws and EU Directive 2010/63/EU, as well as the European Commission recommendation 2007/526/EC concerning the care of animals used for scientific purposes. The experiment was performed in fish experimental facilities of the Ecophysiology and Life History Traits of Marine Organisms Unit (PHYTNESS) of Ifremer (agreement number B29-212-05, Brittany, France). The experimental design was previously approved by the French Ethics Committee for animal testing (CEFEA: Comité d'Éthique Finistérien en Expérimentation Animale, registering code C2EA-74) and the French Ministère de l'Enseignement Supérieur de la Recherche et de l'Innovation (Authorization APAFIS #27510, permit number 2020100911422298_v3). Fish were handled by accredited personnel.

2.1. Fish husbandry and scenario set up

The F1 population used for this experiment was generated through *in vitro* fertilization (IVF) in June 2021, with gametes originating from a F0 population used in a previous experiment (Devergne et al., 2023). Briefly, F0 fish were acclimated to "Current" scenario, corresponding to the current seasonal variations of temperatures and pH of the "Rade de Brest" during 7 months, and to "RCP8.5" scenario, with a warming of 3°C and an acidification of 0,4 pH units as predicted by 2100 (Pörtner et al., 2014). The eggs of 9 Current and 14 RCP8.5 females were fertilised with the sperm solution of 2 males of the same scenario, the IVF procedure was detailed in Devergne et al. (2023). The obtained offspring (F1) were reared, into hatching trays inside 60 L tanks, under the same climatic scenario as their parents (n = 4 tanks per scenario). During the embryo-larval period, half of the tanks were exposed to EE2 contamination (Current-EE2, RCP8.5-EE2, n = 2 tanks per scenario), while the two others were left undisturbed (Current, RCP8.5, n = 2 tanks per scenario). Fish from all experimental groups were fed *ad libitum* with *nauplii artemia* during the whole larval stage and 1 month after reaching the three-spine stage (two months). Afterwards, juveniles were fed with frozen sliced artemia and bloodworms (proportion of 1:4, Antinea, France), enriched twice a week with vitamins (Vitaligo, Geosane, France). At 70 days post-fertilisation (dpf), population densities were adjusted to ~20 fish per tank when fish were transferred from hatching trays to the 60 L tanks (n = 2 tanks per experimental groups from 0 dpf to 70 dpf; afterwards n = 2 tanks for Current-EE2 and RCP8.5, and n = 3 tanks for Current and RCP8.5-EE2 experimental groups, from 71 dpf to 178 dpf). Tanks were filled with natural seawater pumped at 500 m from the coastline from a depth of 20 m, filtered as described previously (Devergne et al., 2023). A flow rate of 30 $L.h^{-1}$ was set for each tank with an artificial light regime following the natural seasonal variations of the photoperiod in the Bay of Brest (42 W halogen lamp, 55-60 lux). For the RCP8.5 climate scenario (RCP8.5 and RCP8.5-EE2) seawater was heated via a tungsten plate heat exchanger (Vicarb, France) and released into two 6 L header tanks of where CO_2 was diffused (Figure 1). For pH regulation, a degassing column was placed within each header tanks to have independent diffusion of carbon dioxide (CO_2). A WTW 3110 pH meter (Xylem Analytics Germany, Weilheim, Germany; with

"This work is licensed under a Creative Commons Attribution 4.0 International License"

This preprint research paper has not been peer reviewed. Electronic copy available at: https://ssrn.com/abstract=4795483

electrode: WTW Sentix 41, NBS scale) was used to measure temperature and pH every day (Figure 2). It was daily calibrated with NBS certified WTW technical buffers pH 4.01 and pH 7.00 (Xylem Analytics Germany, Weilheim, Germany). Total alkalinity was measured twice a month to estimate pCO_2 using the CO2sys macro (Lewis and Wallace, 2012) with the constants from Mehrbach et al. (1973) refit by Dickson and Millero (1987). The same pCO_2 values were used in the macro afterwards to calculate the total scale pH (Table 1) as described in Devergne et al. (2023). Weekly measures of salinity and oxygen were performed with a WTW LF 340 salinometer (Xylem Analytics Germany, Weilheim, Germany; with electrode: WTW Tetracon® 325/C, ‰ scale) and WTW Oxi 340 oximeter (Xylem Analytics Germany, Weilheim, Germany; with electrode: WTW CellOx 325, % scale).

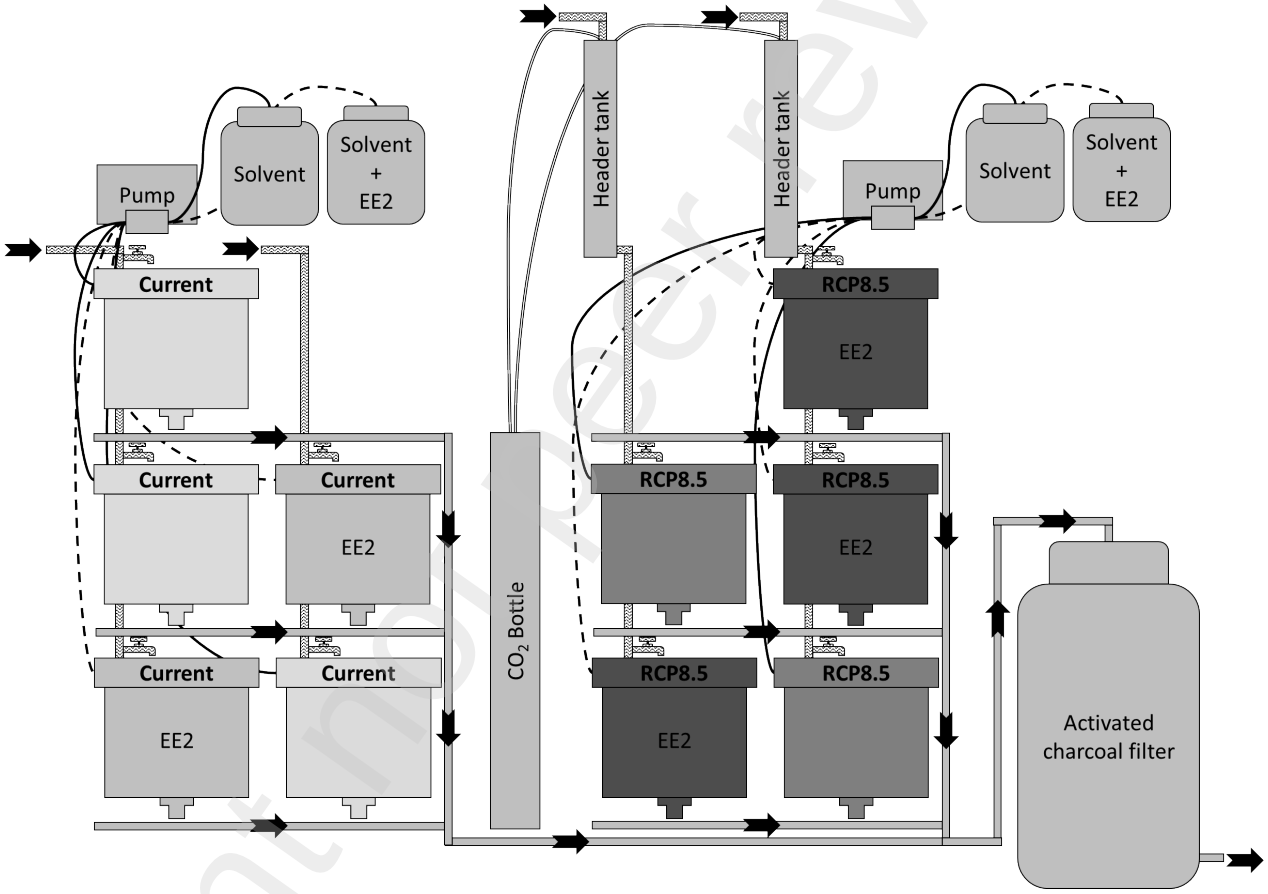


Figure 1: Experimental design at 70 days post-fertilization (dpf), with Current (light grey), Current-EE2 (grey), RCP8.5 (medium grey), and RCP8.5-EE2 (dark grey) experimental groups. In the RCP8.5 scenario, warm water was passed through the header tanks and was acidified by CO_2 diffusion, ensuring homogeneous mixing trough the water flow. EE2 and its solvent were added using peristaltic pumps throughout the embryo-larval developmental stage. Black arrows represent water flow.

2.2. Xenoestrogen exposure

Contamination was done during the first 32 days (i.e. at the embryo-larvae stage), starting from the fertilisation to the appearance of their third dorsal spine (Swarup, 1958). The 17α-ethynylestradiol (EE2, VETRANAL®, Sigma-Aldrich, 46263-250MG, USA) was used at environmental concentrations (0.05

"This work is licensed under a Creative Commons Attribution 4.0 International License"

Preprint not peer reviewed

This preprint research paper has not been peer reviewed. Electronic copy available at: https://ssrn.com/abstract=4795483

nM – 15 ng.L^{-1}). The solvent, dimethyl sulfoxide (DMSO, anhydrous ≥ 99.9%, Sigma-Aldrich, 276855-2L) was used at a final concentration < 0.001 % in all the tanks. This concentration is 10 times lower than the maximum solvent concentration for aquatic tests guidelines, of 0.1 mL.L^{-1} (0.01%), specified by the Organization of Economic Co-operation and Development (OECD, 2019, 2013). The contamination with EE2 and DMSO (15 ng.L^{-1}, current-EE2, RCP8.5-EE2 groups) or DMSO alone (current, RCP8.5 groups) was done by flow through with multichannel peristaltic pumps (Pump models 7520-47 and 7554-95, head model 7535-08, MasterFlexe®, Cole-Parmer Instrument Company, USA) pouring into the respective tanks of the Current and RCP8.5 groups. Contaminated seawater flow was treated with activated charcoal filter (DISPOSORB®, MINIDISPOSORB™, Chemviron, France) before being discharged into the sewer. The contamination levels in seawater from each tank and at the exit of the decontamination columns were verified, in triplicate, once during the contamination phase and post-contamination phase. For measurement of seawater EE2 concentration, a solid phase extraction using Sep-Pak® C18 Plus Short Cartridges (WatersTM, US) was collected. Then EE2 concentration was determined by ELISA kit (Ecologiena®, Tokiwa chemical industries CO.LTD, Japan), according to the protocol described by Wernicke von Siebenthal et al. (2018), and measured using a Multiskan™ GO Microplate Spectrophotometer (Thermo Fisher Scientific, Finland), at the 450 nm wavelength (Table 1).

"This work is licensed under a Creative Commons Attribution 4.0 International License"

Preprint not peer reviewed

This preprint research paper has not been peer reviewed. Electronic copy available at: https://ssrn.com/abstract=4795483

Table 1: Average physico-chemical parameters of the seawater recorded monthly during the experimental period (mean ± S.D.). TA: total alkalinity, pCO_2: partial pressure of CO_2, EE2: ethinylestradiol.

Experimental groups	*Salinity (‰)*	*O_2 (%)*	*pH total*	*TA (µmol.kg$_{sw}^{-1}$)*	*pCO_2 (µatm)*	*EE2 concentration (ng.L^{-1})*	
						Min	*Max*
Current	35.11 ± 0.90	97.41 ± 2.89	7.92 ± 0.02	2389.16 ± 49.51	579.28 ± 27.13	-	-
Current – EE2	35.10 ± 1.20	98.89 ± 0.77	7.92 ± 0.03	2414.32 ± 213.74	595.20 ± 67.96	22.00	24.31
RCP8.5	35.14 ± 1.01	97.86 ± 1.86	7.49 ± 0.07	2415.60 ± 118.72	1786.19 ± 311.81	-	-
RCP8.5 – EE2	35.27 ± 1.09	98.70 ± 0.80	7.52 ± 0.08	2365.36 ± 84.79	1599.48 ± 272.08	11.72	24.70

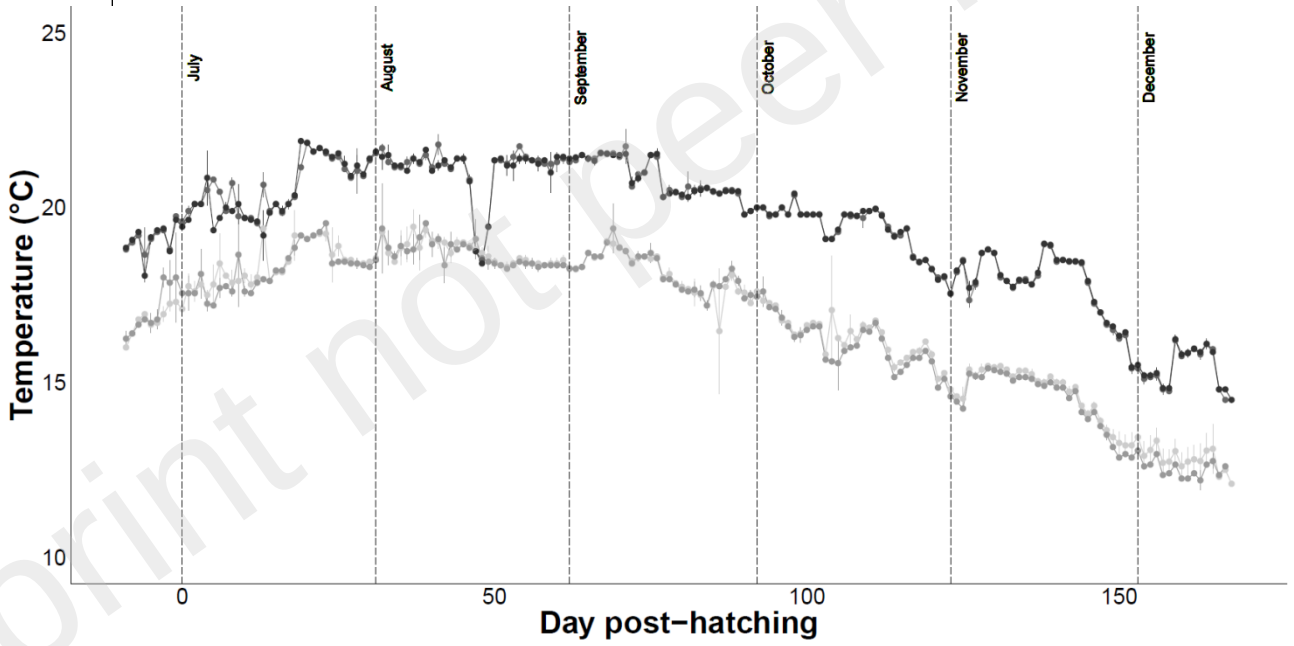


Figure 2: Seawater mean temperature (°C), starting from Current 0 days post-hatching (dph), in the Current (light grey), Current-EE2 (medium grey), RCP8.5 (grey), and RCP8.5-EE2 (dark grey) experimental groups throughout the study (n = 2-3 tanks per experimental groups, mean ± S.D.).

"This work is licensed under a Creative Commons Attribution 4.0 International License"

Preprint not peer reviewed

This preprint research paper has not been peer reviewed. Electronic copy available at: https://ssrn.com/abstract=4795483

1.1. Egg quality:

At 3 dpf, the fertilised eggs (Current: *n* =14, Current-EE2: *n* =8, RCP8.5: *n* =10, RCP8.5-EE2: *n* =8) were photographed (NIKON, D7200, Japan) with a binocular magnifier (Discovery.V8 SteREO, ZEISS, Germany). Their diameter and the perivitelline index were measured individually using ImageJ software (Version 1.53e, Java 1.8.0_172, Schneider et al., 2012) as described in Devergne et al. (2023).

1.2. Biometrics and individual sampling

When individuals reached the juvenile stage (30 dpf), the whole population of each tank was transferred gently into water trays every two weeks and photographed (NIKON, D7200, Japan) before being returned into their respective tanks. The individual standard length was measured with the software ImageJ (Version 1.53e, Java 1.8.0_172; Schneider et al., 2012). The sampling done to evaluate physiological indexes, sex ratio, histological analysis and gene expression profiles were carried out after six months of exposure to the RCP8.5 or Current scenario in prepubertal fish. Prior to sampling, the fish were fasted for 24h, captured, and transferred to an anaesthesia container with 0.125 g·L−1 MS222. Subsequently, they were euthanized with a lethal concentration of 0.250 g·L−1 MS222. Sex ratio, standard length, body mass and organo-somatic indexes were estimated (Sartorius, MC BA 1000, Germany) as described in Devergne et al. (2023): Fulton index (K) = ((*Mass of the fish*)/(*Size of the fish*)^3) × 100; Gonado-somatic index (GSI)= ((*Mass of the gonads*)/(*Mass of the whole body*)) × 100; Hepato-somatic index (HSI)= ((*Mass of the liver*)/(*Mass of the whole body*)) × 100 (*n* in Supplementary Table 6). Fish survival was monitored throughout the experiment at the end of the embryo-larval and the juvenile stages.

1.3. Histological analysis of the gonads

To test the potential presence of intersex individuals, the left gonad was sampled (*n* in Supplementary Table 6) and fixed in 4 % paraformaldehyde (PFA) for 24 h at 4 °C. The tissues were dehydrated, impregnated, embedded, sliced and stained with trichrome Prenant-Gabe method as explained in Devergne et al. (2023). The staining labelled the nucleus in purplish blue, the cytoplasm in red and the conjunctive tissue in green. Then the mounted slides were analysed and photographed (EOS 700D, Canon, Japon) under light microscope (AXIO observer Z1, ZEISS, Germany).

1.4. RNA extraction and purification

The brain and 1 gonad (*n* in Supplementary Table 7) of fish were quickly removed, and the median region containing the hypothalamus was dissected. Tissues were stored in a RNA stabilization reagent (RNAlater, Qiagen, Hilden, Germany) at 4 °C for 24 h and then placed at -20 °C until extraction. The total RNA extraction and purification of median brain was performed with the Nucleospin® RNAXS kit (Macherey Nagel, Germany) according the protocol of the supplier (Version January 2020/Rev.10) as described previously (Devergne et al., 2023). For the gonads, the RNA extraction was performed under chemical and mechanical lysis with Extract-All® (Giagen, Germany) and Polytron® grinder (PT-MR 2100, Kinematic AG, Switzerland). Isolation of nucleic acids was done by phase separation with

"This work is licensed under a Creative Commons Attribution 4.0 International License"

Preprint not peer reviewed

This preprint research paper has not been peer reviewed. Electronic copy available at: https://ssrn.com/abstract=4795483

chloroform, then isopropanol to create a binding condition for purification. The isopropanol-RNA solution was purified with the Nucleospin® RNA kit (Macherey Nagel, Deutschland) according to the protocol of the supplier (Version January 2020/Rev.10). The concentration and purity of the total RNA extracted from brains and gonads were assessed with NanodropTM 2000 (Thermo Scientific Inc., Waltham, MA, USA) and the integrity of the RNA by using an Agilent Tapstation 4150 (Agilent Technologies Inc., Santa Clara, CA, USA). All samples analysed showed an integrity number (RIN) mean of 9.6 for the brain and 8.5 for the gonad. Samples were stored at - 80 °C until use for reverse-transcription quantitative PCR (RT-qPCR) analysis.

1.5. RT-qPCR analysis

The reverse transcription (RT) was carried out with the iScript™ cDNA Synthesis kit (Bio-Rad Laboratories Inc., Hercules, CA, USA) and the ThermalCycler T100™ (Bio-Rad Laboratories Inc.) on all RNA samples with positive and negative (without enzyme) reaction according to the manufacturer's protocol. Then, cDNA was stored at -20 °C until qPCR. The qPCR performed on the brain targeted genes associated to the neuroendocrine regulation of the sexual maturation: Gonadotropin Releasing Hormone 2 and 3 (*GnRH2*, *GnRH3*), brain aromatase (*CYP19A1B*), Kisspeptin 2 (*KISS2*), Gonadotropin-Inhibitory Hormone (*GNIH*). Two other genes associated with the stress axis and development were studied in the brain: respectively Corticotropin-releasing hormone (*CRHβ*), and the thyrotropin-releasing hormone (*TRH*). For the gonads, genes related to the estrogenic regulation where assessed: Gonad aromatase (*CYP19A1A*), Estrogen receptor 1 (*ESR1*), Estrogen receptor 2a (*ESR2A*), Estrogen receptor 2b (*ESR2B*). The primers were specially designed for this project (Table 2) with the primer 3Plus software (Untergasser et al., 2007).

"This work is licensed under a Creative Commons Attribution 4.0 International License"

Preprint not peer reviewed

This preprint research paper has not been peer reviewed. Electronic copy available at: https://ssrn.com/abstract=4795483

248 Table 2: Designed primers used in the qPCR analysis in the median brains and gonads

Targets	*Genes*	*Forward primer*	*Reverse primer*	*GenBank accession number*
Brain	***RPL8***	5'GTGAAGGACATCATCCACGA3'	5'CTCCTCCACACAGCAGATGA3'	ENSGACT00000002668.1
	RPL13A	5'CGCCCTACGACAAGAGGAAG3'	5'CTCTGCCACCTTGGTCAACT3'	ENSGACT00000012382.1
	GNRH2	5'TGTGTTGGAGCTCAGCTGTC3'	5'CTCTGGCTAAGGCATCCAAA3'	ENSGACT00000011943.1
	GNRH3	5'GTTGGTGGTCCAGGTCACTC3'	5'CTCTCTTGGGTCTGGGCACT3'	ENSGACT00000012668.1
	CYP19A1B	5'ATACCCGGTCCGTGCTTCT3'	5'CCCGAATCTGGCTGTGTAGT3'	ENSGACT00000007929.1
	KISS2	5'GGGTCAGTTCTTTCTACGCTCA3'	5'AATGTAGCGTTTCCCAAAGC3'	KT202354.1
	GNIH	5'AGAGCTTCCGCATACTCTCG3'	5'ATGTTGTGGGTCGAACTGGT3'	KT202315.1
	CRHβ	5'ACCGTGATTCTGCTAGTTGC3'	5'CATGGATGAAGATGGGAAAG3'	XM_040167877.1
	TRH	5'GAAGACGGCGGAGACGAG3'	5'CGTCCAAGTATGGTTCATCC3'	ENSGACG00000002980
Gonad	***CYP19A1A***	5'CCTACTACACCAAAGCCCTGA3'	5'TAGCTGAGAAAGACCCGCAT3'	XM_040167072.1
	ESR1	5'GGCATGATGAAAGGAGGTGT3'	5'TTGTCGTTGTCGTTGTCGTC3'	NM_001267672.1
	ESR2A	5'TTCAAGAGGAGCATCCAAGG3'	5'TCCAGTCAAACGGCTTACTC3'	XM_040160794.1
	ESR2B	5'CAACCAATCAATGCACCATC3'	5'TGCTTCACCCGTACTACTT3'	LC006094.1
	FSHR	5'CCTGACACACATCCACCAAG3'	5'TATGCCGAGTGGTCTTTACC3'	XM_040176746.1
	LHR	5'CGTTTCTGAATGCCACCTCT3'	5'GACTTTATCTCGGGTGACGC3'	XM_040179510.1

249

250

Preprint not peer reviewed

"This work is licensed under a Creative Commons Attribution 4.0 International License"

This preprint research paper has not been peer reviewed. Electronic copy available at: https://ssrn.com/abstract=4795483

The qPCRs were performed with the CFX96 Touch Real-Time PCR Detection system (Bio-Rad Laboratories Inc.) and SsoAdvanced Universal SYBR® Green Supermix (Bio-Rad Laboratories Inc.) as explained previously (Devergne et al., 2023). For each sample the reaction was carried out in duplicate with 1/20 dilution for brain cDNA and 1/10 for gonads cDNA. The high-resolution melting curves produced by the protocol were used to verify the amplification of a single product in each reaction. The Gene Expression Module of the CFX Manager program (Bio-Rad Laboratories Inc.) was used to automatically determine the related quantification cycle (Cq) value for each reaction. For the brain, Ribosomal Protein L8 (*RPL8*), Ribosomal Protein L13A (*RPL13A*), and for the gonad, Follicle-stimulating hormone receptor (*FSHR*), Luteinizing hormone receptor (*LHR*), were used as reference genes in the ΔΔCt method to normalized the relative quantity of transcripts. For housekeeping genes, no significant variations in Cq values were found between experimental groups.

1.6. Statistical analysis

The data analysis and graphical representation were performed using the R software (R Core Team, 2018). The significance threshold was set to p-values < 0.05 (see sup. Table 1).

To test the effect of climatic scenario and EE2 contamination on egg quality a two-way ANOVA was run for perivitelline index data since normality of data (Shapiro–Wilk test) and the homogeneity of variance (Bartlett test) were met. For egg diameter data, neither the normality nor the homogeneity of the variance (Levene's test) were met. Therefore, we ran Welch's heteroscedastic F test, followed by a Games-Howell post-hoc test.

To determine the effects of the multi-stress on fish survival and sex ratio a non-parametric Pearson's chi-squared test was performed on the four experimental treatments (Current, Current-EE2, RCP8.5, RCP8.5-EE2) followed by pairwise Pearson's chi-squared test with Holm correction.

To evaluate the effect of the climatic scenario and EE2 contamination on fish size throughout the 6 months of the experiment, a linear mixed effects model was performed using the package "nlme"(Pinheiro and Bates, 2000) with the "climatic scenario" (Current vs RCP8.5), the "xenoestrogen contamination" (non-EE2 vs EE2), and the "time" used as fixed factors and the rearing tank identification code as a random factor. To take into account the non-independence of consecutive measurements time effect, the model considered an autoregressive process of order 1 correlation structure (AR1). This test was followed by the emmeans function for the post-hoc test with a Tukey correction.

Because males and females might respond differently to the contamination, we decided to analyse separately data for males and females for the 6-month measurements. For each sex, the standard length, body mass, physiological indexes and genes expression profiles were analysed using a linear mixed-model using the package "lmer" (Bates et al., 2015) with the climatic scenario (Current *vs* RCP8.5), the xenoestrogen contamination (non-EE2 *vs* EE2) as fixed factors and the tanks as a random

"This work is licensed under a Creative Commons Attribution 4.0 International License"

Preprint not peer reviewed

This preprint research paper has not been peer reviewed. Electronic copy available at: https://ssrn.com/abstract=4795483

factor, after having checked graphically residuals normality and homogeneity of variance. The Anova function was applied to obtain an Analysis of Deviance Table for the fixed factors (Type II Wald chi-square tests). For post-hoc test emmeans function with the Tukey correction was used (Lenth, 2016). To find the most parsimonious model, stepwise backward selections were carried out in all analysis.

2. Results

2.1. Egg quality:

A significant effect of the multi-stress was observed ($W_{3, 16.29} = 12.2$, $p < 0.01$, Supplementary Table 1): the egg diameter was lower under the RCP8.5-EE2 group compared to the Current group ($t = 6.19$, $p < 0.01$; Figure 3A). Moreover, eggs exposed to the RCP8.5 climatic scenario had a higher perivitelline index compared to eggs exposed to the Current scenario ($F_{1, 36} = 4.60$, $p = 0.04$, Figure 3B, Supplementary Table 1). There was no significant effect of contamination on its own or in interaction with the climatic scenario (respectively $F_{1, 36} = 0.77$, $p = 0.39$; $F_{1, 36} = 0.01$, $p = 0.99$, Figure 3B, Supplementary Table 1).

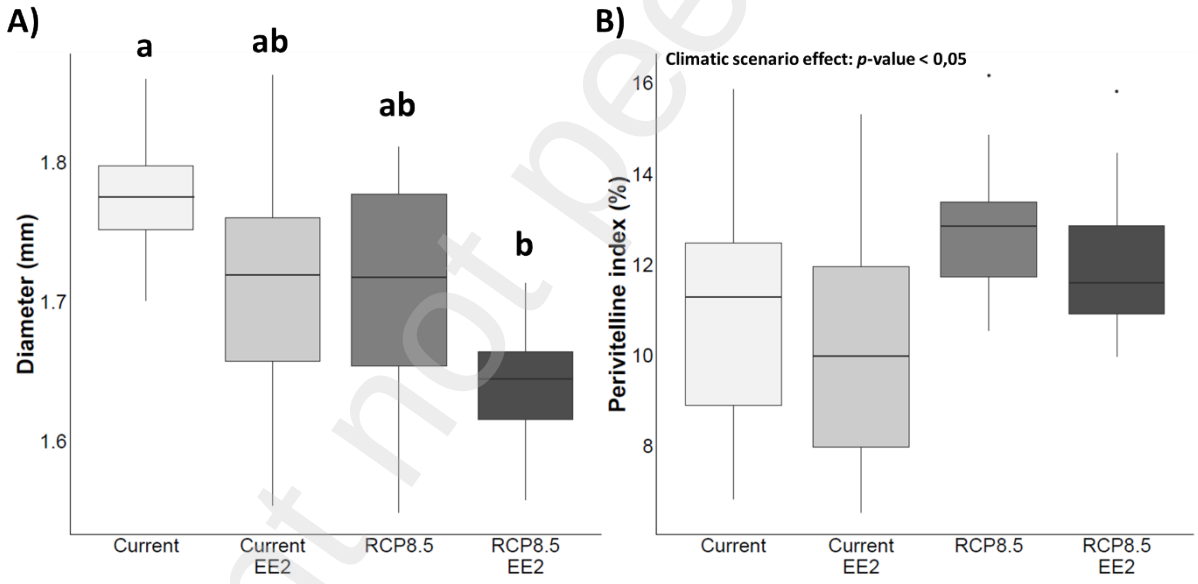


Figure 3: Boxplots representing the diameter (A) and the perivitelline index (B) of eggs after 3 days post-fertilisation under Current (light grey), Current-EE2 (grey), RCP8.5 (medium grey), and RCP8.5-EE2 (dark grey) experimental groups (n = 14 Current, 8 Current-EE2, 10 RCP8.5, 8 RCP8.5-EE2). The 10th and 90th percentiles are represented by the whiskers, the 25th and 75th percentiles by the boxes, the median values by horizontal lines and outliers by points. The letters represent significant differences (Figure 3A; Figure 3B).

"This work is licensed under a Creative Commons Attribution 4.0 International License"

Preprint not peer reviewed

This preprint research paper has not been peer reviewed. Electronic copy available at: https://ssrn.com/abstract=4795483

2.2. Survival and development

The embryo-larval survival was affected across the experimental groups ($\chi^2 = 13.49$, $p < 0.01$; Table 3 and supplementary Table 1) as individuals' survival declined significantly in the RCP8.5 experimental group compared to the Current and Current-EE2 groups (respectively $\chi^2 = 7.03$, $p = 0.04$; $\chi^2 = 12.18$, $p < 0.01$; Table 3 and supplementary Tables 2). Additionally, all embryos from Current and Current-EE2 groups exhibited a hatching time of nine days, in contrast to the RCP8.5 and RCP8.5-EE2 groups, where it was eight days. Thus, the hatching time was shorter by one day in the RCP8.5 scenario compared to the Current scenario. Survival among juveniles varied significantly between the experimental groups ($\chi^2 = 14.03$, $p < 0.01$; Table 3 and supplementary Table 1). The survival was lower in the Current-EE2 experimental group ($\chi^2 = 12.59$, $p < 0.01$; Table 3 and supplementary Tables 2) compared to the Current group. The sex ratio was significantly different among the experimental groups ($\chi^2 = 19.12$, df = 3, $p < 0.01$): fewer males were observed in the juveniles Current-EE2 and RCP8.5-EE2 compared to the Current group (respectively, $p < 0.01$, $p = 0.02$, Supplementary Table 1 & 3).

"This work is licensed under a Creative Commons Attribution 4.0 International License"

Preprint not peer reviewed

This preprint research paper has not been peer reviewed. Electronic copy available at: https://ssrn.com/abstract=4795483

Table 3: Embryo-larval survival and juvenile survival in the experimental groups. The letters represent significant differences.

	Current	Current-EE2	RCP8.5	RCP8.5-EE2
Embryo-larval survival $\frac{number\ of\ juveniles\ at\ the\ appearance\ of\ the\ third\ spine}{number\ of\ stripped\ oocytes\ juvenile\ effectif}$	$\frac{62}{305}$ **a**	$\frac{64}{272}$ **a**	$\frac{35}{292}$ **b**	$\frac{84}{459}$ **ab**
Juvenile survival $\frac{number\ of\ fish\ at\ 6\ months\ of\ age}{number\ of\ juveniles\ at\ the\ appearance\ of\ the\ third\ spine}$	$\frac{57}{62}$ **a**	$\frac{42}{64}$ **b**	$\frac{25}{35}$ **ab**	$\frac{62}{84}$ **ab**

Table 4: Proportion of males in the experimental groups. The letters represent significant differences.

	Current	Current-EE2	RCP8.5	RCP8.5-EE2
$\frac{Male\ effectif}{Total\ effectif}$	$\frac{35}{57}$ **a**	$\frac{8}{40}$ **b**	$\frac{8}{24}$ **ab**	$\frac{19}{57}$ **b**

Preprint not peer reviewed

"This work is licensed under a Creative Commons Attribution 4.0 International License"

This preprint research paper has not been peer reviewed. Electronic copy available at: https://ssrn.com/abstract=4795483

2.3. Growth and individual physiological indexes

An effect of scenario ($F_{1, 6} = 9.04$, $p < 0.01$), contamination ($F_{1, 6} = 11.9$, $p < 0.01$), time ($F_{8, 42} = 9.04$, $p < 0.01$), and interaction between scenario and time ($F_{8, 42} = 2408$, $p = 0.01$) was observed for standard length (Figure 4, Supplementary Table 1). Fish under RCP8.5-EE2 experimental group showed significant lower standard length than those under Current group from the fifth month of exposure until the end of the experiment ($p_{120\text{-dph}} = 0.01$; $p_{139\text{-dph}} = 0.03$; $p_{152\text{-dph}} = 0.01$; Supplementary Table 4).

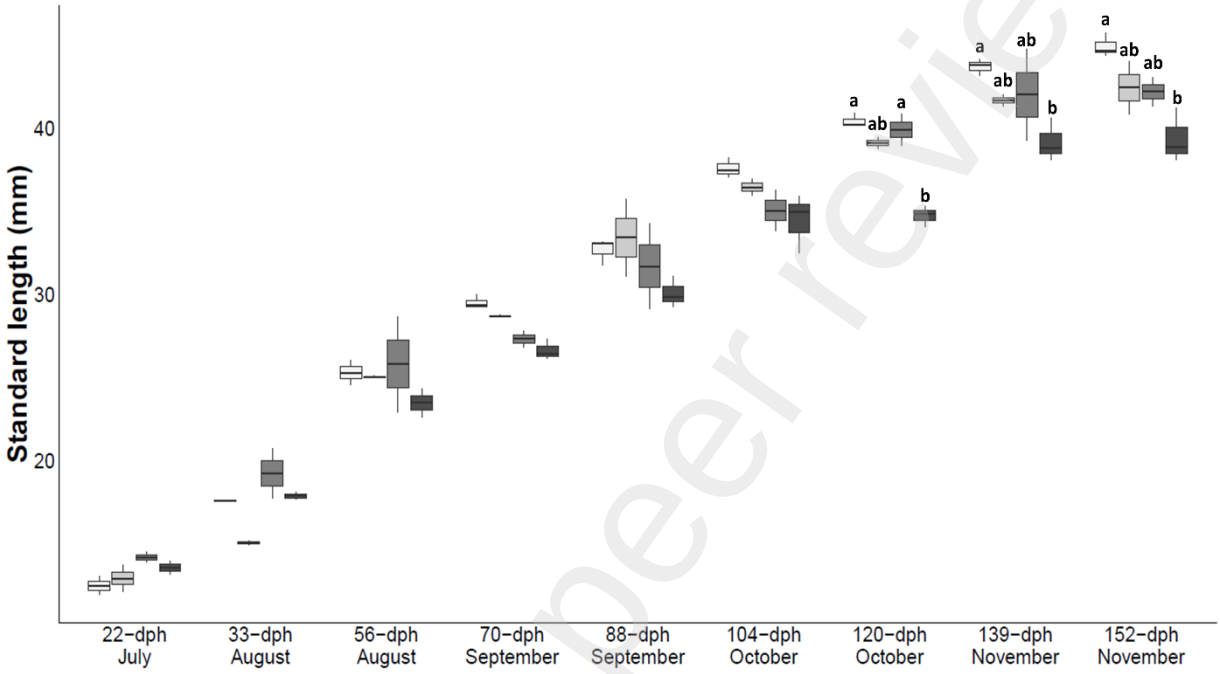


Figure 4: Juvenile stickleback mean standard length per tanks, starting from Current 22 days post-hatching (dph), during the 6 months exposure under Current (light grey), Current-EE2 (grey), and starting from 21 dph under RCP8.5 (medium grey), and RCP8.5-EE2 (dark grey) experimental groups. Letters represent significant differences.

For males (Table 5), no effect of scenario, contamination, or the interaction between scenario and contamination was observed for standard length and for body mass after 6 months of multi-stress ($p > 0.05$; Supplementary Table 1). However, males in RCP8.5 scenario had greater GSI compared to Current scenario ($F_{1, 65} = 5.22$, $p = 0.02$; Supplementary Table 1 & 5) and males exposed to EE2 had lower K and HSI compared to those unexposed (respectively $F_{1, 65} = 4.48$, $p = 0.03$; $F_{1, 41} = 4.88$, $p = 0.03$ Supplementary Table 1 & 5). For females (Table 5), no effect of scenario, contamination, nor their interaction was observed on the standard length, body mass, GSI, and HSI after 6 months of multi-stress (; Supplementary Table 1). However, females show lower K index in RCP8.5 scenario compared to Current scenario ($F_{1, 103} = 9.61$, $p < 0.01$; Supplementary Table 1 & 5).

"This work is licensed under a Creative Commons Attribution 4.0 International License"

This preprint research paper has not been peer reviewed. Electronic copy available at: https://ssrn.com/abstract=4795483

343 Table 5: Stickleback standard body length, body mass and physiological indexes at the sampling. Fulton's index (K); gonado-somatic index (GSI); hepato-
344 somatic index (HIS). Data are presented as mean ± S.D.; #: Climatic scenario effect; **$**: xenoestrogen contamination effect.

Sex	Experimental group	Standard length (mm)	Mass (g)	K	GSI	HSI
♀	*Current*	44.73 ± 4.06	1.14 ± 0.3	1.26 ± 0.14 #	2.11 ± 0.68	5.62 ± 1.73
	Current – EE2	45.12 ± 4.93	1.24 ± 0.4	1.32 ± 0.15 #	2.01 ± 0.58	5.24 ± 1.46
	RCP8.5	45.25 ± 4.31	1.10 ± 0.4	1.17 ± 0.25 #	1.97 ± 0.58	4.93 ± 2.21
	RCP8.5 – EE2	42.53 ± 5.90	0.97 ± 0.4	1.21 ± 0.16 #	1.49 ± 0.81	5.14 ± 1.53
♂	*Current*	47.83 ± 4.66	1.46 ± 0.4	1.31 ± 0.16 **$**	1.33 ± 0.57 #	5.53 ± 1.41 **$**
	Current – EE2	49.38 ± 2.62	1.49 ± 0.3	1.23 ± 0.15 **$**	1.07 ± 0.29 #	4.89 ± 0.90 **$**
	RCP8.5	46.88 ± 3.80	1.47 ± 0.5	1.39 ± 0.10 **$**	1.52 ± 0.38 #	6.25 ± 1.42 **$**
	RCP8.5 – EE2	45.95 ± 4.08	1.29 ± 0.3	1.31 ± 0.09 **$**	1.68 ± 0.75 #	4.81 ± 1.11 **$**

Preprint not peer reviewed

"This work is licensed under a Creative Commons Attribution 4.0 International License"

This preprint research paper has not been peer reviewed. Electronic copy available at: https://ssrn.com/abstract=4795483

2.4. Histological analysis of gonads

The histological analysis of the gonads showed that neither sex reached the final maturation. No aberrant structural modifications were noted within the gonadal tissues in any sex and experimental groups. Nevertheless, oocytes were observed in the testis of 2 individuals: one (out of 5 males analysed) was identified in the Current-EE2 experimental group (Figure 5C), and another one (out of 9 males analysed) in the RCP8.5-EE2 group (Figure 5D).

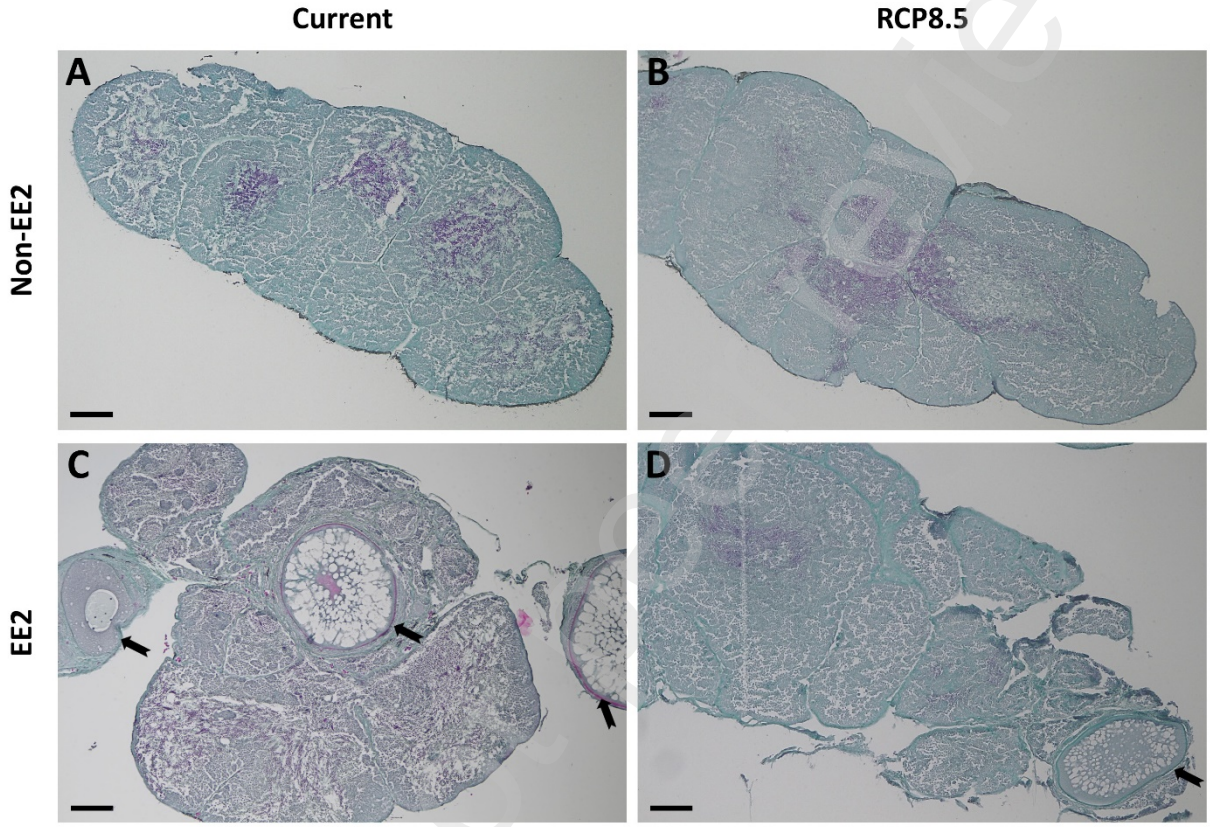


Figure 5: Histological sections of testis from stickleback reared under Current (A), Current-EE2 (C), RCP8.5 (B) and RCP8.5-EE2 (D) experimental groups. Prenant-Gabe trichrome staining: nucleus marked by hematoxylin (purplish blue), cytoplasm marked by eosin Y (pink), connective tissue marked by light green (green). The black arrows show oocytes. Scale bars = 100 µm. Pictures have been edited to increase the brightness (+20 %).

2.5. Genes expression profiles

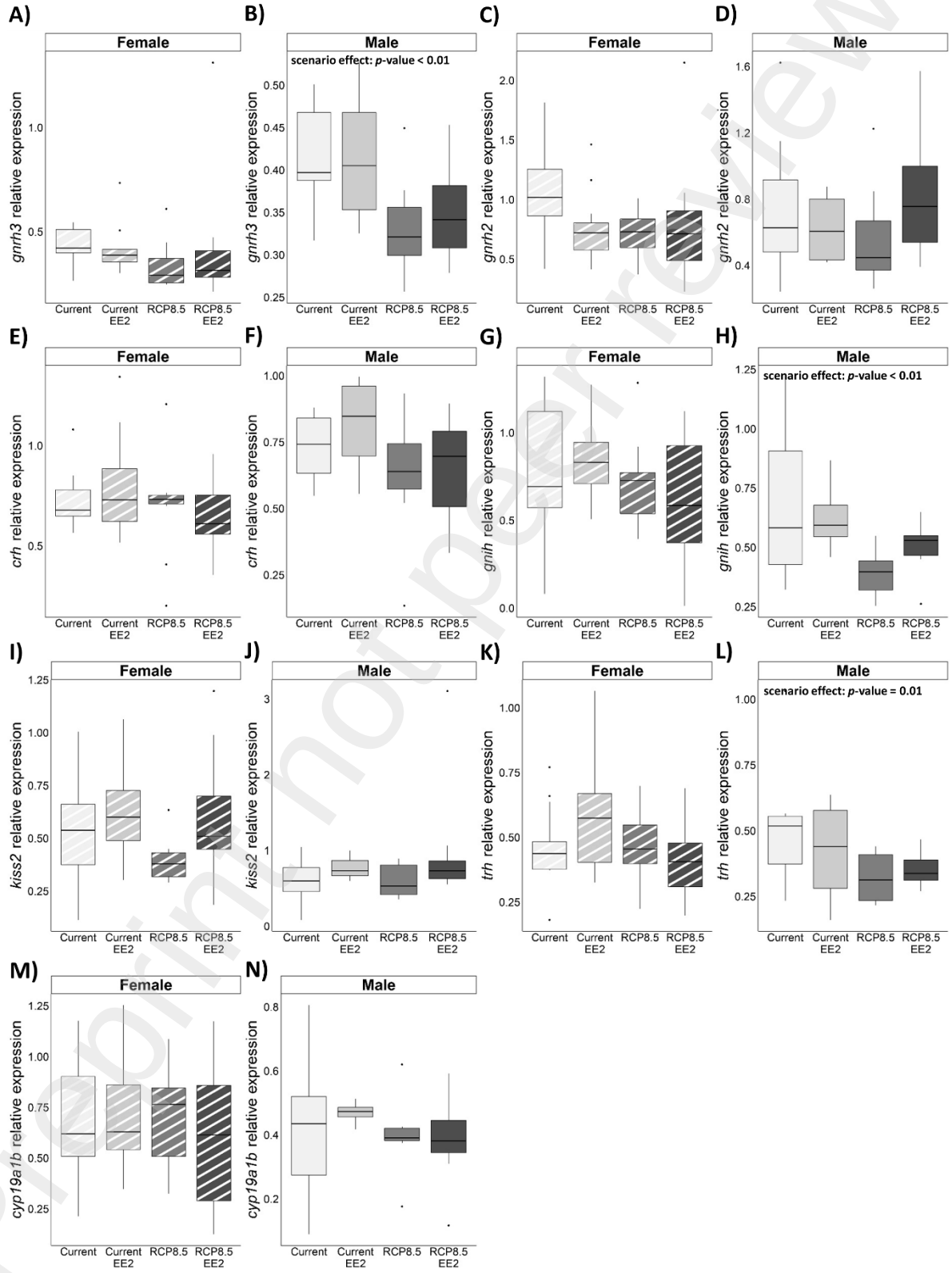
2.5.1. Median brain

The gene expression profiles in the median brain revealed different patterns between females and males (Figure 6). A scenario effect was observed in males with a down-regulation of *gnrh3*, *trh,* and *gnih* expression for juveniles from RCP8.5 scenario compared to the Current scenario (respectively for *gnrh3*, *trh,* and *gnih* expression: $F_{1, 32} = 13.20$, $p < 0.01$; $F_{1, 32} = 6.34$, $p = 0.01$; $F_{1, 32} = 8.93$, $p < 0.01$; Supplementary Table 1). No significant effect of the contamination or the interaction between scenario

"This work is licensed under a Creative Commons Attribution 4.0 International License"

This preprint research paper has not been peer reviewed. Electronic copy available at: https://ssrn.com/abstract=4795483

and contamination was reported (Supplementary Table 1). No significant variations in expression between scenario, contamination or their interaction were observed for the other studied genes (Supplementary Table 1). For females, no scenario, contamination or the interaction between scenario and contamination effects were reported for the gene expression profiles (Supplementary Table 1).



"This work is licensed under a Creative Commons Attribution 4.0 International License"

This preprint research paper has not been peer reviewed. Electronic copy available at: https://ssrn.com/abstract=4795483

Figure 6: Boxplots representing relative expression of *gnrh3* (A-B), *gnrh2* (C-D), *gnih* (E-F), *kiss3* (G-H) and *cyp19a1b* (I-J), *crh* (K-L), and *trh* (M-N) in the median brain of sticklebacks after 6 months under Current (light grey), Current-EE2 (medium grey), RCP8.5 (grey) and RCP8.5-EE2 (dark grey) experimental groups for female (white stripes) and male (no stripes). The 10th and 90th percentiles are represented by the whiskers, the 25th and 75th percentiles by the boxes, the median values by horizontal lines and outliers by points.

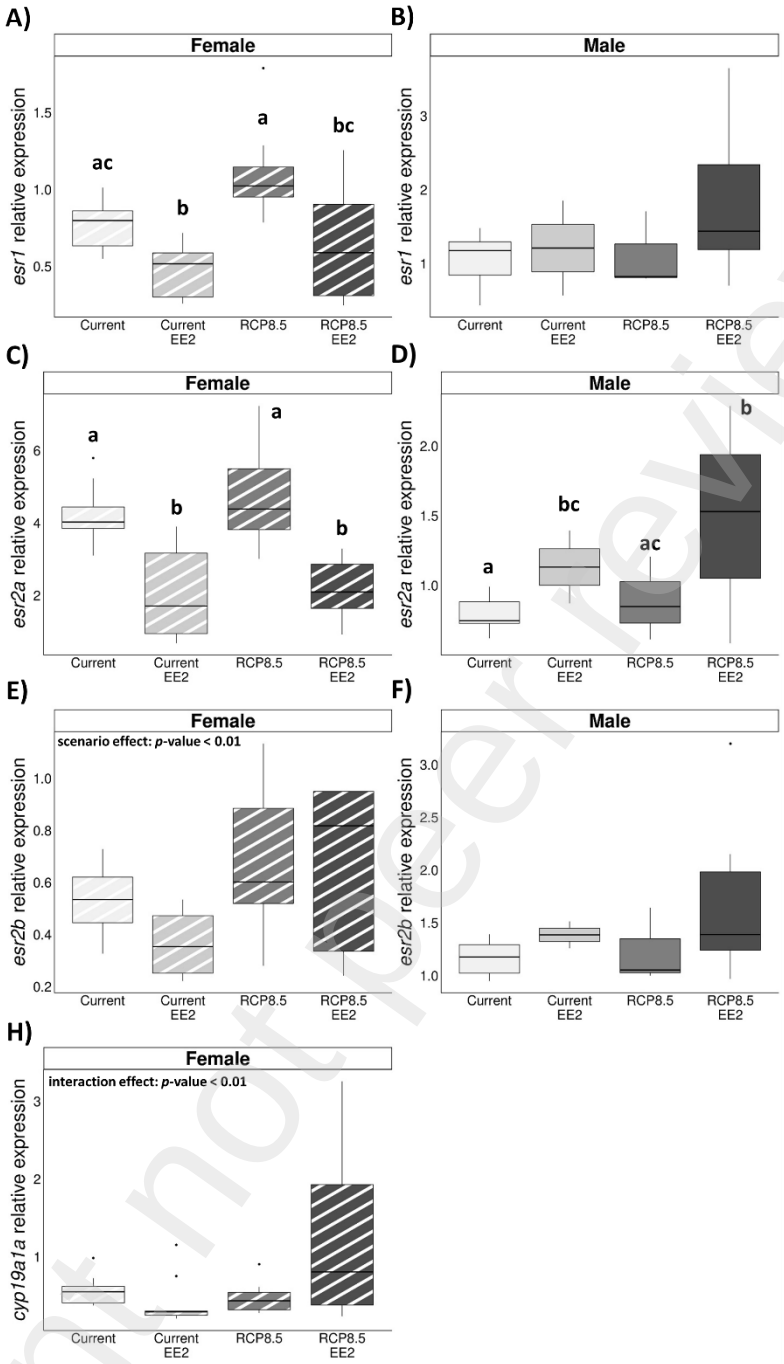
2.5.2. Gonads

The statistical analysis of the gene expression profiles in gonads was done separately in females and males since genes related to sexual maturation can act differently in the two sexes (Figure 7). In ovaries, an effect of the scenario was observed on the gene expression profiles of *esr1* and *esr2b* (respectively: $F_{1, 35} = 8.97$, $p < 0.01$; $F_{1, 31} = 7.33$, $p < 0.01$; Supplementary Table 1), with the RCP8.5 scenario leading to an upregulation of the estrogen receptors compared to the Current scenario. An effect of EE2 contamination was observed on *esr1* and *esr2a* expression (respectively $F_{1, 35} = 20.08$, $p < 0.01$; $F_{1, 35} = 5.75$, $p < 0.01$; Supplementary Table 1) was observed, with a downregulation of *esr1* and *esr2a* expressions in the EE2 exposed groups compared to the unexposed groups. The expression profile of *cyp19a1a* was the only one showing a significant interaction between scenario and contamination ($F_{1, 35} = 7.77$, $p < 0.01$). In testis, a significant contamination effect was observed with an up-regulation of *esr2a* expression in EE2 exposed groups compared with those unexposed ($F_{1, 18} = 7.77$, $p = 0.01$; Supplementary Tables 1 and 11). No effect of scenario, contamination nor interaction was observed for other gene expression profiles ($p > 0.05$; Supplementary Table 1).

"This work is licensed under a Creative Commons Attribution 4.0 International License"

Preprint not peer reviewed

This preprint research paper has not been peer reviewed. Electronic copy available at: https://ssrn.com/abstract=4795483



390

391 Figure 7: Boxplots representing relative expression of *esr1* (A-B), *esr2a* (C-D), *esr2b* (E-F), and
392 *cyp19a1* (G-H) in the gonads of sticklebacks after 6 months under Current (light grey), Current-EE2
393 (medium grey), RCP8.5 (grey) and RCP8.5-EE2 (dark grey) experimental groups for female (white
394 stripes) and male (no stripes). The 10th and 90th percentiles are represented by the whiskers, the 25th
395 and 75th percentiles by the boxes, the median values by horizontal lines and outliers by points. Different
396 letters indicate the significant difference between the boxes.

"This work is licensed under a Creative Commons Attribution 4.0 International License"

Preprint not peer reviewed

This preprint research paper has not been peer reviewed. Electronic copy available at: https://ssrn.com/abstract=4795483

3. Discussion

The overall results of this study suggest that some of the EE2 effects on parameters related to survival, growth and sex determinism/reproductive axis in stickleback can be modulated by the tested future climatic scenario. Furthermore, we found that the impact of EE2 and its climatic modulation can be dependent of the life stage and the sex of the fish.

Often the early life stages, such as embryos and larvae, are the most vulnerable to environmental variations and stressors (Przeslawski et al., 2015). Previous findings described temperature as the principal factor influencing stickleback physiology (Fellous et al., 2022; Ramler et al., 2014; Shama, 2017, 2015). Recent studies suggested that the ocean acidification could also act similarly and reduce egg quality in other fish species (Sganga et al., 2022), as could the combination of warming and acidification in stickleback (Devergne et al., 2023). In this study, a higher perivitelline index was observed in fish of the RCP8.5 scenario, suggesting a decline of resources allocated to the embryo, which may have potential implications for the following development rates. Accordingly, under the RCP8.5 scenario, we also observed a shorter hatching time. Warmer conditions tend to accelerate development, as noted in stickleback species (Shama, 2015) and potentially induce malformations, as can acidification (Pimentel et al., 2014). Previous stickleback studies did not reveal any impact of acidification alone on embryo and larval survival (Glippa et al., 2017). Conversely, a thermal stress alone, i.e a temperature of 21°C, corresponding to the temperature of RCP8.5 scenario in summer in the present study, has been described as a major stress for stickleback survival (Fellous et al., 2022; Hani et al., 2019). Altogether, the higher perivitelline index and the shorter hatching time at warmer temperatures and lower pH, could explain the low embryo-larval survival observed in the RCP8.5 experimental group. Unexpectedly, eggs showing the lowest quality (RCP8.5-EE2), based on the egg quality criteria investigated (egg diameter, perivitelline index, and hatching time), did not exhibit a lower survival. These eggs present the smallest diameters relative to those in the Current group with a higher perivitelline index under the RCP8.5 scenario compared to the Current scenario, which would have further compromised the overall egg quality.

In juveniles, responses to xenoestrogen and RCP8.5 differed as survival was reduced in fish exposed to Current scenario combined with EE2 in comparison to juveniles in Current situation. The lack of impact of the RCP8.5 scenario on juvenile survival may be attributed to the potential selection of the most resistant individuals occurred at embryo-larval stage. The lack of impact of the EE2 contamination under RCP8.5 scenario could be explained in terms of toxicokinetics. It is assumed that the juveniles exposed to RCP8.5 scenario may have an accelerated metabolism, potentially resulting in a higher depuration rate (Blewett et al., 2013). If it is the case, at five-month post-contamination, fish under RCP8.5 condition could have eliminated any residual EE2 effect on survival, in contrast to fish acclimated to lower temperatures. To confirm or refute this hypothesis, quantifying EE2 levels in

"This work is licensed under a Creative Commons Attribution 4.0 International License"

Preprint not peer reviewed

This preprint research paper has not been peer reviewed. Electronic copy available at: https://ssrn.com/abstract=4795483

stickleback target accumulation organs, such as the gall bladder, liver, or whole body (Blewett et al., 2013) at the end of the one-month contamination and five-months after, would have been necessary.

Different patterns of fish growth were observed in the different climatic scenarios during the post-contamination period. Five-months after contamination, the standard length of fish was reduced in RCP8.5 scenario individuals, as well as for the fish with the EE2 contamination during embryo-larval stage. The strongest reduction was observed for the fish exposed to RCP8.5 and EE2 contamination. In a previous study (Devergne et al. (2023), we observed that to achieve equivalent size and mass, sticklebacks under RCP8.5 condition required a greater amount of food. We hypothesised that sticklebacks subjected to higher temperature and lower pH would face greater metabolic cost, resulting in an additional energy requirement, which was compensated when food was not limiting (Devergne et al., 2023). In the present study, fish were fed with the same amount of food in the four experimental groups. This didn't allow compensation for the RCP8.5 scenario increased metabolic cost, resulting thus in lower growth and lower fish size. Xenoestrogens and specifically EE2 contamination have been reported in fish species to reduce the growth, leading to smaller size and mass (Länge et al., 2001; Zha et al., 2007). For instance, at environmental concentrations (around 25 $ng.L^{-1}$) during the early life led to lower body length and weight in *Oreochromis niloticus* (Shved et al., 2008) and *Danio rerio* (Van den Belt et al., 2003). Shved et al. (2008) hypothesized that the growth axis was directly impacted by EE2, resulting in lower mRNA expression in brain, gonads and liver of IGF-1, and a reduced pituitary GH expression level. Given the observed results in the literature, it would be interesting to check whether the growth axis, and particularly the IGF-1 gene signaling, were also disrupted in EE2 treated sticklebacks from the present study. In contrast, Bell (2004) showed in sticklebacks an accelerated growth when contaminated at EE2 higher concentration (100 $ng.L^{-1}$) and over a whole life cycle. The hypothesis of the author was that steroids can have anabolic effects and improve the early growth by increasing food conversion efficiency and leading fishes to reach faster adult size. This highlights that the effects of EE2 exposure on fish growth can vary with life stages, EE2 concentrations and the duration of the exposure/recovery. Ultimately, the smaller size, especially for the fish exposed to early EE2 contamination in interaction with RCP8.5 scenario, can be disadvantageous since smaller individuals show reduction in defense ability with a consequent increasing predation risk (Ramler et al., 2014).

The sex ratio of stickleback populations in the present study appeared unaffected by whole life exposure to warming and acidification (RCP8.5 scenario). However, the contamination of EE2 during the embryo-larval stages led to a higher proportion of females in the population under both climatic scenarios. Xenoestrogenic endocrine disruptors, with EE2 being a preeminent example, are widely recognized for their capacity to induce sex ratio imbalances in fish populations, even at environmentally low concentrations (Länge et al., 2001; Maunder et al., 2007; Porseryd et al., 2019; Shved et al., 2008). Consequently, two plausible hypotheses can be formulated to explain the alteration of the sex ratio observed in this study: 1) an increase in the number of females through feminisation, and/or 2) an

"This work is licensed under a Creative Commons Attribution 4.0 International License"

Preprint not peer reviewed

This preprint research paper has not been peer reviewed. Electronic copy available at: https://ssrn.com/abstract=4795483

increase in male's mortality. The difficulty of accurately determining the phenotypic sex of immature sticklebacks coupled with the lack of genetic sex determination did not enable us to confirm or refute these hypotheses. Yet, the presence of intersexes within the 2 contaminated experimental groups ("Current-EE2" and "RCP8.5-EE2") shows that an early exposure to environmental concentration of EE2, of 15 $ng.L^{-1}$, is actually able to feminise, at least partially, stickleback testis, thus impacting the phenotypic sex of fish. Similarly, previous studies using equivalent levels of contamination in stickleback showed intersexes in their populations (Hahlbeck et al., 2004; Maunder et al., 2007) with persistence of these effects even after several months post-contamination (Porseryd et al., 2019). Furthermore, it has been shown that the larval period is a window of sensitivity for xenoestrogens, during which the phenotypic sex of sticklebacks individuals can be altered (Hahlbeck et al., 2004). Maunder et al. (2007) hypothesised that the presence of oocytes within the testes altered steroidogenesis, disrupting the cycling synthesis and secretion of steroids. This could be due to a diminution of the functional volume of testicular tissue or to the endocrine activity of the oocytes/ovarian tissue in the testes (Maunder et al., 2007). For instance, it has been demonstrated that roach (*Rutilus rutilus*) presenting an intersex condition, after the exposure to sewage, undergoes perturbations in plasma sex steroid concentration profiles (estradiol [E2], testosterone [T], and 11-ketotestosterone [11-KT]) (Jobling et al., 2002a). This supports the hypothesis of altered steroidogenesis in intersex fish, which could ultimately lead to reduced fertility, as shown in intersex *R. rutilus* (Jobling et al., 2002b). It is evident that the sex of the fish plays a role in the accumulation of EE2. Females, *Pimephales promelas*, showed lower uptake of EE2 with a corresponding lower accumulation in their gills compared to males (Blewett et al., 2014). This sex-dependent accumulation may potentially contribute to a greater vulnerability of males to the adverse effects of EE2 exposure. Therefore, assessing the hormone profile and reproductive success of the stickleback of the present study will provide insights of the consequences of environmental contamination on recruitment with potential impact on the dynamics of stickleback populations. Although, according to Sokolowska and Kulczykowska (2006), the GSI values showed in males at the sampling were low in all groups indicating that testis are still immature, even if RCP8.5 scenario's values were significantly lower that Current scenario's ones. This prevents the possibility to assess the real impact of climatic scenarios and the EE2 contamination on fish reproductive performances. Histological analysis of male gonads carried out in this study, confirmed the early stage of spermatogenesis at the sampling time, which is expected for prepubertal sticklebacks during the winter period, in all the experimental conditions. An effect of EE2 contamination was observed on the male K and HSI, both exhibiting lower values in the two conditions with early EE2 contamination. The lower values for the K-index in contaminated males were mainly driven by the reduced mass of the liver due to the EE2 exposure, which also resulted in a lower HSI. In females, the K index exhibited lower values under the RCP8.5 scenario, which may be related to the lower trend observed in the body mass in this scenario. Thus, it would be interesting to estimate the amount of energy reserves when this generation

"This work is licensed under a Creative Commons Attribution 4.0 International License"

This preprint research paper has not been peer reviewed. Electronic copy available at: https://ssrn.com/abstract=4795483

(F1) reaches the spawning time to detect a possible alteration of the energy allocation in RCP8.5 scenario; this was observed for lipid content of their parents (F0) under the same climatic scenario (Devergne et al., 2023). It worth to note that in both sexes any interaction between the exposure of EE2 and the climatic scenario was observed on the physiological indexes.

With the aim of testing whether future climatic scenario affects the neuroendocrine system controlling the implementation of the reproductive axis and the development, and whether exposure to EE2 at early stages could alter this effect, we studied the expression levels of genes involved in reproduction and development at the central level. A general trend was observed suggesting a downregulation in the expression profile of the studied genes in the median brain of males acclimated to RCP8.5 scenario, compared to the Current condition. The expression of *gnrh3*, *gnih* and *trh*, was significantly downregulated in males in the RCP8.5 scenario. Surprisingly, no significant impact of the expression levels of these genes was detected in females exposed to the same climatic scenario. This highlights the sex-specific nature of the impact of environmental stressors on the brain gene profiles in sticklebacks. The genes cited above are involved in the sexual maturation and development, the *gnrh3* being one of the main regulators of maturation in stickleback (Shao et al., 2019) and the *trh* involved in the stimulation of the thyroid axis, playing a role in development and growth (Blanton and Specker, 2007). Interestingly, in our previous study where we evaluated the impact of the combined acidification and warming on adult sticklebacks, we observed the opposite effect (Devergne et al., 2023). The expression level of genes involved in the neuroendocrine control of reproduction showed a general trend of upregulation as well as a higher GSI in males under RCP8.5 scenario. It is worth reminding that fish of this study are still juveniles, approaching puberty (prepubertal), while in the cited previous study fish were adults, with fully developed gonads, sampled during the spawning period (Devergne et al., 2023). The different maturation stage of males (fully mature in the previous study and immature in the present one) could be the reason of the opposite effect of the climatic stress on central expression level and GSI. Another explanation could be that, contrary to fish employed in the previous study (F0), sticklebacks in the present study (F1) are offspring of parents already acclimated to the same climatic conditions, in terms of temperature and pH levels. The parental acclimation to specific environmental conditions can indeed influence the physiological response of offspring exposed to the same environment (Salinas et al., 2013). No persistent effects of EE2 exposure on the neuroendocrine control of sexual maturation and development were observed in this study. This response could be species specific since, one study in zebrafish has indicated effects following contamination during early life, primarily associated with the estrogenic system (Porseryd et al., 2017).

Afterwards, we analysed the expression patterns of genes related to reproduction at the peripheral level, to specifically test whether the early EE2 exposure could alter the endocrine regulation of the estrogenic signaling in gonads acclimated at two climatic scenarios. Interestingly, the effect of scenario alone in females was not significant, even on gonadal aromatase (*cyp19a1a)* expression profile.

"This work is licensed under a Creative Commons Attribution 4.0 International License"

This preprint research paper has not been peer reviewed. Electronic copy available at: https://ssrn.com/abstract=4795483

However, CYP19A1A is known to be a thermo-sensitive enzyme (Alix et al., 2020), exhibiting reduced activity *in vivo* and *in situ*, as well as lower expression levels, under high temperature (Miranda et al., 2013). In this study, the temperature experienced at the sampling (16 °C) was probably low enough to not impact the *cyp19a1a* expression in stickleback's gonads. The expression patterns of the three estrogen receptors (*esr1*, *esr2a*, and *esr2b*) were similar across experimental groups. In teleost the influence of rising temperature on estrogens receptors expression is dependent on various factors such as receptor type, tissue studied, species, sex, developmental stage, and thermal intensity (Bock et al., 2021). In this study, under the RCP8.5 scenario, there was an increase in the expressions of *esr1* and *esr2b* of females, compared to Current scenario. The increase in estrogens receptors expression in females may compensate for the potential decreased affinity of estrogens for their receptors at high temperatures, as demonstrated in other teleost species (Watts et al., 2005). The early exposure to EE2 in Current and RCP8.5 scenario resulted in a reduction of *esr1* and *esr2a* expressions in females. This seems in line with a previous hypothesis of disruption of androgen-estrogen balance in females (Maunder et al., 2007), that would cause a negative feedback and the inhibition of estrogen receptor expression at gonad level. Surprisingly, this effect was still observed several months post-contamination. The effect of EE2 on the gonadal expression of *cyp19a1a* depended on the climatic scenario. EE2 may have amplified the rise of *cyp19a1a* expression levels usually observed at warmer temperatures and probably due to the epigenetic regulation (Voisin et al., 2016). This suggests a long-term, potentially irreversible, effect on ovarian endocrine regulation. In males no alteration of the gene expressions profile of gonads under the RCP8.5 scenario was observed. Nevertheless, the early exposure to EE2, in Current and RCP8.5 scenarios, induced an upregulation of the *esr2a* expression profile. For instance, previous studies have reported that ESR2A gene plays a crucial role in the reproductive processes of female fish (Kayo et al., 2019; Lu et al., 2017), and the knockout of both *esr2a* and *esr2b* resulted in the interruption of follicle development and masculinisation (Lu et al., 2017). This could suggest in the sticklebacks of this study, after an EE2 early contamination, a sign of partial feminization, in line with the presence of oocytes observed in some EE2 contaminated males.

4. Conclusion

This study tested whether the effects of an exposure to xenoestrogen, at environmental concentration (15 $ng.L^{-1}$) and during early life stages, are modulated by a predicted climatic scenario (+3°C; -0.4 pH unit). We investigated the impact of these multiple stressors on stickleback survival, growth and sexual maturation on pre-pubertal juveniles. In this study, sticklebacks (F1) were issued of parents (F0) that were already acclimatized to the same climatic scenario. Experimental data indicated that impact of the EE2 early contamination on juvenile survivals depended on the climatic scenario. In the case of multi-stress condition (RCP8.5-EE2) the body length was significantly lower. Besides, the impact of climatic stress and an early estrogenic exposure on the physiological indexes are sex specific, at least in prepubertal fish (where the phenotypic sex was identified). In the tested experimental conditions, the

"This work is licensed under a Creative Commons Attribution 4.0 International License"

This preprint research paper has not been peer reviewed. Electronic copy available at: https://ssrn.com/abstract=4795483

climatic scenario have a higher impact in the central control of the reproductive axis in males compared to females. Whereas, the early EE2 contamination preferentially affect the gene expression in gonads with an overall upregulation in males and downregulation in females. Interestingly, the only significant interaction between the scenario and the EE2 contamination was evident for the expression level of *cyp19a1a* in the ovaries, suggesting a long-term estrogenic effect in the RCP8.5 scenario. Furthermore, the observed skewed sex ratio, associated with the presence of intersexes in both Current-EE2 and RCP8.5-EE2, clearly indicated a feminization process of the population due to EE2 contamination in both scenarios. Altogether, this could suggest a disruption in sexual maturation and future reproductive success. Indeed, EE2 environmental contamination has been identified as a risk factor for short-lived fish populations in present day conditions, such as the increased mortality of young stages with the consequent collapse of the fish population (Kidd et al., 2007). This raises questions about the sustainability of these populations in future environments. In conclusion, the exposure to a contamination of EE2 during the embryo-larval stage can have long-term effects on stickleback physiology, even after several months post-contamination. This impact can be modulated by the climatic condition, depending on the physiological endpoints considered.

5. Project information

Supplementary Materials: The following supporting information can be downloaded at: (Insert link).

Supplementary Table 1: Statistical tests. Asterisks indicate statistical difference between scenarios.

Supplementary Table 2: Pearson chi-square test with pairwise proportion test on survival for embryo-larval stage and juvenile stage since the formation of the third spine. p: p-value; p.adj: adjusted p-value using Holm method. Asterisks indicate statistical difference between scenarios.

Supplementary Table 3: Pairwise proportion test on sex ratio at the end of the experiment. p: p-value; p.adj: adjusted p-value using Holm method. Asterisks indicate statistical difference between scenarios.

Supplementary Table 4: Pairwise Games Howell post-hoc test on eggs diameter. Statistics: statistic test (t-value) used to compute the p-value; p.adj: adjusted p-value using Tukey method, df: degrees of freedom calculated using Welch's correction. Asterisks indicate statistical difference between scenarios.

Supplementary Table 5: Least-Squares Means pairwise test on K index, gonado-somatic index (GSI), and hepatosomatic index (HSI). p-value: adjusted p-value using Tukey method. Asterisks indicate statistical difference between scenarios.

Supplementary Table 6: Sample size table of individuals used for the estimation of the physiological indexes and histology analysis.

Supplementary Table 7: Sample size table of individuals brain and gonads used for the gene expression profiles analysis.

"This work is licensed under a Creative Commons Attribution 4.0 International License"

This preprint research paper has not been peer reviewed. Electronic copy available at: https://ssrn.com/abstract=4795483

Author Contributions:

Conceptualization, Véronique Loizeau and Arianna Servili;

Data curation, Jimmy Devergne, Sophie Collet, Olivier Mouchel, Marie Lou Fleury, Lauriane Madec, Titouan Brandicourt, and Sylvain Jodet;

Formal analysis, Jimmy Devergne, Véronique Loizeau, Christophe Lebigre, Titouan Brandicourt, Sylvain Jodet, and Arianna Servili;

Funding acquisition, Véronique Loizeau and Arianna Servili;

Methodology, Jimmy Devergne, Véronique Loizeau, Christophe Lebigre, Sophie Collet, Marie Lou Fleury and Arianna Servili;

Project administration, Véronique Loizeau and Arianna Servili;

Validation, Christophe Lebigre, and Sabine Roussel;

Writing – original draft, Jimmy Devergne;

Writing – review & editing, Véronique Loizeau, Christophe Lebigre, Olivier Mouchel Arianna Servili, Sabine Roussel, and Sylvain Jodet.

All authors have read and agreed to the published version of the manuscript.

Funding: This work was supported by the French national program EC2CO-DYCOVI through the ECHANGE project to V.L. (2021-2023) and Ifremer (ICEfish project). J.D. PhD grant was founded by "Université de Bretagne Occidental" and by ISblue project, Interdisciplinary graduate school for the blue planet (ANR-17-EURE-0015) and co-funded by a grant from the French government under the program "Investissements d'Avenir" embedded in France 2030.

Data Availability Statement: The data presented in this study are available in the supplementary materials.

Acknowledgments: Special thanks to Marie-Madeleine Le Gall and all the members of the PHYTNESS unit (Ifremer-LEMAR) for their assistance in the experimental work and fish maintenance.

Conflicts of Interest: The authors declare no conflict of interest.

6. Bibliography

Adeel, M., Song, X., Wang, Y., Francis, D., Yang, Y., 2017. Environmental impact of estrogens on human, animal and plant life: A critical review. Environment International 99, 107–119. https://doi.org/10.1016/j.envint.2016.12.010

Alava, J.J., Cheung, W.W.L., Ross, P.S., Sumaila, U.R., 2017. Climate change-contaminant interactions in marine food webs: Toward a conceptual framework. Glob Change Biol 23, 3984–4001. https://doi.org/10.1111/gcb.13667

Alix, M., Kjesbu, O.S., Anderson, K.C., 2020. From gametogenesis to spawning: How climate-driven warming affects teleost reproductive biology. J Fish Biol 97, 607–632. https://doi.org/10.1111/jfb.14439

"This work is licensed under a Creative Commons Attribution 4.0 International License"

This preprint research paper has not been peer reviewed. Electronic copy available at: https://ssrn.com/abstract=4795483

643 Baag, S., Mandal, S., 2022. Combined effects of ocean warming and acidification on marine fish and
644 shellfish: A molecule to ecosystem perspective. Science of The Total Environment 802, 149807.
645 https://doi.org/10.1016/j.scitotenv.2021.149807

646 Bates, D., Mächler, M., Bolker, B., Walker, S., 2015. Fitting Linear Mixed-Effects Models Using lme4.
647 J. Stat. Soft. 67. https://doi.org/10.18637/jss.v067.i01

648 Bell, A.M., 2004. An endocrine disrupter increases growth and risky behavior in threespined stickleback
649 (Gasterosteus aculeatus). Hormones and Behavior 45, 108–114.
650 https://doi.org/10.1016/j.yhbeh.2003.09.009

651 Bhandari, R.K., Deem, S.L., Holliday, D.K., Jandegian, C.M., Kassotis, C.D., Nagel, S.C., Tillitt, D.E.,
652 vom Saal, F.S., Rosenfeld, C.S., 2015. Effects of the environmental estrogenic contaminants bisphenol
653 A and 17α-ethinyl estradiol on sexual development and adult behaviors in aquatic wildlife species.
654 General and Comparative Endocrinology 214, 195–219. https://doi.org/10.1016/j.ygcen.2014.09.014

655 Björkblom, C., Högfors, E., Salste, L., Bergelin, E., Olsson, P.-E., Katsiadaki, I., Wiklund, T., 2009.
656 Estrogenic and androgenic effects of municipal wastewater effluent on reproductive endpoint
657 biomarkers in three-spined stickleback (gasterosteus aculeatus). Environ toxicol chem 28, 1063.
658 Https://doi.org/10.1897/08-337.1

659 Blaker, E., Sebire, M., Ellis, T., Katsiadaki, I., 2022. The housing, care, and use of a laboratory three-
660 spined stickleback colony, in: Laboratory Fish in Biomedical Research. Elsevier, pp. 349–371.
661 https://doi.org/10.1016/B978-0-12-821099-4.00009-2

662 Blanton, M.L., Specker, J.L., 2007. The Hypothalamic-Pituitary-Thyroid (HPT) Axis in Fish and Its
663 Role in Fish Development and Reproduction. Critical Reviews in Toxicology 37, 97–115.
664 https://doi.org/10.1080/10408440601123529

665 Blewett, T., MacLatchy, D.L., Wood, C.M., 2013. The effects of temperature and salinity on 17-α-
666 ethynylestradiol uptake and its relationship to oxygen consumption in the model euryhaline teleost
667 (Fundulus heteroclitus). Aquatic Toxicology 127, 61–71. https://doi.org/10.1016/j.aquatox.2012.04.009

668 Blewett, T.A., Chow, T.L., MacLatchy, D.L., Wood, C.M., 2014. A species comparison of 17-α-
669 ethynylestradiol uptake and tissue-specific distribution in six teleost fish. Comparative Biochemistry
670 and Physiology Part C: Toxicology & Pharmacology 161, 33–40.
671 https://doi.org/10.1016/j.cbpc.2014.01.004

672 Blewett, T.A., Robertson, L.M., MacLatchy, D.L., Wood, C.M., 2013. Impact of environmental oxygen,
673 exercise, salinity, and metabolic rate on the uptake and tissue-specific distribution of 17α-
674 ethynylestradiol in the euryhaline teleost Fundulus heteroclitus. Aquatic Toxicology 138–139, 43–51.
675 https://doi.org/10.1016/j.aquatox.2013.04.006

"This work is licensed under a Creative Commons Attribution 4.0 International License"

This preprint research paper has not been peer reviewed. Electronic copy available at: https://ssrn.com/abstract=4795483

Bock, S.L., Chow, M.I., Forsgren, K.L., Lema, S.C., 2021. Widespread alterations to hypothalamic-pituitary-gonadal (HPG) axis signaling underlie high temperature reproductive inhibition in the eurythermal sheepshead minnow (Cyprinodon variegatus). Molecular and Cellular Endocrinology 537, 111447. https://doi.org/10.1016/j.mce.2021.111447

Cargouet, M., Perdiz, D., Mouatassimsouali, A., Tamisierkarolak, S., Levi, Y., 2004. Assessment of river contamination by estrogenic compounds in Paris area (France). Science of The Total Environment 324, 55–66. https://doi.org/10.1016/j.scitotenv.2003.10.035

Cosme, M.M., Lister, A.L., Van Der Kraak, G., 2015. Inhibition of spawning in zebrafish (Danio rerio): Adverse outcome pathways of quinacrine and ethinylestradiol. General and Comparative Endocrinology 219, 89–101. https://doi.org/10.1016/j.ygcen.2015.01.013

Devergne, J., Loizeau, V., Lebigre, C., Bado-Nilles, A., Collet, S., Mouchel, O., Iaria, U., Le Gall, M.-M., Madec, L., Turiès, C., Servili, A., 2023. Impacts of Long-Term Exposure to Ocean Acidification and Warming on Three-Spined Stickleback (Gasterosteus aculeatus) Growth and Reproduction. Fishes 8, 523. https://doi.org/10.3390/fishes8100523

Dickson, A.G., Millero, F.J., 1987. A comparison of the equilibrium constants for the dissociation of carbonic acid in seawater media. Deep Sea Research Part A. Oceanographic Research Papers 34, 1733–1743. https://doi.org/10.1016/0198-0149(87)90021-5

Fellous, A., Wegner, K.M., John, U., Mark, F.C., Shama, L.N.S., 2022. Windows of opportunity: Ocean warming shapes temperature-sensitive epigenetic reprogramming and gene expression across gametogenesis and embryogenesis in marine stickleback. Global Change Biology 28, 54–71. https://doi.org/10.1111/gcb.15942

Gattuso, J.-P., Magnan, A., Billé, R., Cheung, W.W.L., Howes, E.L., Joos, F., Allemand, D., Bopp, L., Cooley, S.R., Eakin, C.M., Hoegh-Guldberg, O., Kelly, R.P., Pörtner, H.-O., Rogers, A.D., Baxter, J.M., Laffoley, D., Osborn, D., Rankovic, A., Rochette, J., Sumaila, U.R., Treyer, S., Turley, C., 2015. Contrasting futures for ocean and society from different anthropogenic CO2 emissions scenarios. Science 349, aac4722. https://doi.org/10.1126/science.aac4722

Glippa, O., Brutemark, A., Johnson, J., Spilling, K., Candolin, U., Engström-Öst, J., 2017. Early Development of the Threespine Stickleback in Relation to Water pH. Front. Mar. Sci. 4, 427. https://doi.org/10.3389/fmars.2017.00427

Hahlbeck, E., Griffiths, R., Bengtsson, B., 2004. The juvenile three-spined stickleback (Gasterosteus aculeatus L.) as a model organism for endocrine disruptionI. Sexual differentiation. Aquatic Toxicology 70, 287–310. https://doi.org/10.1016/S0166-445X(04)00265-6

"This work is licensed under a Creative Commons Attribution 4.0 International License"

This preprint research paper has not been peer reviewed. Electronic copy available at: https://ssrn.com/abstract=4795483

Hani, Y.M.I., Marchand, A., Turies, C., Kerambrun, E., Palluel, O., Bado-Nilles, A., Beaudouin, R., Porcher, J.-M., Geffard, A., Dedourge-Geffard, O., 2018. Digestive enzymes and gut morphometric parameters of threespine stickleback (Gasterosteus aculeatus): Influence of body size and temperature. PLoS ONE 13, e0194932. https://doi.org/10.1371/journal.pone.0194932

Hani, Y.M.I., Turies, C., Palluel, O., Delahaut, L., Bado-Nilles, A., Geffard, A., Dedourge-Geffard, O., Porcher, J.-M., 2019. Effects of a chronic exposure to different water temperatures and/or to an environmental cadmium concentration on the reproduction of the threespine stickleback (Gasterosteus aculeatus). Ecotoxicology and Environmental Safety 174, 48–57. https://doi.org/10.1016/j.ecoenv.2019.02.032

Jackson, L.M., Felgenhauer, B.E., Klerks, P.L., 2019. Feminization, altered gonadal development, and liver damage in least killifish (Heterandria formosa) exposed to sublethal concentrations of 17α-ethinylestradiol. Ecotoxicology and Environmental Safety 170, 331–337. https://doi.org/10.1016/j.ecoenv.2018.11.094

Jobling, S., Beresford, N., Nolan, M., Rodgers-Gray, T., Brighty, G.C., Sumpter, J.P., Tyler, C.R., 2002a. Altered Sexual Maturation and Gamete Production in Wild Roach (Rutilus rutilus) Living in Rivers That Receive Treated Sewage Effluents1. Biology of Reproduction 66, 272–281. https://doi.org/10.1095/biolreprod66.2.272

Jobling, S., Coey, S., Whitmore, J.G., Kime, D.E., Van Look, K.J.W., McAllister, B.G., Beresford, N., Henshaw, A.C., Brighty, G., Tyler, C.R., Sumpter, J.P., 2002b. Wild Intersex Roach (Rutilus rutilus) Have Reduced Fertility1. Biology of Reproduction 67, 515–524. https://doi.org/10.1095/biolreprod67.2.515

Jones, F.C., Grabherr, M.G., Chan, Y.F., Russell, P., Mauceli, E., Johnson, J., Swofford, R., Pirun, M., Zody, M.C., White, S., Birney, E., Searle, S., Schmutz, J., Grimwood, J., Dickson, M.C., Myers, R.M., Miller, C.T., Summers, B.R., Knecht, A.K., Brady, S.D., Zhang, H., Pollen, A.A., Howes, T., Amemiya, C., Lander, E.S., Di Palma, F., Lindblad-Toh, K., Kingsley, D.M., 2012. The genomic basis of adaptive evolution in threespine sticklebacks. Nature 484, 55–61. https://doi.org/10.1038/nature10944

Kassambara, A., 2023. rstatix: Pipe-Friendly Framework for Basic Statistical Tests.

Kayo, D., Zempo, B., Tomihara, S., Oka, Y., Kanda, S., 2019. Gene knockout analysis reveals essentiality of estrogen receptor β1 (Esr2a) for female reproduction in medaka. Sci Rep 9, 8868. https://doi.org/10.1038/s41598-019-45373-y

Kibria, G., Nugegoda, D., Rose, G., Haroon, A.K.Y., 2021. Climate change impacts on pollutants mobilization and interactive effects of climate change and pollutants on toxicity and bioaccumulation

"This work is licensed under a Creative Commons Attribution 4.0 International License"

This preprint research paper has not been peer reviewed. Electronic copy available at: https://ssrn.com/abstract=4795483

of pollutants in estuarine and marine biota and linkage to seafood security. Marine Pollution Bulletin 167, 112364. https://doi.org/10.1016/j.marpolbul.2021.112364

Kidd, K.A., Blanchfield, P.J., Mills, K.H., Palace, V.P., Evans, R.E., Lazorchak, J.M., Flick, R.W., 2007. Collapse of a fish population after exposure to a synthetic estrogen. Proc. Natl. Acad. Sci. U.S.A. 104, 8897–8901. https://doi.org/10.1073/pnas.0609568104

Kim, S.-Y., Costa, M.M., Esteve-Codina, A., Velando, A., 2017a. Transcriptional mechanisms underlying life-history responses to climate change in the three-spined stickleback. Evol Appl 10, 718–730. https://doi.org/10.1111/eva.12487

Kim, S.-Y., Metcalfe, N.B., da Silva, A., Velando, A., 2017b. Thermal conditions during early life influence seasonal maternal strategies in the three-spined stickleback. BMC Ecol 17, 34. https://doi.org/10.1186/s12898-017-0144-x

Kwiatkowski, L., Torres, O., Bopp, L., Aumont, O., Chamberlain, M., Christian, J.R., Dunne, J.P., Gehlen, M., Ilyina, T., John, J.G., Lenton, A., Li, H., Lovenduski, N.S., Orr, J.C., Palmieri, J., Santana-Falcón, Y., Schwinger, J., Séférian, R., Stock, C.A., Tagliabue, A., Takano, Y., Tjiputra, J., Toyama, K., Tsujino, H., Watanabe, M., Yamamoto, A., Yool, A., Ziehn, T., 2020. Twenty-first century ocean warming, acidification, deoxygenation, and upper-ocean nutrient and primary production decline from CMIP6 model projections. Biogeosciences 17, 3439–3470. https://doi.org/10.5194/bg-17-3439-2020

Länge, R., Hutchinson, T.H., Croudace, C.P., Siegmund, F., Schweinfurth, H., Hampe, P., Panter, G.H., Sumpter, J.P., 2001. Effects of the synthetic estrogen 17α-ethinylestradiol on the life-cycle of the fathead minnow (Pimephales promelas). Environmental Toxicology and Chemistry 20, 1216–1227. https://doi.org/10.1002/etc.5620200610

Lenth, R.V., 2016. Least-Squares Means: The R Package lsmeans. Journal of Statistical Software 69, 1–33. https://doi.org/10.18637/jss.v069.i01

Lewis, E., Wallace, D.W.R., 2012. MS Excel Program Developed for CO2 System Calculations. Carbon Dioxide Information Analysis Center (CDIAC), Environmental Sciences Division, Oak Ridge, TN, USA. https://doi.org/10.3334/CDIAC/OTG.CO2SYS_XLS_CDIAC105A

Lu, H., Cui, Y., Jiang, L., Ge, W., 2017. Functional Analysis of Nuclear Estrogen Receptors in Zebrafish Reproduction by Genome Editing Approach. Endocrinology 158, 2292–2308. https://doi.org/10.1210/en.2017-00215

Maunder, R.J., Matthiessen, P., Sumpter, J.P., Pottinger, T.G., 2007. Impaired Reproduction in Three-Spined Sticklebacks Exposed to Ethinyl Estradiol as Juveniles1. Biology of Reproduction 77, 999–1006. https://doi.org/10.1095/biolreprod.107.062240

"This work is licensed under a Creative Commons Attribution 4.0 International License"

This preprint research paper has not been peer reviewed. Electronic copy available at: https://ssrn.com/abstract=4795483

Mehrbach, C., Culberson, C.H., Hawley, J.E., Pytkowicx, R.M., 1973. Measurement of the apparent dissociation constants of carbonic acid in seawater at atmospheric pressure. Limnol. Oceanogr. 18, 897–907. https://doi.org/10.4319/lo.1973.18.6.0897

Meinshausen, M., Nicholls, Z.R.J., Lewis, J., Gidden, M.J., Vogel, E., Freund, M., Beyerle, U., Gessner, C., Nauels, A., Bauer, N., Canadell, J.G., Daniel, J.S., John, A., Krummel, P.B., Luderer, G., Meinshausen, N., Montzka, S.A., Rayner, P.J., Reimann, S., Smith, S.J., van den Berg, M., Velders, G.J.M., Vollmer, M.K., Wang, R.H.J., 2020. The shared socio-economic pathway (SSP) greenhouse gas concentrations and their extensions to 2500. Geoscientific Model Development 13, 3571–3605. https://doi.org/10.5194/gmd-13-3571-2020

Miranda, L.A., Chalde, T., Elisio, M., Strüssmann, C.A., 2013. Effects of global warming on fish reproductive endocrine axis, with special emphasis in pejerrey Odontesthes bonariensis. General and Comparative Endocrinology, 7th International Symposium on Fish Endocrinology 192, 45–54. https://doi.org/10.1016/j.ygcen.2013.02.034

OECD, 2019. Test No. 203: Fish, Acute Toxicity Test, OECD Guidelines for the Testing of Chemicals, Section 2. OECD. https://doi.org/10.1787/9789264069961-en

OECD, 2013. Test No. 236: Fish Embryo Acute Toxicity (FET) Test. Organisation for Economic Co-operation and Development, Paris.

OECD, 2011. Test No. 234: Fish Sexual Development Test, OECD Guidelines for the Testing of Chemicals, Section 2. OECD. https://doi.org/10.1787/9789264122369-en

Pimentel, M.S., Faleiro, F., Dionísio, G., Repolho, T., Pousão-Ferreira, P., Machado, J., Rosa, R., 2014. Defective skeletogenesis and oversized otoliths in fish early stages in a changing ocean. Journal of Experimental Biology 217, 2062–2070. https://doi.org/10.1242/jeb.092635

Pinheiro, J., Bates, D., 2000. Mixed-Effects Models in S and S-PLUS, Statistics and Computing. Springer-Verlag, New York. https://doi.org/10.1007/b98882

Porseryd, T., Larsson, J., Kellner, M., Bollner, T., Dinnétz, P., Porsch Hällström, I., 2019. Altered non-reproductive behavior and feminization caused by developmental exposure to 17α-ethinylestradiol persist to adulthood in three-spined stickleback (Gasterosteus aculeatus). Aquatic Toxicology 207, 142–152. https://doi.org/10.1016/j.aquatox.2018.11.024

Porseryd, T., Volkova, K., Reyhanian Caspillo, N., Källman, T., Dinnetz, P., Porsh Hällström, I., 2017. Persistent Effects of Developmental Exposure to 17α-Ethinylestradiol on the Zebrafish (Danio rerio) Brain Transcriptome and Behavior. Front. Behav. Neurosci. 11, 69. https://doi.org/10.3389/fnbeh.2017.00069

"This work is licensed under a Creative Commons Attribution 4.0 International License"

This preprint research paper has not been peer reviewed. Electronic copy available at: https://ssrn.com/abstract=4795483

804 Pörtner, H.-O., Karl, D.M., Boyd, P.W., Cheung, W.W.L., Lluch-Cota, S.E., Nojiri, Y., D.N. Schmidt,
805 P.O. Zavialov, 2014. Ocean Systems, in: Field, C.B., Barros, V.R., Dokken, D.J., Mach, K.J.,
806 Mastrandrea, M.D., T.E. Bilir, M. Chatterjee, K.L. Ebi, Y.O. Estrada, R.C. Genova, B. Girma, E.S.
807 Kissel, A.N. Levy, S. MacCracken, P.R. Mastrandrea, L.L. White (Eds.), Climate Change 2014:
808 Impacts, Adaptation, and Vulnerability. Part A: Global and Sectoral Aspects. Contribution of Working
809 Group II to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change. Cambridge
810 University Press, Cambridge, United Kingdom and New York, NY, USA, pp. 411–484.
811 https://doi.org/10.1017/CBO9781107415379.011

812 Przeslawski, R., Byrne, M., Mellin, C., 2015. A review and meta-analysis of the effects of multiple
813 abiotic stressors on marine embryos and larvae. Glob Change Biol 21, 2122–2140.
814 https://doi.org/10.1111/gcb.12833

815 R Core Team, 2018. R: A language and environment for statistical computing.

816 Ramler, D., Mitteroecker, P., Shama, L.N.S., Wegner, K.M., Ahnelt, H., 2014. Nonlinear effects of
817 temperature on body form and developmental canalization in the threespine stickleback. Journal of
818 Evolutionary Biology 27, 497–507. https://doi.org/10.1111/jeb.12311

819 Saaristo, M., Johnstone, C.P., Xu, K., Allinson, M., Wong, B.B.M., 2019. The endocrine disruptor, 17α-
820 ethinyl estradiol, alters male mate choice in a freshwater fish. Aquatic Toxicology 208, 118–125.
821 https://doi.org/10.1016/j.aquatox.2019.01.006

822 Salinas, S., Brown, S.C., Mangel, M., Munch, S.B., 2013. Non-genetic inheritance and changing
823 environments. Non-Genetic Inheritance 1. https://doi.org/10.2478/ngi-2013-0005

824 Schade, F.M., Clemmesen, C., Mathias Wegner, K., 2014. Within- and transgenerational effects of
825 ocean acidification on life history of marine three-spined stickleback (Gasterosteus aculeatus). Mar Biol
826 161, 1667–1676. https://doi.org/10.1007/s00227-014-2450-6

827 Schneider, C.A., Rasband, W.S., Eliceiri, K.W., 2012. NIH Image to ImageJ: 25 years of image analysis.
828 Nat Methods 9, 671–675. https://doi.org/10.1038/nmeth.2089

829 Sganga, D.E., Dahlke, F.T., Sørensen, S.R., Butts, I.A.E., Tomkiewicz, J., Mazurais, D., Servili, A.,
830 Bertolini, F., Politis, S.N., 2022. CO2 induced seawater acidification impacts survival and development
831 of European eel embryos. PLoS ONE 17, e0267228. https://doi.org/10.1371/journal.pone.0267228

832 Shama, L.N.S., 2017. The mean and variance of climate change in the oceans: hidden evolutionary
833 potential under stochastic environmental variability in marine sticklebacks. Sci Rep 7, 8889.
834 https://doi.org/10.1038/s41598-017-07140-9

"This work is licensed under a Creative Commons Attribution 4.0 International License"

Preprint not peer reviewed

This preprint research paper has not been peer reviewed. Electronic copy available at: https://ssrn.com/abstract=4795483

Shama, L.N.S., 2015. Bet hedging in a warming ocean: predictability of maternal environment shapes offspring size variation in marine sticklebacks. Glob Change Biol 21, 4387–4400. https://doi.org/10.1111/gcb.13041

Shao, Y.T., Roufidou, C., Chung, P.C., Borg, B., 2019. Changes in kisspeptin, GnRH, and gonadotropin mRNA levels in male threespine stickleback (Gasterosteus aculeatus) during photoperiod-induced sexual maturation.

Shved, N., Berishvili, G., Baroiller, J.-F., Segner, H., Reinecke, M., 2008. Environmentally Relevant Concentrations of 17α-Ethinylestradiol (EE2) Interfere With the Growth Hormone (GH)/Insulin-Like Growth Factor (IGF)-I System in Developing Bony Fish. Toxicological Sciences 106, 93–102. https://doi.org/10.1093/toxsci/kfn150

Sokolowska, E., Kulczykowska, E., 2006. Annual reproductive cycle in two free living populations of three-spined stickleback (Gasterosteus aculeatus L.): patterns of ovarian and testicular development. Oceanologia 48.

Sridevi, P., Chaitanya, R.K., Prathibha, Y., Balakrishna, S.L., Dutta-Gupta, A., Senthilkumaran, B., 2015. Early exposure of 17α-ethynylestradiol and diethylstilbestrol induces morphological changes and alters ovarian steroidogenic pathway enzyme gene expression in catfish, *Clarias gariepinus*: EE $_2$ and Des Affect Ovarian Steroidogenesis. Environ. Toxicol. 30, 439–451. https://doi.org/10.1002/tox.21920

Swarup, H., 1958. Stages in the Development of the Stickleback *Gasterosteus aculeatus* (L.). Development 6, 373–383. https://doi.org/10.1242/dev.6.3.373

Thorpe, K.L., Cummings, R.I., Hutchinson, T.H., Scholze, M., Brighty, G., Sumpter, J.P., Tyler, C.R., 2003. Relative Potencies and Combination Effects of Steroidal Estrogens in Fish. Environ. Sci. Technol. 37, 1142–1149. https://doi.org/10.1021/es0201348

Untergasser, A., Nijveen, H., Rao, X., Bisseling, T., Geurts, R., Leunissen, J.A.M., 2007. Primer3Plus, an enhanced web interface to Primer3. Nucleic Acids Research 35, W71–W74. https://doi.org/10.1093/nar/gkm306

Van den Belt, K., Verheyen, R., Witters, H., 2003. Effects of 17α-ethynylestradiol in a partial life-cycle test with zebrafish (Danio rerio): effects on growth, gonads and female reproductive success. Science of The Total Environment 309, 127–137. https://doi.org/10.1016/S0048-9697(03)00046-9

Voisin, A.-S., Fellous, A., Earley, R.L., Silvestre, F., 2016. Delayed impacts of developmental exposure to 17-α-ethinylestradiol in the self-fertilizing fish Kryptolebias marmoratus. Aquatic Toxicology 180, 247–257. https://doi.org/10.1016/j.aquatox.2016.10.003

"This work is licensed under a Creative Commons Attribution 4.0 International License"

Preprint not peer reviewed

This preprint research paper has not been peer reviewed. Electronic copy available at: https://ssrn.com/abstract=4795483

Watts, M., Pankhurst, N.W., King, H.R., Geraghty, D.P., 2005. Differential effects of temperature and maturity stage on hepatic estrogen receptor characteristics of Atlantic salmon. Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology 140, 377–383. https://doi.org/10.1016/j.cbpb.2005.02.002

Wernicke von Siebenthal, E., Rehberger, K., Bailey, C., Ros, A., Herzog, E.L., Segner, H., 2018. Trade-Offs Underwater: Physiological Plasticity of Rainbow Trout (Oncorhynchus mykiss) Confronted by Multiple Stressors. Fishes 3, 49. https://doi.org/10.3390/fishes3040049

Zha, J., Wang, Z., Wang, N., Ingersoll, C., 2007. Histological alternation and vitellogenin induction in adult rare minnow (Gobiocypris rarus) after exposure to ethynylestradiol and nonylphenol. Chemosphere 66, 488–495. https://doi.org/10.1016/j.chemosphere.2006.05.071

Preprint not peer reviewed

"This work is licensed under a Creative Commons Attribution 4.0 International License"

This preprint research paper has not been peer reviewed. Electronic copy available at: https://ssrn.com/abstract=4795483