Impacts of Long-Term Exposure to Ocean Acidification and Warming on Three-Spined Stickleback (Gasterosteus aculeatus) Growth and Reproduction

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Abstract: The warming and acidification of surface waters as predicted by the IPCC leads aquatic species to face major multifaceted changes in their environment. Although teleosts have efficient regulatory systems to cope with these changes, such changes clearly have the potential to impact their physiological functions. Hence, it is crucial to estimate the ability of teleost fishes to cope with multi-stresses to predict how they will deal with future environments. In this context, we investigated the joint effect of warming and acidification on three-spined stickleback (*Gasterosteus aculeatus*) from the juvenile stage to adulthood, focusing on parameters linked to growth, sexual maturation, and reproduction. Juvenile sticklebacks were split in 2 climate scenarios: a "Current" scenario corresponding to the current seasonal physico-chemical parameters of the water of the "Rade de Brest" in France, and a "RCP8.5" scenario with a warming of 3 °C and an acidification of 0.4 pH units. After 7 months, fish in the RCP8.5 scenario reached the same size and mass as those in the Current scenario, but they needed greater amounts of food to reach satiety. Furthermore, the mortality rate over the experiment was higher in the RCP8.5 scenario. Muscle lipid content, an indicator of energy reserves, was lower in females in the RCP8.5 scenario, suggesting an increased need for energy to maintain homeostasis and other physiological functions or a divergence in energy allocation strategy. Moreover, females exhibited lower sexual maturation and egg quality under the RCP8.5 scenario, which could have contributed to the lower fertilisation rate observed. Males were more resilient to the RCP8.5 scenario, exhibiting only a trend for lower kidney somatic index scores. Altogether, these results suggest a delay and/or an inhibition of gametogenesis and maturation in fish in warmed and acidified waters. The analysis of blood sex steroid concentrations, brain gene expression profiles, and physiological indexes did not allow us to discriminate between a delay and an inhibition of maturation in the RCP8.5 scenario. Overall, these findings clearly indicate that there is a long-term global impact of combined acidification and warming on the mortality and reproductive performance of three-spined stickleback.

Keywords: *Gasterosteus aculeatus*; climate change; life cycle; sexual maturation; long-term acclimation; lipids; multi-stress

Key Contribution: (1) In a warming and acidification experiment (+3 °C; −0.4 pH), conducted over 7 months, with an *ad libitum* feeding, the growth of stickleback (*G. aculeatus*) was not affected but the mortality rate was higher. (2) The lower fertilisation rate was associated with lower muscle lipid content in females and poorer egg quality.

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1. Introduction

Since the beginning of the industrial revolution in the 19th century that generated the expansion of anthropic activities, the levels of greenhouse gas emissions have never stopped increasing, with for instance, the rise in the partial pressure of carbon dioxide (pCO_2) in the atmosphere from 280 µatm during the preindustrial period to 380 µatm nowadays [1]. The increased atmospheric CO_2 has led to alterations in multiple physicochemical parameters of seawater (temperature, pH, oxygen and salinity [1,2]). The most pessimistic scenario of the Intergovernmental Panel on Climate Change (IPCC), named representative concentration pathway 8.5 (RCP8.5), predicts a warming of the sea surface temperature of 3.04 ± 0.62 °C and an acidification of 0.38 ± 0.005 pH units by 2100 [1,2].

Such changes in environmental conditions can affect the physiology and behavior of aquatic organisms as most of them are poikilothermic (i.e., unable to regulate their internal temperature), including a majority of fish species [3–5]. As a result, all of their physiological functions can be directly influenced by environmental temperature fluctuations [6]. The effects of warming on fish physiology have been widely studied, showing, for example, that even a rise of few degrees in the environment can lead to an increase in growth and metabolic rates in several fish species [7–12]. Warming is known to also impact fish sexual maturation [4,13,14], steroidogenesis [14–17] and development [3,18] by modifying molecular structures and rates of biochemical reactions with an end impact on enzymatic activities [19,20], gene expression profiles [14,16,17,21] and plasma hormone concentrations [4,15,22], as reviewed by many studies [23–25]. Ocean acidification is another major process directly related to increased pCO_2. This environmental hypercapnia leads to acidosis of the body/fluids of aquatic fauna by accumulation of carbonic acid (H_2CO_3) [26]. In teleosts, who possess an efficient acid–base regulation, body acidosis is compensated by increased gill bicarbonate concentration (HCO_3^-), which could potentially drive an alteration in internal ionic concentrations, with possible consequences on neurotransmission [27,28]. The end result could be impacts on key physiological functions (basal metabolism, growth, reproduction) [29–32], behaviour (swimming activity, foraging, etc.) [31,33], and ultimately increase the vulnerability of the species [30,31].

As ocean warming and acidification will occur simultaneously under future climate change circumstances, it is essential to consider the combination of these factors and determine how organisms will cope with future environments. Results from previous studies evaluating the influence of several stressors combined are increasing. Most of these studies reported the effects of ocean warming and acidification on the early stages of physiological functions of the commercially important species. For instance, several studies about the futures projections of warming (4–5 °C) and acidification (0.3–0.4 pH unit) showed negative impacts on growth [34,35], survival [34], malformations [34,35], and digestive enzymes activity [35] in *Solea senegalensis*. In similar experimental designs, decreases in larval survival of Atlantic cod [36], gilthead seabream, and meagre [31] were also reported, associated in the two latter cases with increased malformation rates and behavioural impairment [31]. Regarding the combined effect of warming and acidification on the metabolism of early stages of marine fish, the reported results exhibit different physiological responses depending on the species [35,37,38], indicating inter-specific variations in sensitivity. Altogether, this reveals the need of further studies on different species (i.e., including non-commercially important species) and longer exposure duration also covering the most advanced life stages to enable more realistic predictions of the impact of climate change on fish physiology. Indeed, (i) over focus on a few model species makes it difficult to extrapolate results to other context and (ii) limiting the evaluation to short-term effects at early stages can be misleading since the main consequences of warming and acidification may occur in later life stages.

The three-spined stickleback (*Gasterosteous aculeatus*, Linnaeus, 1758) is an amphihaline teleost fish which is widely distributed over the northern part of the globe. It has been heavily used as a model species, providing a strong background knowledge of its physiology and ecology [39–41]. Furthermore, the full sequencing of its genome provides

tools to investigate the effects of environmental variations in its physiology at the molecular level [42,43]. Its small size and short life cycle make this species easy to rear in lab facilities, enabling the possibility to follow all life stages in a relatively short time span (sexual maturity at one year). These characteristics make the stickleback a good model for long-term studies of environmental variations in fish physiology.

Several studies have already shown direct effects of increased temperatures in stickleback, such as lower growth and mass [44], lower fecundity [10], smaller egg diameter and faster egg development [45–47]. Metabolic and redox processes were also shown to be upregulated at higher temperatures, as was the expression of certain genes involved in reproduction and growth, such as *smtlb*, *pomca* and *tshba* [10,48]. Previous studies have also reported the impacts of acidified water, with higher fecundity under high acidification [32] and no effect of pH on egg survival and diameter [45]. However, to our knowledge, the resilience of this species to multiple stressors of climatic and chemical origin is still unknown.

In this context, we aimed at testing whether ocean acidification and warming together could negatively impact the growth and reproductive performance of three-spined sticklebacks. For this purpose, juvenile sticklebacks were subjected to multi-stress (pH and temperature), as predicted by the IPCC RCP8.5 to occur by 2100, for 7 months, until adulthood. According to the new set of scenarios, the shared socio-economic pathways (SSP), which combine pathways of atmospheric greenhouse gas concentration (as in the previous RCP scenarios) to socio-economic projections [49], the experimental scenario tested in the present study, named RCP8.5, would correspond to both the SSP3-7.0 and the SSP5-8.5 scenarios [50].

During this multi-stress experiment, and in addition to estimating mortality rates, we evaluated the impact of acidification and warming on growth by estimating size, mass and physiological indexes as well as the feed conversion ratio in each experimental scenario. The potential disruption of the individuals' sexual maturation was tested by combining hormonal, histological, and molecular approaches (at the final sampling point, after 7 months of the experiment). Finally, we investigated the impact of ocean warming and acidification on the individuals' reproductive success by comparing eggs and sperm quality as well as the fertilisation rates. We are aware that without a complete cross-factorial design, it is not possible to distinguish whether the observed effects on fish physiology are due to just one factor or to their interaction. However, in the present study, the goal is to evaluate the global impact of ocean acidification and warming together to obtain a more realistic understanding of what will occur in nature.

2. Materials and Methods

2.1. Animals and Experimental Design

A population of freshwater juvenile three-spined stickleback (*Gasterosteus aculeatus*), born in July 2020, were obtained on the 7th of October 2020 from the experimental freshwater mesocosms of the *Institut national de l'environnement industriel et des risques* (E60-769-02, INERIS, Verneuil-en-Halatte, France). The stabling of the fish was performed within Ifremer's experimental facilities in Brest (Brittany, France), at the aquaculture facility of the laboratory of Ecophysiology and Life History Traits of Marine Organisms (agreement number B29-212-05). On arrival at the Brest experimental facility, these fish were placed for a week in water at a salinity level of 10‰, then gradually acclimated to seawater (increasing the salinity by 10‰ per week) until the conditions reached the 34‰ salinity level of Brest Bay. The fish were fed once a day with frozen Artemia and bloodworms (proportion 1:4, Antinea, France). The food was distributed progressively into the tank until satiety was reached. The satiety of the fish population in a tank was determined by observation as a cessation of eating behaviour. The theoretical food consumption was calculated as the difference between mass of food available and mass of food not distributed, once satiety was reached. This amount was used to adjust the ration per tank for the next day. One month after salinity acclimation, the juvenile population, then around 4 months old, was

randomly divided into 2 tanks corresponding to 2 experimental groups of 557 fish per tank. Then, on 12 December 2020, after acclimating to these new conditions for around 3 weeks, during which no mortality was observed, the 5-month-old fish were subjected to two experimental scenarios. The rearing conditions of the 1st group (labelled "Current scenario") correspond to the current temperature and pH of the Bay of Brest. The second group (labelled "RCP8.5 scenario") was subjected to warming (+3 °C) and acidification (−0.4 pH units). These scenarios simulate the IPCC RCP8.5 scenario predicted for 2100 [26]. The experimental tanks were supplied with flow-through seawater directly pumped at a location 500 m from the coastline from a depth of 20 m in the Bay of Brest. The water was filtered by passing through a sand filter (Waterco Lacron, Australia), heated via a tungsten plate heat exchanger (Vicarb, Sweden), degassed through a column packed with plastic rings, refiltered with a 2 µm membrane, and sterilized by a UV lamp (PZ50, 75W, Ocene, France). The RCP8.5 scenario was supplied with sea water via a header tank where gas CO_2 was diffused in. The water temperature and pH were monitored daily (Figure 1) with a WTW 3110 pH meter (Xylem Analytics Germany, Weilheim, Germany; with electrode: WTW Sentix 41, NBS scale). The devices were daily calibrated with NBS certified WTW technical buffers pH 4.01 and pH 7.00 (Xylem Analytics Germany, Weilheim, Germany; the values are summarized in Table 1). Juveniles and adults experienced a temperature range from 11.3 to 17.9 °C in the Current scenario and from 13.2 to 20.6 °C in the RCP8.5 scenario, following the natural temperature fluctuation of the Bay of Brest. The photoperiod regime was also set to simulate the natural photoperiod of the Bay of Brest (Halogen lamp at 42W, 55–60 lux).

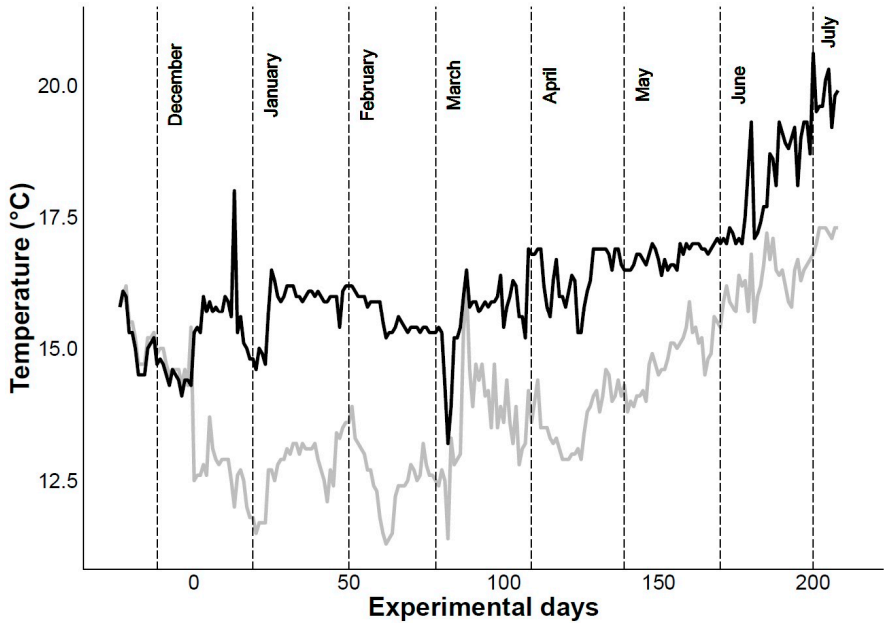

Figure 1. Temperature (°C) in the Current (in grey) and RCP8.5 (in black) scenarios throughout the experiment.

Table 1. Mean physico-chemical conditions recorded twice a month during the total rearing period (mean ± S.D.). TA: total alkalinity, pCO_2: partial pressure of CO_2, mean ± se calculated on the monthly (7 months) measure of alkalinity.

Rearing Scenarios	Salinity (‰)	O_2 (%)	Temperature (°C)		pH Total	TA ($\mu mol \cdot kg_{sw}^{-1}$)	pCO_2 (µatm)
			Min	*Max*			
Current	32.70 ± 0.98	96.54 ± 4.49	11.90	17.90	7.95 ± 0.06	2184.07 ± 86.59	527.78 ± 135.91
RCP8.5	32.79 ± 0.99	96.67 ± 3.82	15.40	20.30	7.53 ± 0.05	2193.56 ± 138.72	1509.03 ± 265.52

The total alkalinity of the seawater was measured twice a month according to the protocol described in Cohen-Rengifo et al. [51] adapted from the protocol of Anderson and Robinson [52] and Strickland and Parsons [53]. Salinity and Oxygen (O_2) were measured simultaneously with a WTW LF 340 salinometer (Xylem Analytics Germany, Weilheim, Germany; with electrode: WTW Tetracon® 325/C, ‰ scale) and a WTW Oxi 340 oximeter (Xylem Analytics Germany, Weilheim, Germany; with electrode: WTW CellOx 325, % scale). Then, the pCO_2 was calculated through the CO2sys macro [54] with the constants from Mehrbach et al. [55] and the refit by Dickson and Millero [56]. The total scale pH was afterwards calculated using pCO_2 values from the same macro (Table 1).

2.2. Non-Invasive Biometric Approaches

During the 7-month rearing period, a sample of 144 fish were collected (and returned) from each experimental scenario every two months to assess their mass and size and subsequently calculate the thermal growth coefficient of each experimental group. Sedated (0.05 $g{\cdot}L^{-1}$ of Tricaine methane sulfonate 222 (MS222), Pharmaq, Fordingbridge, Hampshire, UK) fish were weighed (Mettler-Toledo, MS4002TSDR, Switzerland) and photographed (NIKON, D7200, Japan) in 12 batches of 12 fish. Because MS222 residues are eliminated rapidly from the tissues of fish medicated by bath administration, even when used repeatedly [57], and due to the low doses used here, we exclude any long-term impact of MS222 on fish physiology. To measure the individuals' size, pictures of each batch were taken in front of a scale and measurements were carried out using the ImageJ software (Version 1.53e, Java 1.8.0_172; [58]). The feed conversion ratio (FCR; [59]), mortality rate, and thermal growth coefficients (TGC; [60]) were measured using the following formulas: FCR = (Total mass of food consumed in the tank)/(Biomass gain in the tank); Mortality rate = (Total number of dead fish)/(Total number of fish); TGC = $(Mass_{final}{}^{1/3} - Mass_{initial}{}^{1/3})/(\sum(\text{Temperature}))$, where $\sum$(Temperature) corresponds to the accumulation of temperature in degrees Celsius experienced by the stickleback.

2.3. Final End-Points

2.3.1. Individual Physiological Indexes

In July, after 7 months of acclimation to the climate conditions, a sampling was carried out on 33 fish in total. The remaining adult sticklebacks were preserved for longer exposure for use in further studies. The fish were fasted for 24 h to reduce faecal contamination of measures and risk of regurgitation. Before sampling, the fish were anaesthetised with 0.125 $g{\cdot}L^{-1}$ MS222 in the tank, and then fished and euthanized with a lethal concentration of 0.250 $g{\cdot}L^{-1}$ MS222. Individual biometric data, including the standard body length, body mass, Fulton index corrected from the mass of the gonads (K_{corr}), gonado-somatic index (GSI), hepato-somatic index (HSI) and kidney-somatic index (KSI), were estimated (Sartorius, MC BA 1000, Germany) using the following formulas [61]: K_{corr} = ((Mass of the fish − Mass of the gonads)/(Size of the fish)^3) × 100; GSI = ((Mass of the gonads)/(Mass of the whole body)) × 100; HSI = ((Mass of the liver)/(Mass of the whole body)) × 100; and KSI = ((Mass of the kidney)/(Mass of the whole body)) × 100.

2.3.2. Lipids Extraction and Analysis

The total lipid content of fish muscle was assessed individually. During the extraction, the atmosphere in the tube was replaced by azote gas as far as possible to preserve the lipids. White muscle tissue was flash frozen in liquid nitrogen after sampling and kept at −80 °C until extraction. The total lipid content of each muscle sample was extracted following a modified Folch method [62]. The micro-extraction was initiated by homogenising 100 to 500 mg of tissues, with the addition of an equivalent amount of Na_2So_4, using a polytron in Dichloromethane:Methanol (98:2) solution [62]. Afterword, the following extraction process was repeated thrice: after shaking for 3 min with a vortex, the homogenate was run through an ultrasonic tank for 5 min and centrifuged at 4 °C for 10 min at 1.500 G to collect the supernatant in a separate tube. The samples were concentrated in a SpeedVac

at 45 °C for 1 h. The concentrates were then transferred to clean, tared, round-bottomed reaction tubes and all the remaining solvent was removed by impinging with a stream of nitrogen. The total lipid extracts (TL) were measured to the nearest 0.01 mg using an analytical balance (Sartorius, MSE225P-100-DU, Germany).

2.3.3. RNA Extraction and Purification

The fish brains were quickly removed and the median region containing the hypothalamus was dissected and stored in an RNA stabilization reagent (RNAlater, Qiagen, Hilden, Germany) at 4 °C for 24 h and then placed at −20 °C until the extraction. The total RNA extraction and purification of the median brain was performed with the Nucleospin® RNA_{XS} kit (Macherey Nagel, Germany) according the protocol of the supplier (Version January 2020/Rev.10). The concentration and purity of the RNA were assessed with Nanodrop™ 2000 (Thermo Scientific Inc., Waltham, MA, USA) and the integrity of the RNA was assessed using Agilent Tapstation 4150 (Agilent Technologies Inc., Santa Clara, CA, USA). All the samples showed an integrity number (RIN) higher than 7.4 and were stored at −80 °C until used in reverse-transcription quantitative PCR (RT-qPCR) analysis.

2.3.4. RT-qPCR Analysis

The reverse transcription (RT) of cDNA was performed on all the RNA samples with positive and negative (without enzyme) reaction by following the protocol recommended by the supplier, with 500 ng of RNA, using the iScript™ cDNA Synthesis kit (Bio-Rad Laboratories Inc., Hercules, CA, USA) and run in ThermalCycler T100™ (Bio-Rad Laboratories Inc.). Then, cDNA was stored at −20 °C until qPCR.

The qPCR was performed on the following genes of interest: Gonadotropin Releasing Hormone 2 and 3 (*gnrh2*, *gnrh3*), aromatase (*cyp19a1b*), Kisspeptin 2 (*kiss2*), Gonadotropin-Inhibitory Hormone (*gnih*), and 2 housekeeping genes (Ribosomal Protein L8 (*rpl8*), and Ribosomal Protein L13A (*rpl13a*)). The primers were specially designed (Table 2) for this study with the primer 3Plus software [63].

Table 2. Designed primers used in the qPCR analysis in the median brain.

Gene Symbol	Forward Primer	Reverse Primer	GenBank Accession Number
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

The qPCR was performed with the CFX96 Touch Real-Time PCR Detection system (Bio-Rad Laboratories Inc.). The efficiency of the primer pairs was calculated by using serial dilution (1/5, 1/10, 1/20 and 1/40) of a cDNA pool. Each well was filled with 5 µL of cDNA (1/20 dilution) to conduct the qPCR in duplicate, followed by 10 µL of reaction mix made up of 333 nM of each primer pair, 7.5 µL of SsoAdvanced Universal SYBR® Green Supermix (Bio-Rad Laboratories Inc.) and completed with of RNase/DNase-free water. The qPCR program initial activation step started at 98 °C for 2 min, followed by 40 cycles of 5 s at 95 °C and 20 s at 60 °C. The protocol ended with the generation of a high-resolution melting curve after the amplification phase in order to confirm 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 sample. *rpl8* and *rpl13a* were used as reference genes in the ΔΔCt method to normalize the relative quantity of transcripts. No significant differences in Cq values were observed for housekeeping genes between experimental scenarios.

2.3.5. Histological Assessment of the Gonadal Maturation Stage

After sampling, and weighing, gonads were fixed in paraformaldehyde (PFA) 4% for 24 h at 4 °C. Then, the tissues were washed in phosphate-buffer saline (PBS) solution and 0.85% sodium chloride (NaCl), each for 15 min. Next, 5 washes of increasing ethanol solution (70% to 100%) were performed at 4 °C to dehydrate the samples. The samples were lightly shaken overnight in 100% ethanol, then in 2 xylene baths. Finally, they were impregnated into warm filtrate paraffin. The gonads were then embedded into cassettes, the anterior part closest to the cut. A microtome (RM 2035, Leica, UK) was used to make 5 µm thick slices starting at 200 to 250 µm from the apical lobe of the gonad. The slices were dried on glass slides at 37 °C overnight and warmed at 53 °C for 20 min before the trichrome Prenant–Gabe staining. The slides were dewaxed into xylene and gradually rehydrated in decreasing ethanol solutions (100% to 70%) and distilled water. Staining of the nucleus, in purplish blue, was obtained with 1% Weigert's Haematoxylin (1 min), cleared by tap and distilled water. The cytoplasm was stained in red with 1% aqueous 1% Eosin Y (8 min) and the conjunctive tissue in green after an aqueous Phosphomolybdique acid (30 s) bath by 0.05% aqueous Light Green (30 s). The tissues were dehydrated by 2 baths of 100% ethanol and xylene. The slides were mounted with Eukitt® medium (ORSAtec, Royaume-Unis). Analysis and photography (EOS 700D, Canon, Japan) were performed under a light microscope (AXIO observer Z1, ZEISS, Germany). In total, 5 different regions of 8 gonads per sex and scenario were analysed to estimate the maturity of gametes identified. To evaluate maturation, the stages described by Sokolowska and Kulczykowska [64] and Furin et al. [65] were pooled into 5 and 4 groups for females and males, respectively. This resulted in the following classification of maturation stages in ovaries: "Early" (stages 1 and 2), "Midterm" (stages 3 to 4), "Late" (stages 5 to 6), "Mature" (stages 7 to 8), and "Regression" stages (stage 9). In the testis, the classification groups analysed were "Early" (stages 6 and 7), "Midterm" (stages 8 to 9), "Late" (stages 1 to 2), "Mature" (stages 3 to 4), and "Spawning" stages (stage 5). For statistical purposes, to evaluate the maturation of females, an advanced maturation oocytes stage corresponding to the "Late" and "Mature" groups was created. The "Spawning" group was used in the statistical analysis to test differences in maturation in males.

2.3.6. Steroid Blood Analysis

After sampling, the blood samples were preserved at −80 °C in a storage solution (50% PBS, 30% heparin, 20% glycerol, 1% protein inhibitor). The quantification of blood 11-ketotestosterone and 17-β-estradiol in males was carried out by commercial Cayman ELISA kits (Cayman Chemical, Lexington KY, 11-ketotestosterone (Cat # 582751), 17-β-estradiol (Cat # 501890)), following the supplier's recommendations. These assays have been previously validated for fish blood samples [66,67]. The intra- and inter-assay variations reported were 13.4% and 7.7%, respectively, for a concentration of 12.5 pg·mL^{-1} of 11-ketotestosterone and 33.8% and 27.8%, respectively, for a concentration of 2.4 pg·mL^{-1} of 17-β-estradiol.

2.3.7. Gamete Quality, Reproduction and Fertilisation Rate

During the reproduction period (May–July), the fish exhibited secondary sexual traits such as a large belly showing the shape of the eggs and the red ventral pigmentation of the mature males [64]. To perform in vitro fertilisation (IVF), males were anesthetised and euthanised before extraction and dilaceration of their gonads in 200 µL of 300 mOsmol·kg^{-1} Hank Balanced saline solution (HBSS). The semen was stored on ice until a gravid female fish was sampled, anaesthetised (0.125 g·L^{-1} MS222) and smoothly stripped to extract the eggs. The eggs of each female were counted and fertilised with the sperm solution of 2 males. The sperm viability and spermatozoa concentration were carried out by flow cytometry (MACSQuant X, Miltenyi Biotec, Germany) by using the kit LIVE/DEAD™ Sperm Viability (L7011, ThermoFisher, USA). Briefly, 1 µL of SYBR™ 14 (50-fold diluted) and 1 µL of propidium iodide were incubated with 200 µL of spermatozoa suspension

(diluted 1/1000 with HBSS) for 10 min in the dark at room temperature. Then, the viable and dead cells expressed green (FL1) and red (FL3) fluorescence, respectively. The assessment of the fertilisation rate was carried out 24 h after fertilisation under a binocular magnifier (Discovery.V8 SteREO, ZEISS, Germany). The fertilised eggs were photographed (NIKON, D7200, Japan) with a binocular magnifier (Discovery.V8 SteREO, ZEISS, Germany) at 1 and 3 days post fertilisation (Dpf). Their diameter and the perivitelline index were measured individually using ImageJ software (Version 1.53e, Java 1.8.0_172, [58]) with the following formulas: Fertilisation rate = ((Fertilised eggs)/(Total of eggs)) × 100; and Perivitelline index = ((Perivitellin width)/(Diameter of the fertilised)) × 100.

3. Statistical Analysis

Data analyses were performed using the R software [68]. The significance threshold was set to p-value < 0.05 (See Supplementary Table S4). Prior to the ANOVA analysis, the assumptions of normality of the data and homogeneity of variance were tested by the Shapiro–Wilk test and Bartlett test. Levene's test was used to determine the homogeneity of variance when the assumption of normality was not fulfilled. If the assumption of homogeneity was met, the Kruskal–Wallis rank sum test followed by a pairwise Wilcoxon rank sum post hoc test was used, with Holm's p-value adjustment method [69]. If none of these assumptions for the ANOVA were met, the Welch's heteroscedastic F test, followed by the Games Howell post hoc test, was used.

Pearson's chi-squared test was used to determine the difference in mortality between the two scenarios.

The function glmmPQL from the r package "MASS" was used [70] to identify differences in gonadal maturation with the scenario as the fixed factor and the slide and the observer as random factors. Furthermore, the residuals normality and homogeneity of variance were checked graphically.

In addition, the analyses of the standard length, body mass, K_{corr}, GSI, HIS and KSI were performed for the males and females separately.

4. Results

4.1. Mortality and Biometric Study

Over the 7-month experimental period, mortality occurred homogeneously under both scenarios. A significantly higher mortality rate was observed in the RCP8.5 scenario (p-value = 0.01; Table 3). The final feed conversion ratio (FCR) was 24 $mg_{food} \cdot mg_{body}{}^{-1}$ in the RCP8.5 scenario and 20 $mg_{food} \cdot mg_{body}{}^{-1}$ in the Current scenario, with a mass thermal growth coefficient (mTGC) of 0.27 and 0.31 mg·degree $days^{-1}$, respectively. Table S3 in Supplementary Materials shows the evolution of the stickleback FCR, TGC and mortality rates throughout the 7 months of the experiment.

Table 3. Stickleback mortality and biometric data after 7 months of acclimation to the RCP8.5 scenario. The asterisk indicates a significance between the scenarios.

	Mortality (%)	Standard Length (mm)			Mass (g)		
		Mean ± S.D.	Min	Max	Mean ± S.D.	Min	Max
Current	24.0	40.5 ± 5.8	22.1	55.0	1.4 ± 0.3	0.6	2.1
RCP8.5	31.8 *	41.3 ± 6.2	25.3	51.0	1.4 ± 0.4	0.8	2.5

At the beginning of the experiment (November), the mean standard length and the mass of the fish population were 26.3 ± 4.5 mm and 381.5 ± 191.7 mg, respectively (Figure 2). After 7 months of exposure to the RCP8.5 scenario, the mass of the fish increased significantly with time (p-value < 0.01) in a comparable manner in the two experimental scenarios (Supplementary Table S1), and no significant differences in the final body mass (p-value = 0.80) and standard length (p-value = 0.17) were found.

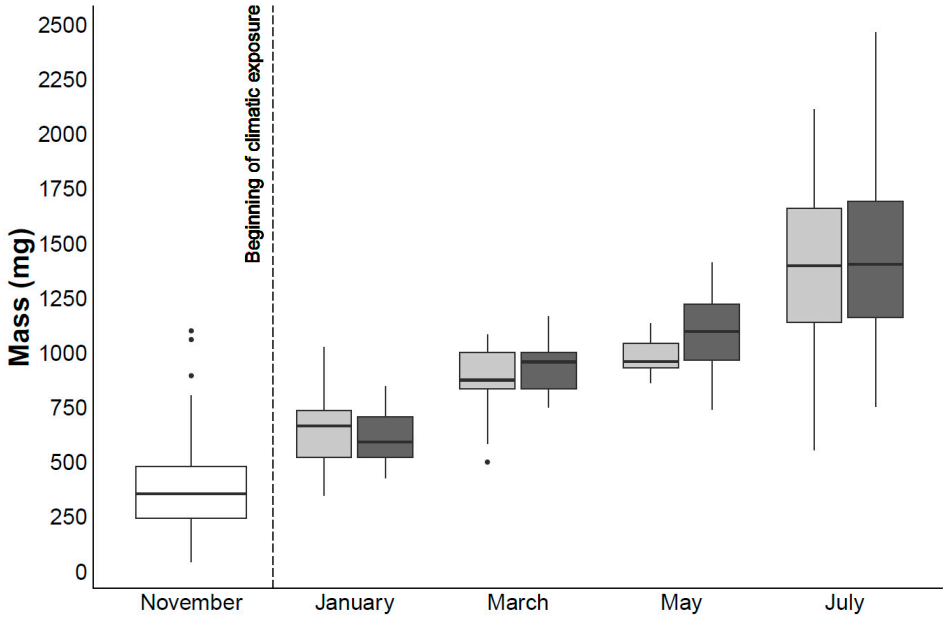

Figure 2. Stickleback mass values under Current (in light grey) and RCP8.5 (in dark grey) scenarios (n = 144 per scenario). The white box represents the mean mass of the population sample before the beginning of the experiment (n = 125). The 10th and 90th percentiles are represented by the whiskers, the 25th and 75th percentiles by the boxes, the median values by horizontal lines and outliers by points.

At the sampling day, the individual body standard length and mass as well as the physiological indexes, including the corrected Fulton (Kcorr), gonado-somatic (GSI), hepato-somatic (HSI), and kidney-somatic (KSI) indexes, were estimated for each gender (Table 4). Male fish reared in the RCP8.5 scenario tended to have lower KSI (males: p-value = 0.06; females: p-value = 0.94) and GSI (males: p-value = 0.80; females: p-value 0.37) than those reared in the Current scenario. There were no significant differences between scenario groups in the other biometric parameters measured, such as the standard length (males: p-value = 0.74; females: p-value 0.09), body mass (males: p-value = 0.70; females: p-value 0.35), Kcorr (males: p-value = 0.99; females: p-value = 0.25), and HSI (males: p-value = 0.32; females: p-value = 0.77).

Table 4. Stickleback standard body length and mass and physiological indexes at the sampling in July, after 7 months of acclimation to the RCP8.5 scenario. K_{corr}: Fulton's index corrected of the mass of the gonads; GSI: gonado-somatic index; HSI: hepato-somatic index; KSI: kidney-somatic index (n = 5–9 per scenario and sex). Data are presented as mean ± S.D.

	Sex	Standard Length (mm)	Mass (g)	K_{corr}	GSI	HSI	KSI
Current	♀	45.0 ± 3.3	1.5 ± 0.2	1.4 ± 0.2	14.6 ± 7.4	4.9 ± 0.7	0.4 ± 0.3
	♂	44.5 ± 2.4	1.3 ± 0.2	1.4 ± 0.1	0.6 ± 0.2	3.6 ± 0.7	2.8 ± 1.0
RCP8.5	♀	47.5 ± 2.1	1.6 ± 0.2	1.3 ± 0.1	11.1 ± 7.6	5.1 ± 1.8	0.4 ± 0.2
	♂	44.9 ± 1.9	1.3 ± 0.2	1.4 ± 0.2	0.8 ± 0.5	4.4 ± 1.9	1.7 ± 0.7

4.2. Muscle Lipids Storage

The total lipid content of the white muscle of stickleback under each scenario (Figure 3) revealed a significantly lower percentage of lipids in the muscle of the females of the RCP8.5 scenario compared with females of the Current scenario and to males, irrespective of the scenario (p-value = 0.01; see Supplementary Table S2).

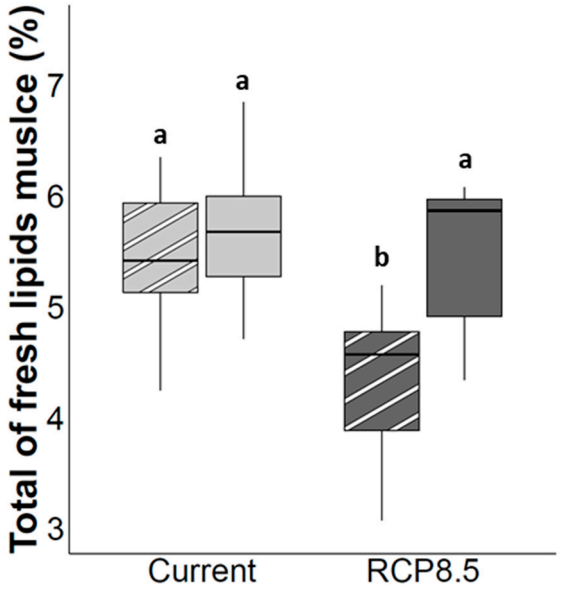

Figure 3. Stickleback muscle lipid storage after 7 months under Current (in light grey) and RCP8.5 (in dark grey) scenarios for female (white stripes) and male (no stripes) (n = 8). The 10th and 90th percentiles are represented by the whiskers, the 25th and 75th percentiles by the boxes, the median values by horizontal lines and outliers by points. Different letters indicate a significant difference between the boxes.

4.3. Genes Expression Profiles in the Brain

The analysis of the gene expression in the median brain (Figure 4) revealed a downregulation of *cyp19a1b* in males under both Current and RCP8.5 scenarios (sex: p-value < 0.01). There were no significant differences between scenarios and sexes in all the other genes analysed (*gnrh2*: p-value = 0.24; *gnrh3*: p-value = 0.11; *kiss2*: p-value = 0.49; *gnih*: p-value = 0.42), although an overall trend suggesting an upregulation under RCP8.5 scenario is observed for all these genes related to reproductive processes. Furthermore, except for *cyp19a1b*, the analysis of the variance for data concerning the expressions of *gnrh2*, *gnrh3*, *kiss2* and *gnih* showed significantly higher interindividual variability in the RCP8.5 scenario (*gnrh2*: p-value = 0.02; *gnrh3*: p-value = 0.02; *kiss2*: p-value = 0.03; *gnih*: p-value = 0.01).

4.4. Gonad Maturation

The evaluation of the ovarian maturation by histology revealed a shift in the proportion of developmental stages between RCP8.5 and Current scenario (Figure 5A), with a significantly lower proportion of advanced maturation oocytes (Figure 5B) in RCP8.5 scenario (p-value = 0.01). In males, the analysis of testicular maturation (Figure 6B) did not reveal any difference in the proportion of tubules in the Spawning stage between the scenarios (p-value = 0.46). The Mature stage was predominant in the testis (Figure 6A) and in the ovary in both scenarios (Figure 5A). The histological structure of the gonadic tissues did not show any alterations.

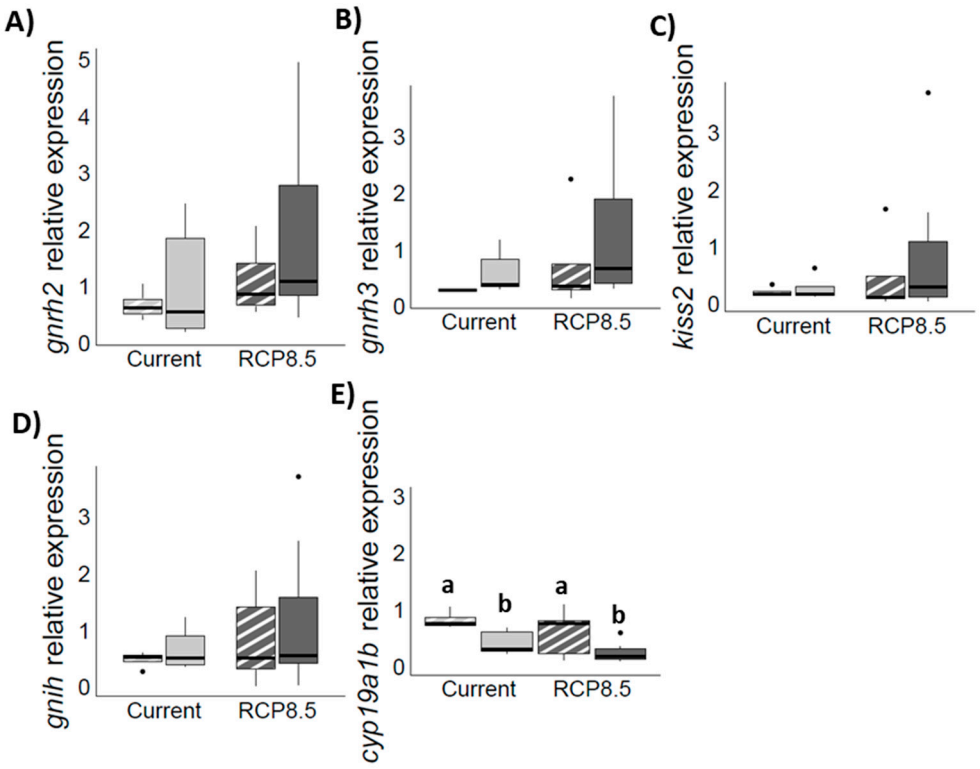

Figure 4. Boxplots representing relative expression of *gnrh2* (**A**), *gnrh3* (**B**), *kiss2* (**C**), *gnih* (**D**) and *cyp19a1b* (**E**) in the median brain of sticklebacks after 7 months of exposure to Current (in light grey) and RCP8.5 (in dark grey) scenarios for female (white stripes) and male (no stripes) (females: $n = 4$ and 5 for Current and RCP8.5 scenarios, respectively; males: $n = 5$ and 7 for Current and RCP8.5, respectively). 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. Different letters indicate a significant difference between the boxes.

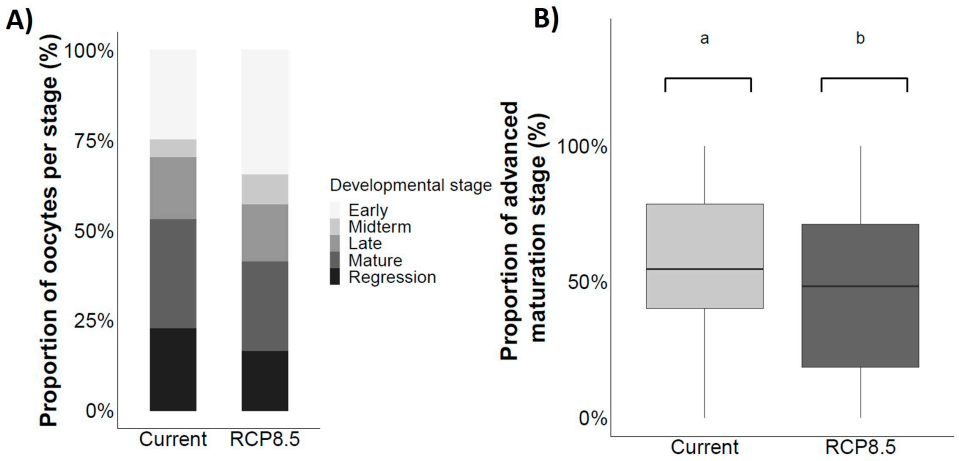

Figure 5. Proportions of oocytes in different maturation stages in stickleback ovaries (**A**) and proportion of advanced maturation oocytes (**B**) under Current (light grey) and RCP8.5 (dark grey) scenarios ($n = 8$). The 10th and 90th percentiles are represented by the whiskers, the 25th and 75th percentiles by the boxes, and the median values by horizontal lines. Different letters indicate a significant difference between the boxes.

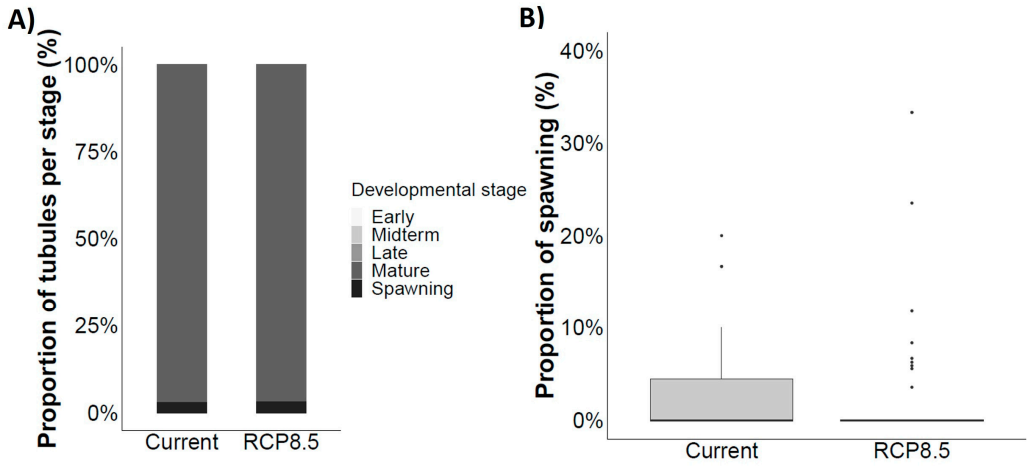

Figure 6. Proportions of tubules in different maturation stages in the stickleback testis (**A**) and proportion of spawning tubules (**B**) under Current (light grey) and RCP8.5 (dark grey) scenarios (n = 8). The 10th and 90th percentiles are represented by the whiskers, the 25th and 75th percentiles by the boxes, the median values by horizontal lines and outliers by points.

4.5. Steroid Hormones

The blood concentrations of 11-keto-testosterone (11-KT; Figure 7A) and 17 β-estradiol (E2; Figure 7B) at the July sampling point did not show any differences between scenarios in males (11-KT: p-value = 0.28; E2: p-value = 0.14). Furthermore, the variance of data revealed a trend in E2 blood concentration, showing higher interindividual variability in the RCP8.5 scenario (p-value = 0.06).

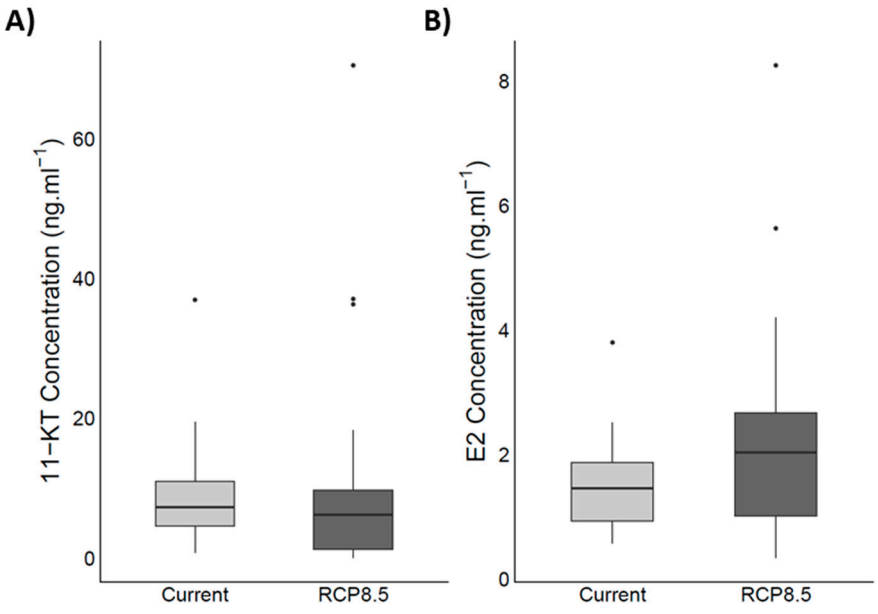

Figure 7. Blood concentrations of 11-keto-testosterone (11-KT; panel (**A**)) and 17-β-estradiol (E2; panel (**B**)) in stickleback males under Current (light grey; n = 17) and RCP8.5 (dark grey; n = 26) scenarios. The 10th and 90th percentiles are represented by the whiskers, the 25th and 75th percentiles by the boxes, the median values by horizontal lines and outliers by points.

4.6. Reproductive Success

The number of eggs per fish obtained was 66 ± 23 for the Current scenario and 57 ± 18 for RCP8.5 (p-value = 0.28). The fertilisation rate was significantly lower in RCP8.5 scenario (63 ± 21.3%) than in the Current scenario (81.8 ± 4.5%; p-value = 0.02). In the scenario RCP8.5

at 1 and 3 Dpf, the size of the eggs was significantly smaller (scenario: p-value < 0.01, Dpf: p-value < 0.01; scenario/Dpf: p-value = 0.68; Figure 8A) and the perivitelline index significantly higher (scenario: p-value < 0.01, Dpf: p-value < 0.01; scenario/Dpf: p-value = 0.58; Figure 8B). The sperm quality was assessed, and no significant differences in spermatozoa concentration (p-value = 0.92) and mortality (p-value = 0.38) were observed between scenarios (Table 5).

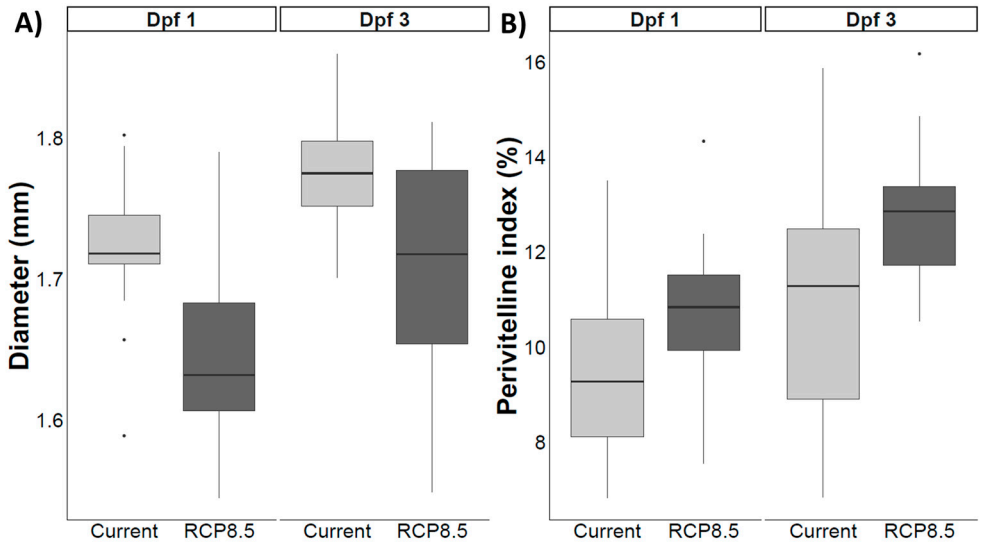

Figure 8. Boxplots representing the diameter (**A**) and the perivitelline index (**B**) at 1 and 3 days post fertilisation (Dpf) under Current (light grey) and RCP8.5 (dark grey) scenarios (Current, Dpf 1 n = 19, Dpf 3 n = 14; RCP8.5, Dpf 1 n = 22, Dpf 3 n = 10). The 10th and 90th percentiles are represented by the whiskers, the 25th and 75th percentiles by the boxes, the median values by horizontal lines and outliers by points. Different letters indicate a significant difference between the boxes.

Table 5. Summary of sperm quality markers of the three-spined stickleback after 7 months of acclimation to RCP8.5 (n = 8) or Current (n = 7) scenarios. Data are presented as mean ± S.D.

	Current	RCP8.5
Spermatozoa concentration (10^7 cells/mL)	1.68 ± 1.25	1.62 ± 1.08
Spermatozoa mortality (%)	7.45 ± 2.70	5.81 ± 4.03

5. Discussion

Overall, the findings from this study indicate that a long acclimation to combined ocean warming and acidification, as predicted by the RCP8.5 IPCC scenario by 2100, could impact the survival and reproductive performance of three-spined stickleback.

Given the freshwater origin of the sticklebacks used in this study, one concern could be related to the additional stress the fish suffered in seawater, which could compromise their resilience to warming and acidification, although during the gradual acclimatisation to the salinity of Brest Harbour (34‰), no mortality was observed and the fish showed a behaviour and feeding rate consistent with and equivalent to individuals of the same stage in fresh water [71,72]. These observations are similar to the results obtained by Grotan et al. [73] on this same species. Their study, conducted on sticklebacks from populations locally adapted to saline, brackish and freshwater environments at three different salinities (0, 15 and 30‰), indicated that the standard metabolic rate did not increase in non-indigenous salinity environments. This means that marine sticklebacks paid no additional metabolic cost in freshwater, and freshwater sticklebacks paid no additional metabolic cost in marine waters. Altogether, it indicates that sticklebacks are able to rapidly acclimate to seawater, suggesting that it would not alter their physiological response to the exposure to a different climatic scenario.

In each scenario, the mortality was globally homogenous for the whole duration of the exposure, suggesting that it was likely natural mortality. No increase in mortality events was observed during the breeding season, meaning that the experimental set-up and fish density succeeded in avoiding the typical aggressive behaviour of dominant stickleback males. However, the cumulative mortality at the end of this experiment (from November to July) was higher in the RCP8.5 scenario, even if the lowest upper lethal temperature (21.6 °C) of stickleback (as previously reported) was not reached in this study [39]. Despite numerous studies on the effects of ocean acidification on the survival of early developmental stages of fish [74], no studies have examined the impact of acidification on stickleback survival at juvenile and adult stages. In our experimental conditions, we cannot attribute the increased vulnerability observed in sticklebacks directly to acidification conditions rather than to the interaction between acidification and warming. According to Pörtner [26], the physiological response to this multi-stress could require higher energy expenditure. Indeed, metabolic rates increase with temperature until the temperature level of intolerance for the species is reached [26]. This leads to an increase in food consumption and overall growth of individuals when food is not limiting [7,9,75], with a potential indirect impact on other key physiological functions.

In this study, biometric monitoring (body mass and standard length) during the 7 months of the experiment showed no difference between the fish subjected to the two experimental scenarios. However, the RCP8.5 scenario fish needed larger amounts of food to reached satiety. In this work, the RCP8.5 scenario represented a combination of warming and acidification, and the potential faster growth of fish reared in warmer water temperatures might have been counter-balanced by the costs associated with their simultaneous exposure to lower pH [76,77]. Moreover, and in contrast to many other studies, sticklebacks were not exposed to constant temperatures during this experiment [61], but rather to the thermal seasonal variations of the "Rade de Brest" in France, meaning that a positive effect at a given temperature might have been offset by a negative one (especially in warmers periods). This led to the same standard body size and mass in the two scenarios, but with a FCR of 24 $mg_{food} \cdot mg_{body}{}^{-1}$ in the RCP8.5 scenario and of 20 $mg_{food} \cdot mg_{body}{}^{-1}$ in the Current scenario. This finding suggests that at higher temperature and lower pH, the sticklebacks needed to increase their food intake to reach the same body size, indicating an extra energetic demand in the RCP8.5 scenario. For instance, Cominassi et al. [59] observed that in European sea bass, the decreased growth rate at higher temperature and pCO_2 levels was associated with a lower specific activity of digestive enzymes. Altogether, these findings suggest that sticklebacks facing warming and acidification would exhibit a lower FCR and/or higher metabolic cost. Previous studies hypothesised that a high CO_2 scenario leads to increased metabolic costs due to hypercapnia and the consequent acid–base regulation, which could compromise the energy available for growth and other physiological functions of the fish [26,78–80]. However, the effect of CO_2 on growth depends on the temperature [80–83]. To estimate how warming and acidification impact the energy absorption efficiency and metabolism, digestive enzyme activities should be determined, as well as oxygen consumption under swimming challenge.

The amount of reserves that fish can accumulate, particularly for reproduction, is likely to be impacted by such increases in FCR and metabolic costs. In fish, lipids, along with proteins, play a major role as energy sources for growth and reproduction [84]. Thus, their reproductive cycle includes consecutive phases of energy storage in the form of lipids, mobilisation, and transfer from the lipid deposition sites to the liver for metabolization and transport to the gonads [85]. In sticklebacks, the energetic requirements associated with reproduction may be met in two ways: stored energy or energy from food consumed as reproduction proceeds [86]. For instance, as the energy content of sticklebacks ovaries increases considerably between March and June, a parallel depletion in liver and carcass lipid and glycogen is observed [87]. In our study, the analysis of the total lipids in the muscle was carried out during the reproductive period. It showed that females under the RCP8.5 scenario had significantly lower lipid levels, suggesting a smaller amount of energy available for reproduction at the time of sampling.

This is in line with the observation of a lower proportion of mature oocytes in females under the RCP8.5 scenario. In July, during the spawning period, mature oocytes were the most common stage in both scenarios, but a smaller number of advanced maturation oocytes (late and mature stages) was observed in the RCP8.5 scenarios. It has already been reported that warmer environments can lead to a shift, a partial or even total inhibition in gametogenesis, depending on the species [24]. The slower oocyte maturation in the RCP8.5 scenario suggests that a delay and/or a partial inhibition of the maturation process might be occurring in these fish when warming occurred simultaneously with acidification. Keeping in mind our hypothesis concerning the increase in metabolic rate in the RCP8.5 scenario, we could assume that the reproductive capital of females under the RCP8.5 scenario is inadequate at the time of sampling to ensure the energy flow necessary for maturation [88,89]. What are the consequences of this potential delay? Fish exposed to the RCP8.5 scenario might reach their peak reproductive activity with a similar delay, which could have major consequences for their offspring if they hatch in suboptimal conditions. Future studies would be needed to understand the fate of the offspring of RCP8.5 fish, but such a mismatch (commonly reported in the literature [90–92]) and the decline in reproductive success, which itself comes after a significant decline in individuals' survival, suggest that the RCP8.5 scenario could have major effects on the fitness of three-spined stickleback. Secondly, the quality of the eggs might be impaired, and this is what our data indicates. Indeed, egg quality also appeared to be affected in the RCP8.5 scenario, with eggs of smaller diameter and a higher perivitelline index at 1 and 3 Dpf. Similar observations, i.e., smaller diameters, have been reported by numerous studies on eggs of seawater sticklebacks with a constant warmer condition of 4 °C [46,47,93,94], and as reported by Glippa et al. [45], who showed a negative effect of temperature on stickleback egg diameter. Another study hypothesised lower egg quality in freshwater sticklebacks (i.e., small eggs) under a constant rise of 5 °C [61]. Other fish species also show smaller diameters after warming exposure (short winter), such as *Perca flavescens* [95] and *Gadus chalcogrammus*, under constant temperature (8 °C) increase [96]. Indeed, in many marine fish species, it is often observed that an increase in water temperature is associated with a decrease in egg size [97]. No effect of acidification has been found on the size of sticklebacks eggs [32,45], but in European eel (*Anguilla anguilla*), acidification induced a decline in both egg diameter and perivitelline space. The authors hypothesised that the entrance of acidified water into eggs could impact the development of the embryos and compromise their survival [98]. Then, if we assume the effects observed in European eel's egg under acidification would occur also in stickleback, the reduction in egg diameter could be an additive or synergic effect of warming and acidification. Overall, our findings show smaller diameters of eggs with higher perivitelline index scores at 1 and 3 Dpf in the RCP8.5 scenario, suggesting lower availability of reserves for the embryo. This could potentially increase embryo vulnerability, which could result in suboptimal hatching conditions that substantially reduce the survival rate of larvae.

Males seemed to be less impacted by the RCP8.5 scenario, with muscle lipid levels and testicular maturation stable between scenarios. However, males' KSI scores tended to be lower in the RCP8.5 scenario after 7 months of exposure. Since no difference in body mass was observed between scenarios, our finding indicates a decrease in the mass of the kidney, which is in line with other studies showing similar results in male sticklebacks under warmer conditions [44,61,99]. Kidney hypertrophy is one of the secondary sexual characters of the males in the reproductive period of sticklebacks, which results from the kidney's physiological function in producing the spiggin, a protein glue that allows males to make the nest required for reproduction in nature. This hypertrophy is directly related to the androgen concentration in the blood and specifically 11-KT [100]. The potential hypotrophy of the male kidney under the RCP8.5 experimental scenario is not associated with a decrease in blood 11-KT, with blood 11-KT levels being similar in the two experimental groups at the sampling time. However, the same value for 11-KT concentration in the two scenarios may not correspond to the same point of the seasonal cycle of 11-KT levels in the blood. Therefore, we cannot exclude a delay or an inhibition of maturation in the RCP8.5 scenario, even in males. There were no significant differences in the concentration or mortality of

spermatozoa between scenarios. Nevertheless, we could not evaluate the direct or indirect effects of the climate scenario on the motility of spermatozoa, which is directly linked to the fertilisation potential [24,101,102]. An impact of the RCP8.5 scenario on sperm motility is likely, since reduction in fish sperm velocity has been already reported under higher temperature [13] and acidified water [102]. However, to our knowledge, the combined effect of acidification and warming on fish sperm motility is not clear yet. Overall, although the impacts of the RCP8.5 scenario on stickleback males were less clear than those on females, we cannot rule out a decline in their reproductive performance or at least a short delay in their maturation. To resolve this, monitoring of the kinetics of physiological indexes and hormone concentrations during the reproductive cycle would be needed. This would enable us to better decipher the impact of the RCP8.5 scenario in both sexes and, notably, to discriminate between a shift in and an inhibition of gonad maturation in this scenario. Similarly, kinetic assessments of lipids in the liver and the muscle could help to evaluate the energy status of fish in the context of climate change and contribute to distinguishing between potential delay or inhibition of maturation.

The neuroendocrine control of reproduction showed only a trend in the impact of the RCP8.5 scenario. The expression levels of genes involved in fish reproduction (*gnrh2*, *gnrh3*, *kiss2*, *gnih*) did not show any significant differences between the scenarios in males or females. However, there was a general trend suggesting a slight up-regulation in the RCP8.5 scenario in most genes. More specifically, the genes *gnrh2*, *gnrh3*, and *kiss2* in fish are all involved in the triggering of reproduction, and *gnrh3* is also involved in the maturation of fish [103–107]. Altogether, these data could suggest that sticklebacks under RCP8.5 would undergo central stimulation of the reproductive axis. This central stimulation could represent a positive feedback coming from the gonad and returning to the brain, aiming to increase the maturation of gonads in both sexes. This could be further evidence of a delay or a slight inhibition of fish maturation in this scenario in July at the time of the sampling. Interestingly, a previous study of the eurythermal sheepshead minnow (*Cyprinodon variegatus*) showed that the transcripts of genes encoding for *gnih* and *gnrh3* were higher in relative abundance in the hypothalamus of both sexes at higher temperature when a global inhibition of reproduction (reduced gonadal steroidogenesis and oogenesis) is observed [108]. The higher expression of *cyp19a1b* in female sticklebacks is not surprising, due to the role of *cyp19a1b* in converting androgens to oestrogens. Brain aromatase did not show any response to the experimental RCP8.5 scenario, which is in line with the lack of effect on blood sex steroid levels. This study also provided information on the reproductive success of sticklebacks, with lower fertilisation rates observed in the RCP8.5 scenario than in the Current scenario. This could be explained by a potential decline in gamete quality, for example, a possible decrease in spermatozoa motility and/or change in egg morphology (size and composition).

Interestingly, the RCP8.5 scenario exhibited increased variability in interindividual responses for most of the analysed end points related to reproduction, suggesting a potential amplification of the phenotypic variation in this scenario. Higher variance in the RCP8.5 scenario was observed for the expression levels of genes related to reproduction and blood sex steroids concentrations, notably 17-β-estradiol. Phenotypic plasticity is the capacity of a single genotype to express multiple phenotypes in response to environmental stimuli [109–111], allowing organisms to cope with environmental change [112]. Plasticity can occur within a generation and across generations (transgenerational plasticity). When rapid change in environmental conditions occurs, as in the context of climate change, within-generation plasticity can be important to mitigate the immediate impacts of the changing environment on fish individuals [94,113]. Most of the within-generation plasticity is driven by the developmental plasticity, defined as the phenotypic variation in later life stages explained by the environmental conditions experienced in early life [114,115]. In the present study, the exposure to environmental changes began when fish were already at the juvenile stage, excluding any chance of developmental plasticity. Nevertheless, the exposure lasted until adulthood and the gametogenesis process, which has been described as a

sensitivity window towards environmental changes that generates plasticity in fish [116]. The increased variation in hormonal responses and the neuroendocrine regulation of reproduction in the RCP8.5 scenario could lead, for instance, to a higher variation in spawning period starting and duration, with consequences for hatching time that could potentially impact offspring survival in the changed environment. Previous works have reported both increases [117] and decreases [118,119] in phenotypic variability in fish under warmer temperatures or acidification. According to the proposition of Rodriguez-Dominguez and collaborators [118], this discrepancy could be explained by a difference in phenotypic variation in response to changing environments in studies based on wild populations versus laboratory experiments. The authors noticed that most of environmental reduction in phenotype variation is observed in wild populations that face whole-life exposure to the changing environments, while increases in the phenotypic responses are observed mostly in laboratory experiments following short exposures. One explanation could be that in short-term laboratory experiments, the frequency of rare phenotypes that would unlikely survive in the wild is potentially increased [118]. The present study showed higher response variability in a laboratory experiment in which sticklebacks were facing warming and acidification at the same time after a relatively long exposure (7 months). The amplification of variations in phenotypes related to reproductive function is consistent with observations in experimental studies focusing only on warming impacts [117]. This could suggest that the stickleback population is still in the initial phase of selection for beneficial traits that provide advantages in the new environment. Evaluating the impact of changing environments on phenotypic plasticity is crucial to identifying fish acclimation ability in future environments.

6. Conclusions

The aim of this study was to estimate the global effect of the combination of warming (+3 °C) and acidification (−0.4 pH unit) on individuals' mortality, growth, and reproductive performance in three-spined stickleback. The experimental design of this study enabled us to monitor the biometric parameters (size and mass) over 7 months and thus to estimate the growth of sticklebacks under the two scenarios from the juvenile stages to the first reproduction. Stickleback size and mass did not change with exposure to warming and acidification when feeding was not limited, but food consumption was greater under the RCP8.5 scenario. This suggests an increased need for energy to maintain homeostasis and other physiological functions. The measurement of lipid content in muscle, an indicator of energy reserves, revealed lower levels in females in the RCP8.5 scenario, supporting the hypothesis of a divergence in energy allocation strategy between scenarios. This experiment also enabled us to characterise the first reproduction of sticklebacks in the two different scenarios. After 7 months of exposure, the females showed lower maturation and egg quality under the RCP8.5 scenario, which could have contributed to the lower fertilisation rate observed. This suggests a delay and/or inhibition of gametogenesis and maturation in warmed and acidified waters. The analysis of blood sex steroid concentrations and physiological indexes at one single time point did not allow us to discriminate between a delay and an inhibition of maturation in the RCP8.5 scenario. Altogether, these results highlight the importance of studying the effects of combined stresses, since resilience and physiological responses of organisms to multi-stress can differ from those observed when organisms of the same species face just one stress at a time. This experiment is not based on a complete cross-factorial design and does not allow to distinguish the effects of single stressors and the potential interaction between them, but instead, shows the resulting global effect of the two stresses when applied together.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/fishes8100523/s1. Supplementary Table S1: Pairwise Games Howell post hoc test on stickleback growth in mass between Current and RCP8.5 condition through the 7 months of acclimation; Supplementary Table S2: Pairwise Wilcoxon post hoc test on stickleback fresh lipids content between sexes and experimental scenarios at the final sampling in July after 7 months

of acclimation to RCP8.5 scenario; Supplementary Table S3: Sticklebacks feed conversion ratio (FCR), thermal growth coefficients (TGC) and mortality rate through the 7 months of experiments; Supplementary Table S4: Statistical tests and scores of data analysis.

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