Article

Differential Effects of Food Restriction and Warming in the Two-Spotted Goby: Impaired Reproductive Performance and Stressed Offspring

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Abstract: Climate change is a growing threat to marine organisms and ecosystems, and it is already modifying ocean properties by, for example, increasing temperature and decreasing pH. Increasing water temperature may also lead to an impairment of primary productivity and an overall depletion of available zooplankton. Understanding how the crossover between warming and zooplankton availability impacts fish populations has paramount implications for conservation and mitigation strategies. Through a cross factorial design to test the effects of ocean temperature and food availability in a temperate marine teleost, *Pomatochistus flavescens*, we showed that hindered feeding impacted sheltering and avoidance behaviour. Also, low food availability impaired fish reproduction, particularly male reproduction, as the expression of cyp11b1, a gene with a pivotal role in the synthesis of the most important fish androgen, 11-ketotestosterone, was significantly reduced under a low food regime. In contrast, temperature alone did not affect reproductive success, but offspring showed increased saturated fatty acid content (embryos) and increased lipid peroxidation (larvae). Altogether, food availability had a stronger effect on fitness, showing that coping with elevated temperatures, an ability that may be expected in shallow-water fish, can be indirectly impacted, or even overwhelmed, by the effects of ocean warming on primary productivity and downstream ecological processes.

Keywords: ocean warming; food availability; behaviour; reproduction; *Pomatochistus flavescens*

1. Introduction

For ectotherms, temperature is a key environmental factor that influences several fundamental processes such as behaviour [1,2], metabolism [3,4], reproduction [5,6], growth, and development [7,8]. In a world of rapid warming, with ocean temperature projected to increase up to 4 °C until the end of the century [9,10], it is imperative to understand the overall consequences to marine life. In fish, warming has been shown to induce changes in swimming performance [11], activity [12,13], and risk-taking related behaviours such as foraging, predator-prey interactions [14,15], predator escape responses, and boldness [2,16].

Temperature is known to be one of the most important cues for triggering fish reproductive activity [7,17]. Climate change has the potential to induce temperature variations which can have implications on the timing of reproduction, anticipating, delaying, or even interrupting it [18]. Warming can also influence several behavioural and physiological processes related to reproduction, such as parental care [6], spawning frequency, clutch size, egg area, and duration of the embryonic phase [5,19,20]. Moreover, temperature will act on gonads, mainly interfering with steroidogenesis [17], and responsible for the differentiation of gonads, their growth and maturation [21]. Elevated temperatures can also lead to gonadal degeneration and partial or complete sterility [22] and modify gonadal sex in early development [23,24], with further cascading effects on offspring fitness and quality [25].

Ocean warming is also expected to lead to increased metabolism and disrupted metabolic activity in fishes [26,27], triggering an increased production of reactive oxygen species (ROS), such as the singlet oxygen, the superoxide anion, the hydrogen peroxide, and the hydroxyl radical, during the aerobic metabolism [28], causing protein, lipid and DNA damages. Moreover, temperature is responsible for influencing the fatty acid (FA) content in marine fishes [29,30], which are the "building blocks" of lipids, with important roles in cellular membrane formation, protein modification, energy storage, among others [31]. Due to their biosynthesis specificity, FA can act as biological markers of specific taxa [32,33]. In fact, while saturated FA (SFA) and monounsaturated FA (MUFA) are usually synthetized or genetically inherited by most marine organisms [31,34,35], others, such as polyunsaturated FA (PUFA), are usually restricted to phytoplankton [35,36], and are therefore obtained through feeding [37].

As a response to higher temperature, fish will increase their energetic demands [38] and consequently increase food intake [8]. However, in the natural environment, food is never unlimited, and warming is expected to impact plankton communities [39] due to stratification of the water column and the subsequent depletion of nutrients, which result in poor nutritional quality zooplankton [40] with reduced abundance [41], namely a drastically decreased of the production of omega-3 in the phytoplankton that will decrease availability of this fatty acid in the above levels of the trophic chain [42]. Food shortage can affect organisms' dynamic energy budgets [43], reducing the energy available for key biological processes, such as reproduction, with carry-over effects on number, size, energy and biochemical composition of eggs, and overall quality of offspring (see review by Green [44]). Despite this link between warming and food availability, few studies have addressed their interactive effects on marine organisms' behaviour and physiology [45–48], with most experimental studies following an *ad libitum* feeding regime, which may provide fish with enough energy to compensate for the negative effects of other stressors.

Here, it was proposed to explore the effect of increasing water temperature and low food availability on individual behaviour, metabolism, stress responses and reproduction in temperate coastal teleosts, deploying the two-spotted goby, *Pomatochistus flavescens*, as a model. This is a semi-pelagic fish that distributes along the European coast and inhabits shallow rocky shores and seaweed beds [49]. Previous studies already demonstrated that the species' reproductive abilities can be affected by realistically increased water temperature, which led to the hypothesis that fish of temperate waters can be particularly vulnerable to forecasted warming regimes [5], rendering it a promising surrogate for other shallow-water teleosts in this biogeophraphical area.

2. Materials and Methods

2.1. Fish Collection and Experimental Setup

Adult male and female (totaling 150 individuals) two-spotted goby were collected via SCUBA diving at Arrábida Marine Park, Portugal (38°28′ N; 8°59′ W), in February 2019. Upon arrival at the lab facilities, fish were placed in a 140-L tank, with a continuous supply of recirculating seawater, enriched with substrate and artificial algae, with temperature (15 °C) and salinity (35 PSU) matching the conditions at the collection site. The fish

photoperiod at the lab facilities varied according to light/dark changes in the wild for the duration of the experiment. Fish were left to acclimate to captivity conditions in this tank for 10 days and were fed daily unto satiation with a mixture of frozen Artemia (Gamma Slice Brine shrimp, Tropical Marine Centre™ Hertfordshire, UK) and Copepods (Gamma Slice Copepods, Tropical Marine Centre™). Subsequently, fish were weighted, measured and randomly assigned to 30 L tanks, at a density of four fish of the same sex per tank. To minimize potential aggressive behaviours, particularly between males, each tank was supplied with artificial nests (pvc tubes) and artificial algae. Water in the 30 L tanks was supplied at a total of eight 200 L sumps (two sumps for each of the four treatments), equipped with biological, mechanical, chemical and ultraviolet filtration. Each sump supplied water to five 30 L tanks. Temperature was controlled using both chillers and heaters. Temperature and salinity were measured daily, and other water quality parameters, such as ammonia, nitrates and nitrites, were monitored weekly and kept below critical levels. Excess feed was removed from tanks daily.

Fish were then assigned to one of four treatments: control temperature (CT) + high ration (HR); control temperature (CT) + low ration (LR); high temperature (HT + 3 °C) + high ration (HR); and high temperature (HT + 3 °C) + low ration (LR). The temperature in HT treatments was increased stepwise at a ratio of about 1 °C per week, using heaters, until +3 °C above the control temperature treatment was reached. As this study involves a prolonged exposure to treatments, temperatures in both control and high temperature conditions varied monthly to match local seasonality (Supplementary Table S1), and the variation followed an average of the last 10 years at the collection site. Once the experimental temperature was reached, the food treatments started (day 0 of treatments) (Figure 1). Fish in the high ration treatments were fed daily with 8 g of frozen Artemia and Copepods, divided by two meals, while fish in low ration treatments were fed the same amount of food once every two days. The choice of the high ration was selected based on preliminary observations that allowed us to conclude that 8 g per day would match fish needs. The choice of low ration was selected while taking into consideration that even lower feeding levels would lead to higher mortality rates within the first few days, which we avoided. The amount of feed was readjusted to mortality.
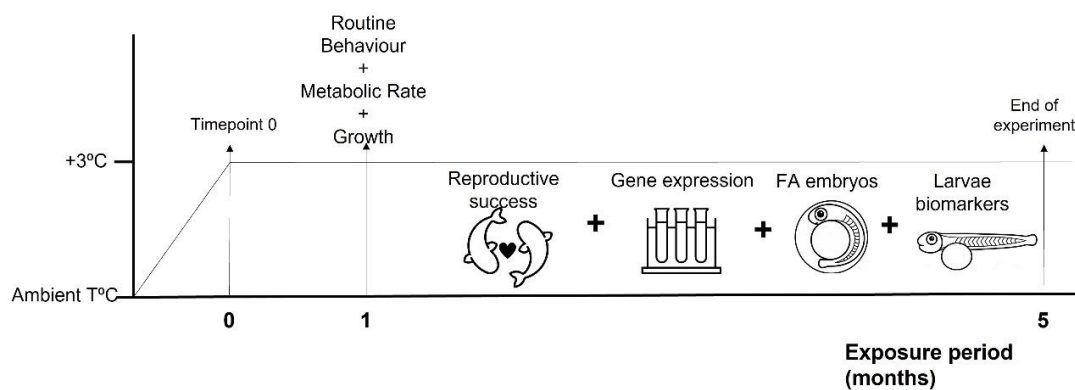

Figure 1. A diagram of the experimental design. Two-spotted goby, *Pomatochistus flavescens* were exposed to 4 treatments: CT + HR-control temperature and high ration; CT + LR control temperature and low ration; HT + HR high temperature (+3 °C) and high ration; and HT + LR high temperature (+3 °C) and low ration. At Timepoint 0, temperature in the "High" temperature treatments was reached, and the food treatments started. After 1 month of exposure to treatments, a subset of fish was tested for routine behaviour, metabolic rates and growth. Another subset of fish was exposed to treatments for a total of 5 months, which included the breeding season. During this period, reproductive success and gene expression of reproduction-related genes were determined in adult fish; fatty acid (FA) content was determined in recently fertilized eggs and biomarker analysis determined in recently hatched larvae. Fish under LR treatments did not reproduce, thus FA content and biomarkers were not determined.

2.2. Temperature and Food Availability Effects on Behaviour and Physiology

A total of 48 fish (12 fish per treatment), with 3.7 ± 0.045 cm and 0.675 ± 0.027 g (mean $\pm$ s.e.m.), were exposed for a 1-month period to treatment conditions and, subsequently, routine activity, metabolic performance and growth were determined (Figure 1). Fish size (SL) and weight (W) did not differ among the fish randomly assigned to the treatments at the start of the experiment (SL: $F_{(3:36)} = 0.063$, $p = 0.980$; W: $F_{(3:35)} = 0.409$, $p = 0.747$).

2.2.1. Routine Behaviour

An ethogram was built based on [50,51] to assess routine behavioural activity (Table 1). Behavioural descriptions were made using focal observations (sensu Martin and Bateson [52]), which were conducted between 10:00 and 13:00 a.m., approximately 30 min after feeding, to avoid pre-prandial bias, by a motionless observer standing in front of the aquarium (always the same observer). Time taken by the fish to ignore the observer was registered (determined as the time taken for the fish to turn away from the observer, as initially the fish expected to be fed again and would swim towards the observer). During the focal observations, the behavioural categories displayed by the focal fish were registered. Each focal observation lasted 2 min, and this procedure was repeated for 10 to 12 fish per treatment. Each fish was observed in three sessions throughout a one-week period to confirm consistency in behaviour and outwit punctual behaviours. Fish were not tagged, but small differences in coloration on body and fins of fishes kept in the same tank considerably reduced the risk of misidentifying each individual.

Table 1. List of behaviours displayed by two-spotted goby, *Pomatochistus flavescens* during the routine behaviour experiment.

Behavioural Category	Behaviour
Aggression (frequency)	Charge-Quick movement towards a second fish (conspecific), while the conspecific avoids the first fish. Chase-Individual swimming after conspecific.
Hide (frequency)	Fish attempting to hide, either in shelter or in corner of the tank.
Swim (time, s)	Active swimming-Movement against current using pectoral fins and caudal body movement/Movement up and down the side of the tank using active swimming technique. Beat-and-glide-Intermittent swimming and drifting. Surface touching–fish swimming and touching the surface of the water.
Rest (time, s)	Motionless horizontally on bottom of aquarium.

2.2.2. Routine Metabolic Rate

Fish were fasted for 24 h prior to analysis to ensure a post-absorptive state, as metabolic rates tend to increase during digestion [53]. Metabolism was assessed by measuring routine metabolic rate (RMR), using a closed respirometry system, for 11 to 12 fish per treatment. Each individual was tested only in a single assay. One individual was placed into a 100 mL syringe filled with 60 mL of UV filtered aerated water, matching salinity and temperature of the corresponding treatment. All air bubbles were removed from the syringe, parafilm was wrapped around the tip and a cap was used to ensure there were no leaks of water or air from the syringes. The syringe was placed in a water bath to ensure that fish were kept at the same temperature of the treatment and covered with black plastic to avoid external disturbances. After 1 min of acclimation, oxygen levels inside the syringe were measured for 10 min using contactless oxygen sensor spots (OXSP5, Pyroscience, Aachen, Denmark) and monitored using the software Pyro Oxygen Logger (Pyroscience,). The oxygen concentration was maintained above 80% during experimental tests. Four 100 mL syringes were used simultaneously, three with fish, and one without fish, which served as

"blank" to account for bacterial respiration. Between trials, all syringes were washed with distilled water and cleaned for particles with a sterilized cotton swab before being filled again for the following fish. After each trial, fish were removed from the syringe, measured and weighted to determine body condition. Experimental trials were conducted over a period of 1 week in a temperature-controlled room to minimize temperature fluctuations during trials. At the end of the 1-month exposure period, all fish were euthanized with an overdose of MS-222 (100 mg L^{-1}).

2.3. Temperature and Food Availability Effects on Reproduction

Another 80 fish were assigned as breeding pairs (male-female), at a density of two breeding pairs per 30 L tank and a total of 10 pairs per treatment. Pairs in each tank were separated by a partition to keep track of the identity of each parental pair. The partitions in the tank allowed constant renewal of the water and the maintenance of water temperature and quality on both sides. Size (3.68 ± 0.029 cm, mean ± s.e.) did not differ between fishes assigned to the different treatments ($F_{(3:80)}$ = 1.369, p = 0.258). Weight (0.633 ± 0.016 g, mean ± s.e.) was also not different at the start of the experiment for fish assigned to the treatments ($F_{(3:80)}$ = 0.716, p = 0.545). These pairs were exposed to treatment conditions for a 5-month period, which included the breeding season. Reproductive success was evaluated by measuring the number of clutches produced by breeding pairs, the average number of eggs, average egg area, and larval size at hatching and adult body condition. Additionally, the expression of reproduction related genes was measured in adults' gonads, fatty acid content was measured in recently fertilized eggs and oxidative stress and energy metabolism-related biomarkers were measured in recently hatched larvae (Figure 1).

2.3.1. Reproductive Success

Breeding couples were provided with a pvc tube (10 cm, Ø1.3 cm) for shelter and nesting. Each pipe was lined inside with a removable acetate sheet where the spawning females could attach their eggs. Egg clutch presence was checked daily, and when present, the acetate sheet was carefully removed and placed in a Petri dish filled with seawater from the respective tank and photographed for egg counting. Approximately 10 eggs were removed from the clutch and placed in a smaller Petri dish, filled with seawater and photographed under a stereomicroscope for measurements of egg area. The first clutch from each breeding pair was entirely sampled and stored in a 2 mL cryotube at −80 °C for the determination of fatty acid content (see below). Pairs were not affected by the brief removal of the acetate sheet, as fish resumed normal activity after the acetate sheet was put back in place. Reproductive output was determined by multiplying the total number of eggs per breeding pair by the average egg area (mm^2). Close to hatch date, the acetate sheet was removed and placed in a maternity cage (16.5 × 12.5 × 11.5 cm) inside a 30 L tank, matching the parental treatment. A total of 10–15 larvae were collected at hatching, euthanized using an overdose of MS-222 and immediately photographed under a stereomicroscope to determine morphometric traits (mm): standard length (SL), head height (HH), pre-caudal body length (BL), and muscle mass depth at vent (MDV). Approximately 150 larvae were collected at hatching and stored in 2 mL cryotubes at −80 °C, in pools of 50 larvae per tube, for a biomarker analysis (see below).

At the end of the experiment, adults were measured, weighted and euthanized with an overdose of MS-222 (100 mg L^{-1}). Fish were dissected, gonads were removed, weighted to determine gonadosomatic index (GSI) and stored at −80 °C for quantification of expression of reproduction related genes. Gonadosomatic index was measured using the formula GSI = (gonad mass (g)/fish mass (g)) × 100. If a fish died in the tank during the experimental period, no morphometric traits were determined, and gonads were not dissected, as the time of death was not possible to determine. This occurred in 22 out of the 80 fishes (3 in the Control, 7 in the Low ration, 2 in the High T°C and 10 in the High T°C × Low ration treatments).

2.3.2. Gene Expression of Reproduction-Related Genes

Total RNA was extracted using the RNeasy Lipid Tissue Mini Kit (Qiagen, Germantown, ML, USA), according to manufacturer instructions, with minor adaptations. A DNase digestion step was included to prevent DNA contamination. Yield was assessed using a NanoDrop® spectrophotometer (ThermoFisher Scientific, Waltham, MA, USA), and RNA integrity was confirmed with Bioanalyzer (Agilent). RNA was stored at −80 °C and then reverse transcribed using the iScript cDNA synthesis kit (Bio-Rad, Heracles, CA, USA).

mRNA levels of genes encoding the steroidogenic enzymes P450 11b hydroxylase (cyp11b1) were quantified in males and both P450 aromatase (cyp19a1) and carbonyl reductase/20α-hydroxysteroid dehydrogenase (20α-hsd) for females. The selected housekeeping gene was β-actin. Primers used for β-actin are already described in the literature [54]. For the other genes, we obtained cDNA sequences for some teleost fish at the National Center for Biotechnology Information (NCBI) (cyp11b1: XM_029128166.2; cyp19a1: AY684254.1; 20α-hsd: KT932711.1) and searched for homologous sequences in a non-annotated transcriptome of a very closed species (*Pomatoschistus minutus*, the sand goby [55]). For 20α-hsd, no blast result on the goby transcriptome was obtained, so we considered the above-mentioned NCBI sequence for the following step. Primers were designed with Primer3 software [56,57] using the sequences obtained earlier, and possible primer dimer formation was checked with FastPCR v5.4 software [58]. Primers were tested with a pool of cDNA in a qRT-PCR, and PCR products were confirmed via sequencing. Primer sequences and the size of amplification products are available in Supplementary Table S2. qRT- PCR was performed using the QuantStudio 7 Real-Time PCR Systems (ThermoFisher Scientific). The reaction mix included Power SYBR Green PCR Master Mix (ThermoFisher Scientific), 50 μM of each primer and 2 μL of cDNA in an 8 μL reaction volume. Cycling parameters were: (i) denaturation: 5 min at 95 °C; (ii) amplification and quantification: 40 cycles (30 s at 95 °C, 30 s at the annealing temperature of 56 °C, 30 s at 72 °C); and (iii) dissociation curve assessment (30 s at 95 °C, 30 s at 55 °C, 30 s at 95 °C). The dissociation curve was performed to confirm a single melting curve, proving the inexistence of primer-dimer formation and/or plate contamination. All samples were run in triplicate and no-template controls were also included in each assay. The relative initial template concentration normalized to the housekeeping gene β-actin was determined from the equation: $2^{\hat{}}$(Ct housekeeping gene − Ct target gene), assuming that the efficiency of the genes was approximately 100%. To validate this assumption, the efficiency of each gene was determined using serial dilutions of stock cDNA and only primers with efficiencies above 90% were used. Mean values for gene β-actin did not differ between treatments, thus confirming its suitability to be used as a reference gene in this study.

2.3.3. Fatty Acids Content in Recently Fertilized Eggs

FA profile of egg clutches, produced by adults exposed to the above-mentioned conditions, was determined byfreeze-drying the samples for 24 h at −50 °C under low pressure (~10–1 atm), after which they were grinded and weighted. Subsequently, 10–50 μL of C21:0 internal standard with a concentration of 10 mg/mL was added according to each sample weight (10–60 mg of dry mass per sample). Afterwards, samples were dissolved in 5 mL of acetyl chloride/methanol (1:19 *v/v*; Merck), shaken and heated at 80 °C for 1 h, after which they were left to cool on ice. After cooling, we added 1 mL of Milli-Q distilled water and 2 mL of n-heptane pro-analysis (Merck) to each sample. Following a 5 min centrifugation process at 2300× *g*, we collected the upper phase content and added it to an anhydrous sodium sulphate column (Panreac) to remove the moisture content. Finally, 2 μL of filtered sample was injected in a gas chromatograph (Varian Star 3800 Cp, Walnut Creek, CA, USA) equipped with an auto-sampler and fitted with a flame ionization detector at 250 °C for fatty acid methyl ester (FAME) analysis. The separation was carried out with helium as the carrier gas at a flow rate of 1 mL min^{-1} in a capillary column DB-WAX (30 m length × 0.32 mm internal diameter; 0.25 mm film thickness; Hewlett-Packard, Minneapolis, MN, USA) programmed at 180 °C for 5 min, raised to 220 °C at 4 °C/min and

maintained at 220 °C for 5 min with the injector at 250 °C. FAME identification (% total FA) was accomplished through comparison of retention times with those of Sigma, Nu Check Preap and Larodan Fine Chemicals standards.

2.3.4. Biomarker Analysis in Recently Hatched Larvae

Biomarkers were selected based on their molecular roles in thermal and oxidative stress responses: (i) Hsp70 is a marker of reversible thermal damage to proteins (e.g., protein unfolding), acting as a chaperone [59–61], (ii) TAC measures the concentration of antioxidants in tissues, including both enzymatic (e.g., catalase, superoxide dismutase, glutathione peroxidase, etc.) and non-enzymatic antioxidants (e.g., vitamins and pigments) [62] and (iii) LPO is a marker of oxidative damage to cell membranes that arises from the interaction of reactive oxygen species with unsaturated fatty acids, producing lipid radicals and reactive aldehydes such as malondialdehyde [63].

Samples from whole body larvae were homogenised in 1 mL of Phosphate Buffer Saline solution (PBS, pH 7.4, 140 mM NaCl, 3 mM KCl, 10 mM NA2HPO4, 2 mM KH2PO4) using an electric homogenizer (Fisherbrand 150 Handheld Homogenizer 220V EU cord, 150 W) to extract soluble proteins. The homogenates were centrifuged at 4 °C for 10 min at $10,000 \times g$, and the supernatant fraction was collected and transferred to new microtubes (1.5 mL) and immediately frozen at -80 °C for later analysis.

Total protein in samples was quantified using a NanoDrop® ND-1000 spectrophotometer (ThermoFisher Scientific, by measuring sample absorbance at 280 nm and calculating protein concentration based on the Beer–Lambert equation, using the molar extinction coefficient of BSA (bovine serum albumin) 43,824 $cm^{-1}M^{-1}$ and a path length of 10 mm. Total proteins were used to normalize all biomarker results.

Heat Shock Protein 70 (HSP70) was quantified using an indirect Enzyme Linked Immunosorbent Assay (ELISA). Protein extracts from skeletal muscle were diluted 1:5 in PBS to optimize the signal-to-noise ratio, and duplicates of 50 μL of each sample were pipetted to the microplates (96-well flat bottom high-binding plates from Greiner, Greinerstraße, Kremsmünster Austria) and then incubated overnight at 4 °C. The microplates were washed (3×200 μL PBS 0.05% Tween-20), and then blocked by adding 200 μL of 1% BSA (Albumin Bovine Fraction V (BSA), Nzytech, Lisbon, Portugal) in PBS. The microplates were incubated at 37 °C for 90 min and washed again. Fifty μL of primary antibody (Mouse monoclonal Hsp70/Hsc70 Antibody, ref #TA326357, OriGene Technologies, Inc., Rockville, ML, USA), diluted to 1 μg.mL^{-1} in a solution of 1% BSA in PBS were added to each microplate wells and incubated at 37 °C for 90 min and washed. Fifty μL of secondary antibody (Anti-mouse IGG (FC fragment) alkaline, ref # A1418, Sigma-Aldrich, Taufkirchen, Germany) was diluted to 1 μg mL^{-1} in a solution of 1% BSA in PBS and added to the microplate and at 37 °C for 90 min and washed. After the final washing stage, 100 μL of alkaline-phosphatase substrate (100 mM Tris-HCl, 100 mM NaCl, 0.5 mM $MgCl_2$, 27 mM PnPP, pH 8.5) was added to each microplate well and incubated for 30 min at room temperature. Fifty μL of STOP solution (3M NaOH) was added to each well and the absorbance was read in the microplate reader (Multiskan Sky Thermoscientific, Massachusetts, USA) at 405 nm. Quantification was done through a calibration curve constructed using serial dilutions of HSP70 standards (Recombinant Human HSP70 (active), ref #AR03018PU-S, OriGene Technologies, Inc., USA) in order to obtain a protein concentration, range from 0 to 2 μg mL^{-1}. Hsp70 concentrations were normalized per total proteins in each sample (given in μg mg^{-1} of total protein).

The Total Antioxidant Capacity (TAC) assay was adapted from [62,64], for 96 well-microplates, according to manufacture instructions. Duplicates of 10 μL were pipetted from each sample (protein extracts) into the wells, 10 μL of 90 μM myoglobin solution (lyophilized myoglobin from equine heart, Sigma-Aldrich, in 5 mM phosphate potassium, 0.9% NaCl, pH 7.4) and 150 μL of 600 μM ABTS (2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)) were added the wells. The reaction was started by adding 40 μL of 500 mM hydrogen peroxide (H_2O_2) followed by an incubation at room temperature for 5 min. The

absorbance was read in the microplate reader (Multisky Scan, Thermoscientific) at 405 nm. To determine the concentration of total antioxidants (enzymatic and non-enzymatic) present in the samples, a calibration curve was constructed using serial dilutions of Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) standards to obtain a concentration range from 0 to 0.330 mM. Total antioxidants concentration was normalized per total proteins in each sample (given in mM mg^{-1} of total protein).

The Lipid Peroxidation assay was adapted from the thiobarbituric acid reactive substances (TBARS) protocol [65]. Five µL of each sample was added to 45 µL of 50 mM Phosphate buffer (pH 7.0–7.4). 12.5 µL of SDS 8.1% and 93.5 µL of trichloroacetic acid (TCA 20%) was added for deproteination of the samples. For LPO detection, 93.5 µL of thiobarbituric acid (TBA 1%) and 50 µL of MQ-water were added to the microtubes. After samples were mixed in the vortex, the microtubes were incubated in boiling water for 10 min and placed on ice to cool down right after. 62.5 µL of ultra pure water was added, duplicates of 150 µL of each sample were pipetted in the microplate and the absorbance was read at 532 nm. To quantify the lipid peroxides, an eight-point calibration curve (0–0.3 µM) was constructed using malondialdehyde (MDA) as standard. MDA concentration in samples was normalized per total proteins in each sample (given in nmol mg^{-1} of total protein).

2.4. Statistical Analysis

Data normality was assessed using Shapiro–Wilk's test and homogeneity of variance using Bartlett's and Levene's tests. Routine activity was assessed using a generalized mixed model "glmm" with Temperature, Food and Day of observation as fixed factors and ID of individual as a random factor to account for the repeated measures, following a Poisson distribution. Data was tested for over dispersion, and if a significant over or under dispersion was found, a negative binomial distribution was applied. A specific generalized mix model "glmmTMB" was used to explore possible relations between behaviours and treatments, if zero inflation was detected the model would be adjusted with ziformula = ~1. All behaviours followed the negative binomial distribution except Locomotion, which showed a gaussian distribution. For the observed behaviours, different models were fitted and compared, and the best model was selected based on the lowest AIC value (Supplementary Table S3). Impacts of temperature and food availability were assessed on RMR, using a 2-factor "glm". Reproductive success, gonadosomatic index, fatty acid content on the eggs and biomarkers on the larvae were assessed using a "glm" with temperature as the fixed factor, following a normal distribution. The number of clutches laid and successfully hatched were also measured with a glm but followed a Poisson and negative binomial distribution, respectively. Differences in the larvae morphometric measures were also accessed with a "glm", with temperature as the fixed factor. For the gene expression analysis, outlier observations were identified and replaced by missing values using a generalized extreme studentized deviate procedure (e.g., Jain [66]), with a *p*-value of 0.05 and a maximum number of outliers set at 20% of the sample size. Gene expression levels were logarithmically transformed [log10 (x + 1)] to meet parametric test assumptions. A generalized linear model "glm" was used to check for differences between treatments with temperature and food availability as fixed factors. Size and weight of the fish measured in the different time points were assessed with a 2 factor "glm", and survival was assessed with a χ^2 test. All statistical analyses were performed with R [67]. Results were considered statistically significant at $p \leq 0.05$.

3. Results

3.1. Temperature and Food Availability Effects on Behaviour and Physiology

3.1.1. Routine Activity

The time it took for the fish to ignore the observer was significantly lower for fish kept under higher temperatures (Table 2); throughout the 3 days of observations, fish also took less time to ignore the observer (Table 2).

Table 2. Model coefficients for the generalized mixed model: time (in s) two-spotted goby, *Pomatochistus flavescens* took to ignore the observer (Time to Ignore), spent moving in the aquarium (Locomotion), or were resting, not swimming (Rest); frequency the fish hide in the aquarium (Hide), or showed aggressive behaviours (Aggression), during the observation period for the treatment conditions (temperature and ration) and day of observation. Bold text indicates significant treatment effects ($p \leq 0.05$) as compared to CT + HR.

Conditional Model–Time to Ignore	Estimate	z Value	Pr (<|z|)
CT + LR	−0.871	−1.590	0.112
HT + HR	−1.422	−2.507	**0.012**
HT + LR	0.010	0.012	0.991
Day	−0.370	−2.068	**0.039**
CT + LR × Day	0.356	1.433	0.152
HT + HR × Day	0.469	1.813	0.070
HT + LR × Day	0.053	0.142	0.887
Zero-inflation model			
(Intercept)	−0.914	−4.764	**<0.001**
Conditional model-Locomotion	**Estimate**	**z value**	**Pr (<|z|)**
CT + LR	7.842	0.930	0.353
HT + HR	0.592	0.073	0.942
HT + LR	−19.633	−1.673	0.094
Zero-inflation model			
(Intercept)	−1.938	−7.468	**<0.001**
Conditional model–Rest			
CT + LR	−0.826	−0.558	0.577
HT + HR	0.831	0.574	0.566
HT + LR	−1.855	−0.865	0.387
Conditional model–Hide	**Estimate**	**z value**	**Pr (<|z|)**
CT + LR	−1.843	−2.251	**0.024**
HT + HR	−1.540	−1.925	0.054
HT + LR	3.517	3.000	**0.003**
Conditional model-Aggression			
CT + LR	1.464	1.217	0.224
HT + HR	1.265	1.052	0.293
HT + LR	−1.795	−1.070	0.284

During the observation period, fish were found either actively swimming in the tank or resting on the bottom; however, neither behaviour (locomotion nor rest) was statistically significant in the different treatments (Table 2). The frequency of hiding behaviours, observed when fish hide in or below the nests, or between algae, was significantly affected by exposure to low rations, with fish spending less time hiding when under food shortage (Table 2). Contrarily, fish exposed to the treatment interaction (high temperature x low ration) spent significantly more time hiding (Table 2). The frequency of aggressive behaviours during the observational period did not differ among treatments (Table 2).

3.1.2. Metabolic Rate

Routine metabolic rate was not affected by 1 month of exposure to treatments (Table 3), although average oxygen consumption levels tended to be lower under high temperature treatments (MO2Ambient = 507.57 $\pm$ 108.10 mg^{-1} kg^{-1} h^{-1}; MO2Low-ration = 441.89 $\pm$ 75.48 mg^{-1} kg^{-1} h^{-1}; MO2HighT°C = 304.08 $\pm$ 85.13 mg^{-1} kg^{-1} h^{-1}; MO2HighT°CxLow-ration = 386.35 $\pm$ 111.96 mg^{-1} kg^{-1} h^{-1}).

Table 3. Effect of temperature and food availability on two-spotted goby, *Pomatochistus flavescens* routine metabolic rate (MO2 mg^{-1} kg^{-1} h^{-1}).

Conditional Model	Estimate	t Value	Pr (>|t|)
CT + LR	−65.68	−0.449	0.656
HT + HR	−203.53	−1.392	0.173
HT + LR	147.99	0.759	0.453

At the end of 1 month of exposure to treatments, there was no difference in the size and weight of the fish, among treatments (Table 4).

Table 4. Effect of temperature (CT, HT) and food availability (HR, LR) on two-spotted goby, *Pomatochistus flavescens* size (SL) and weight (W) after one month in the treatments.

Conditional Model–SL	Estimate	t Value	Pr (>|t|)
HT + LR	−0.120	−0.915	0.366
HT + HR	−0.003	−0.027	0.979
HT + HR	0.053	0.285	0.777
Conditional model-W			
HT + LR	−0.140	−1.662	0.105
HT + HR	0.028	0.348	0.730
HT + HR	−0.006	−0.047	0.963

3.2. Temperature and Food Availability Effects on Reproduction

3.2.1. Reproductive Success

Couples under high ration reproduced throughout the entire breeding season, regardless of the temperature. A total of 65 clutches were produced by 20 breeding pairs: 9 out of the 10 couples in the ambient temperature and high ration treatment laid 32 clutches, of which 10 successfully hatched; 10 out of 10 couples in the high temperature and high ration treatment laid 31 clutches, of which 7 successfully hatched. High temperature did not affect the number of laid clutches ($Z_{(1,20)}$ = −0.083, p = 0.935) or hatched clutches ($Z_{(1,20)}$ = −0.466, p = 0.641). Low ration treatments, though, had detrimental impacts on reproductive activity of the two-spotted goby, as fish under these conditions did not reproduce throughout the experimental period. The average number of eggs per clutch did not differ between ambient and high temperature ($t_{(1,56)}$ = 0.869, p = 0.389, Figure 2), however, eggs were significantly smaller under high temperature ($t_{(1,56)}$ = −2.008, p = 0.050 Figure 2). Nevertheless, the overall reproductive output (number of eggs $\times$ mean egg area) was not affected by high temperatures ($t_{(1,56)}$ = 0.217 p = 0.829). Embryonic development lasted for 8–10 days, regardless of the treatment. Despite smaller eggs, larval size at hatch did not differ between treatments, as well as all the other morphometric traits (SL: $t_{(1,157)}$ = −0.332, p = 0.74; HH: $t_{(1,157)}$ = −0.755, p = 0.451; BL: $t_{(1,157)}$ = 0.055, p = 0.956; MDV: $t_{(1,157)}$ = −0.653, p = 0.515), although there was a tendency for smaller larvae under high temperature treatment (Figure 2). GSI was significantly affected by elevated temperatures and low rations on males of two-spotted gobies (Supplementary Table S4, Figure 3), but no effect of the treatments was found on the females (Supplementary Table S4, Figure 3).
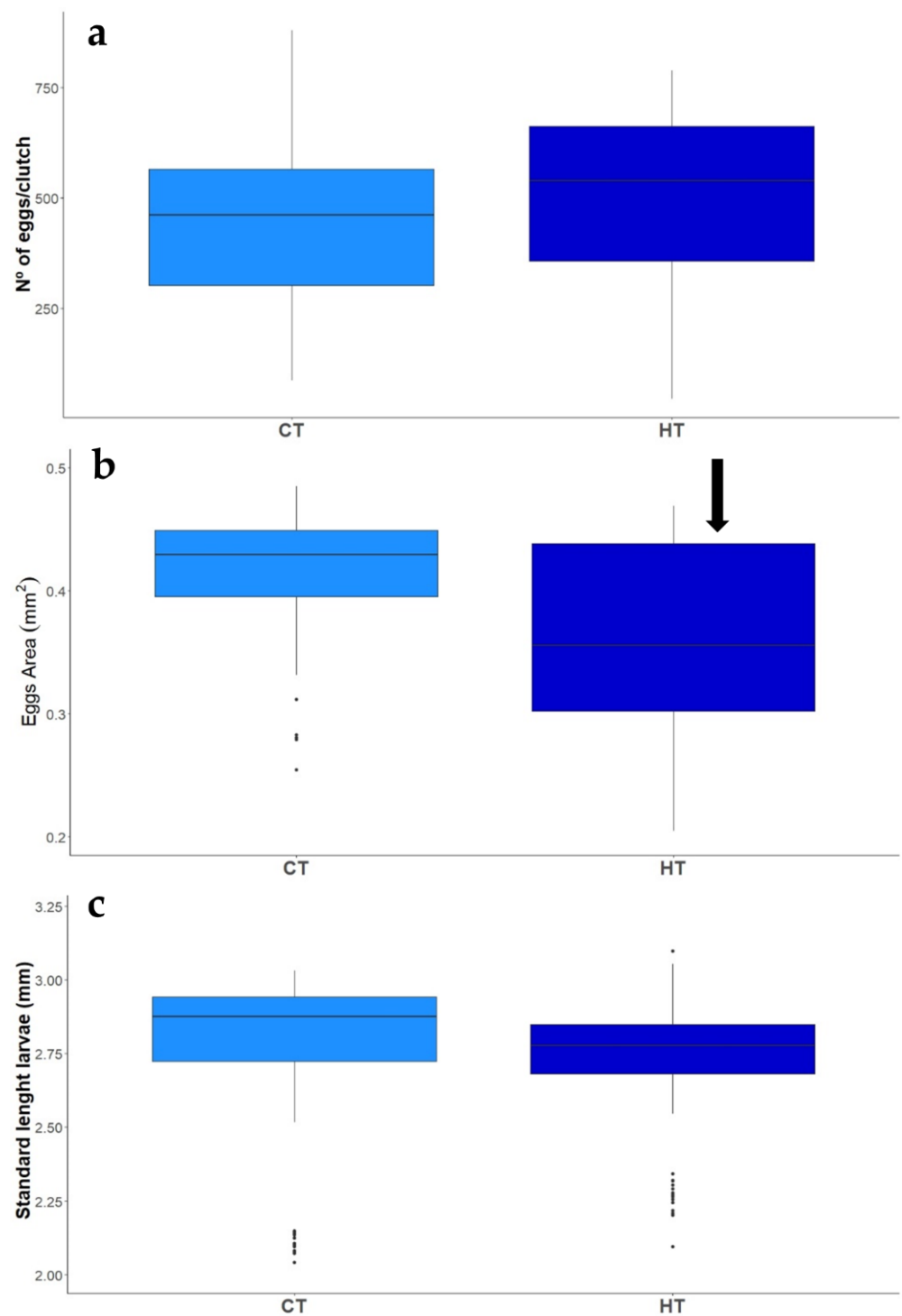
Figure 2. Effect of exposure to Control (CT) and High (HT) temperature treatments on the average number of eggs per clutch (**a**), egg area (**b**) and the size of larvae at hatch (**c**) of two-spotted goby, *Pomatochistus flavescens*. Black arrow indicates significant treatment effects ($p \leq 0.05$), as compared to CT + HR treatment.

3.2.2. Expression of Reproduction-Related Genes

Significant differences on gene expression were only detected for males. The levels of cyp11b1 changed significantly with food availability ($t_{(2,26)} = -3.527$, $p = 0.001$) and temperature ($t_{(2,26)} = -2.479$, $p = 0.020$) but were not significantly affected by the interaction between these factors ($t_{(2,26)} = 1.896$, $p = 0.069$). The levels of female genes, cyp19a1 and

20α-hsd, did not change with food availability (cyp19a1: $t_{(2,18)} = -1.562$, $p = 0.136$; 20α-hsd: $t_{(2,18)} = -0.07$, $p = 0.939$; Figure 4) or temperature (cyp19a1: $t_{(2,18)} = -1.193$, $p = 0.248$; 20α-hsd: $t_{(2,18)} = -0.895$, $p = 0.383$; Figure 4). The interaction between these factors was also non-significant (cyp19a1: $t_{(2,18)} = 0.988$, $p = 0.336$; 20α-hsd: $t_{(2,18)} = -0.146$, $p = 0.886$; Figure 4).
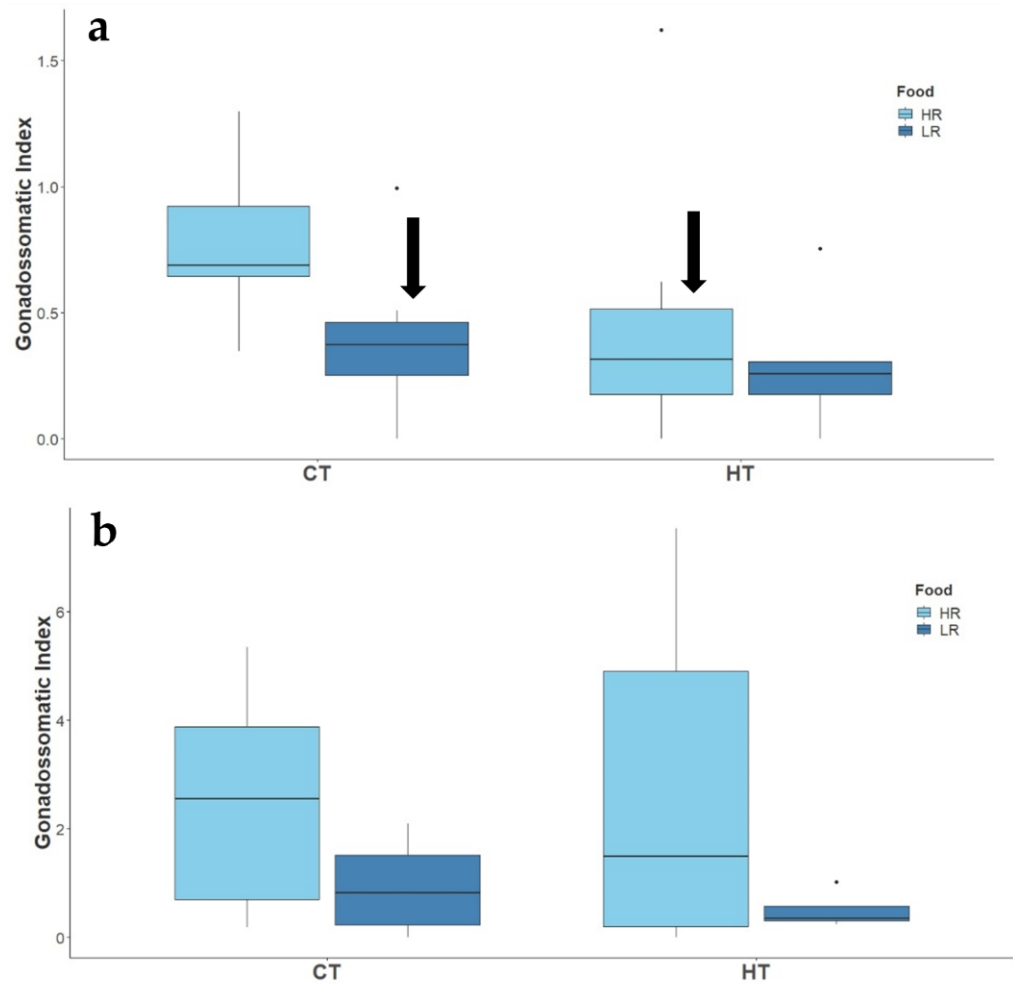

Figure 3. Effect of temperature (CT and HT) and food availability (HR, LR) on the gonadosomatic index of males (**a**), and females (**b**), of adult two-spotted goby, *Pomatochistus flavescens*. Black arrows indicate significant treatment effects ($p \leq 0.05$) as compared to CT + HR treatment.

By the end of the exposure period, fish under the low ration treatment were significantly smaller and leaner than controls (Figure 5). Fish under the interaction of high temperature and low ration treatment, however, did not show statistical significance when compared to fish in control (Supplementary Table S5), possibly due to the several outliers found on their size and weight (Figure 5). At the end of the experiment, the survival of fish on control and high temperature treatments was 85% and 90%, respectively. Fish under the low ration and high temperature × low ration treatment had a survival of 65% and 50%, respectively; however, these values were not statistically significant. ($\chi^2 = 2.828$, df = 3, $p = 0.419$).
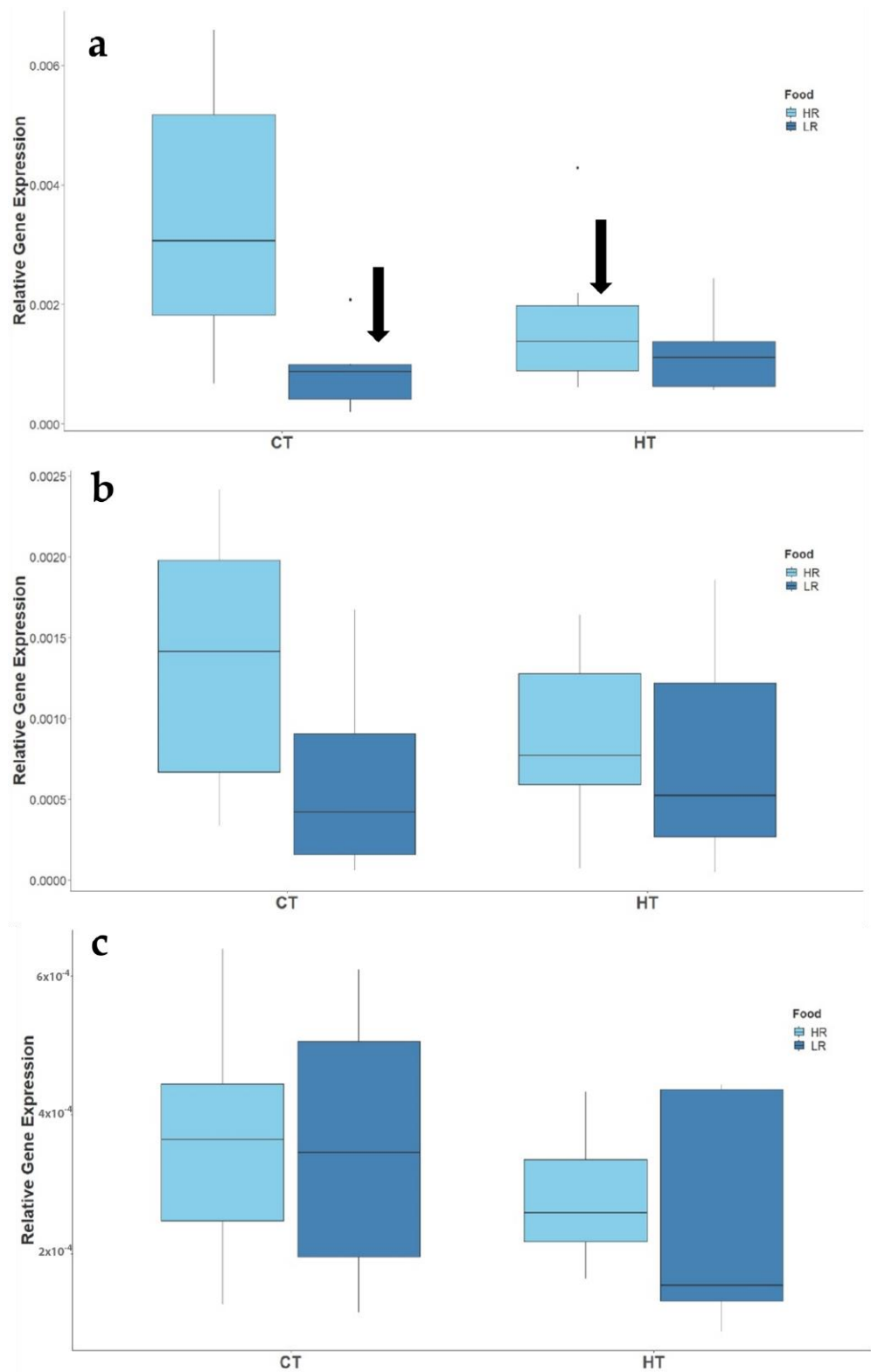
Figure 4. Effect of temperature (CT and HT) and food availability (HR, LR) on the expression of gonadal steroidogenic enzymes *cyp11b1* (**a**); *cyp19a1* (**b**); *20β-hsd* (**c**) of two-spotted goby, *Pomatochistus flavescens*. Black arrows indicate significant treatment effects ($p \leq 0.05$) as compared to CT + HR treatment.
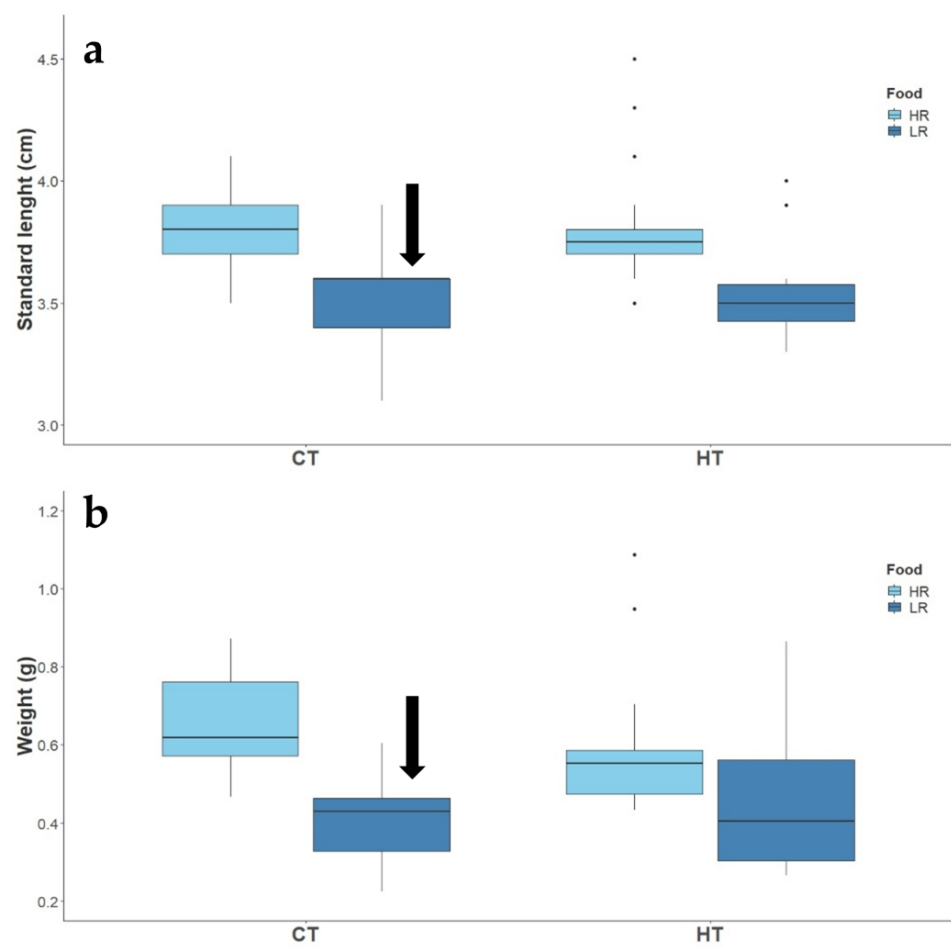
Figure 5. Effect of temperature (CT and HT) and food availability (HR, LR) on the standard length (**a**), and weight (**b**), of adult two-spotted goby, *Pomatochistus flavescens,* at the end of the exposure period. Black arrows indicate significant treatment effects ($p \leq 0.05$) as compared to CT + HR treatment.

3.2.3. Fatty Acid Content

Eggs exposed and descended from couples at higher temperature treatment presented a significantly higher percentage of SFAs than the ones at the control temperature (25% against 24%, respectively; $p = 0.027$), as shown in Table 5 (see also Supplementary Table S6). The higher level of SFAs under the high temperature scenario was mainly related to the higher levels of palmitic acid (16:0; 12% against 13%, in control and high temperature, respectively; $p = 0.012$, Supplementary Table S6). Regarding monounsaturated fatty acids (MUFA), no significant differences were found with temperature, with higher levels for oleic and vaccenic acids (18:1 n-9/n-7; 20% for both temperatures), and lower for 7-hexadecenoic and palmitoleic acids (16:1 n-9/n-7; 5% for both temperatures). We only found significant differences in polyunsaturated fatty acids (PUFA) with temperature for the eicosapentaenoic acid (EPA, 20:5 n-3) with higher levels in the high temperature scenario, 6% compared with 5% under control temperature ($p = 0.013$, Supplementary Table S6). Nonetheless, and even though not statistically different ($p < 0.05$), there is also a higher level of total PUFA with temperature, 41% against 37% under control, representing a p-value of 0.09. The same trend is found for alpha linolenic acid (18:3 n-3; $p = 0.058$, Supplementary Table S6), total n-3 (24% versus 29%, $p = 0.063$, Supplementary Table S6), n-3/n-6 ratio (2% versus 3%, $p = 0.063$, Supplementary Table S6) and unknown FAs (13% versus 8%, $p = 0.101$, Supplementary Table S6).

Table 5. Fatty acid composition (% of total FA) on eggs of two-spotted goby, *Pomatochistus flavescens* in Control (CT) and High (CT) temperature treatments (mean ± s.e.). Fish under low ration did not reproduce. Bold text indicates significant differences between temperatures in fatty acid composition.

Fatty Acids	Control Temperature	High Temperature
Saturated (SFA)		
14:0	0.59 ± 0.26	0.70 ± 0.07
16:0	**11.84 ± 0.71**	**12.68 ± 0.55**
18:0	7.32 ± 0.55	7.36 ± 0.29
Other SFA	4.41 ± 0.25	4.35 ± 0.30
∑ SFA	**24.15 ± 0.98**	**25.08 ± 0.64**
Monounsaturated (MUFA)		
16:1 *n*-9/*n*-7	5.25 ± 0.71	5.20 ± 0.48
18:1 *n*-9/*n*-7	19.61 ± 5.28	19.88 ± 3.21
Other MUFA	0.61 ± 0.09	0.64 ± 0.04
∑ MUFA	25.47 ± 4.84	25.71 ± 3.47
Polyunsaturated (PUFA)		
18:2 *n*-6	7.61 ± 0.67	6.97 ± 0.64
18:3 *n*-3	8.87 ± 3.69	9.00 ± 0.92
20:4 *n*-6	2.18 ± 0.13	2.24 ± 0.20
20:5 *n*-3	**4.93 ± 0.71**	**5.68 ± 0.42**
22:5 *n*-3	1.30 ± 0.39	2.66 ± 3.64
22:6 *n*-3	5.86 ± 2.62	7.29 ± 3.60
Other PUFA	6.24 ± 0.46	7.37 ± 4.67
∑ PUFA	36.99 ± 4.09	41.21 ± 5.50
∑ *n*-3	24.42 ± 3.92	29.37 ± 6.05
∑ *n*-6	11.83 ± 1.01	11.38 ± 0.63
***n*-3/*n*-6**	2.08 ± 0.39	2.61 ± 0.66
Total	86.60 ± 7.57	91.99 ± 5.50
100-TFA	13.40 ± 7.57	8.01 ± 5.50

3.2.4. Biomarkers

Total antioxidant capacity (TAC) levels in recently hatched larvae did not differ between temperature treatments, with average levels of $0.38 ± 0.14$ mM mg^{-1} total protein under control temperature and $0.38 ± 0.11$ mM mg^{-1} total protein for the high temperature treatment (Table 6, Figure 6). Regarding HSP70 levels, temperature did not increase HSP70 levels, $0.59 ± 0.30$ µg mg^{-1} total protein and $0.50 ± 0.25$ µg mg^{-1} total protein, under control and high temperature treatments, respectively (Table 6, Figure 6). Lipid peroxidation levels, measured as MDA levels, however, significantly increased with temperature, from an average of $0.01 ± 0.006$ nmol mg^{-1} total protein under control temperature, to an average of $0.02 ± 0.009$ nmol mg^{-1} total protein in the high temperature treatment (Table 6, Figure 6).

Table 6. Effect of Control (CT) and High (HT) temperature treatments on larvae of two-spotted goby, *Pomatochistus flavescens*MDA (nmol mg^{-1} total protein), TAC (mM mg^{-1} total protein) and HSP70 (µg mg^{-1} total protein). Bold text indicates significant treatment effects ($p \leq 0.05$) as compared to CT + HR treatment.

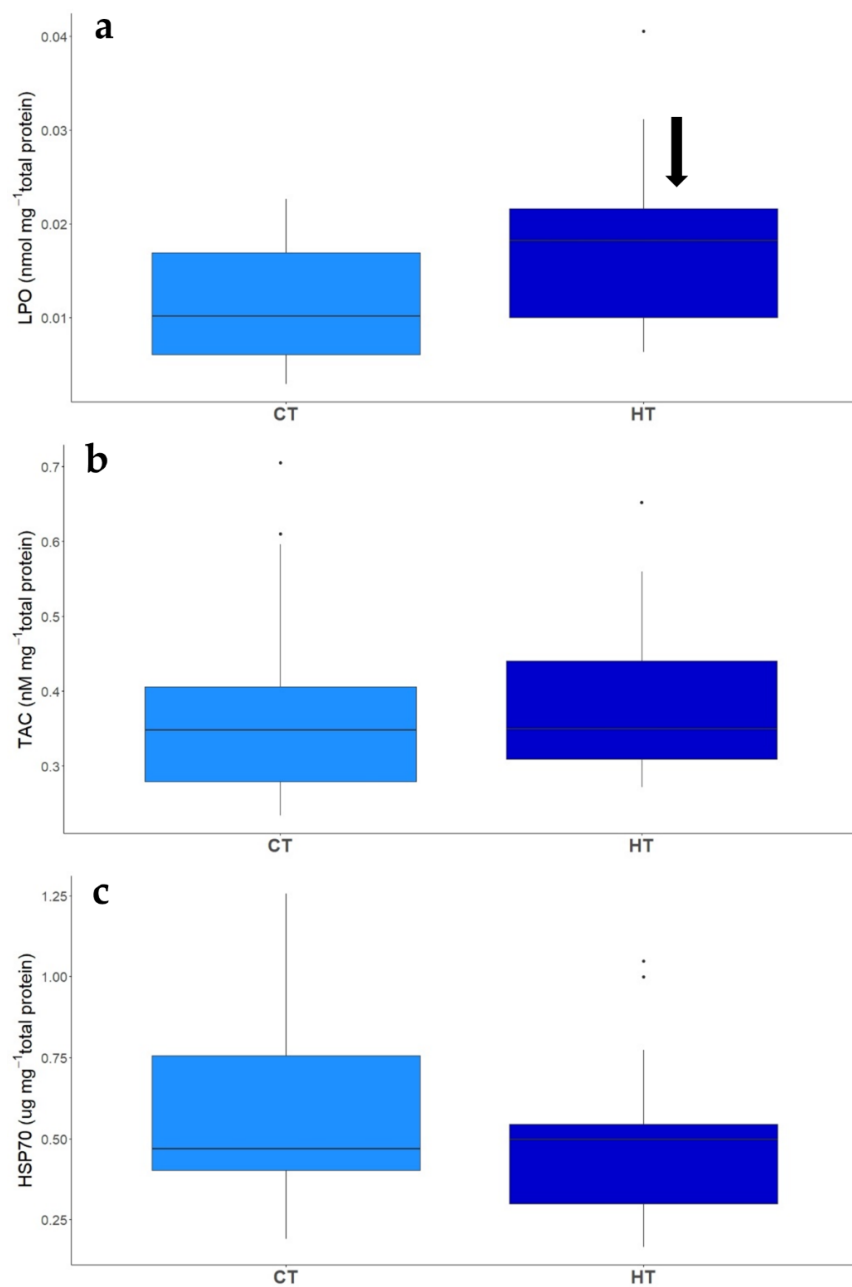| Conditional Model	Estimate	t Value	Pr (>|t|)
LPO	0.006	2.239	**0.033**
TAC	0.007	0.155	0.878
HSP70	−0.094	−0.953	0.348

Figure 6. Effect of exposure to Control (CT) and High (HT) temperature treatments on LPO (**a**), TAC (**b**) and HSP70 (**c**) of larvae of two-spotted goby, *Pomatochitus flavescens*, at hatching. Black arrows indicate significant treatment effects ($p \leq 0.05$) as compared to CT + HR treatment.

4. Discussion

The current results demonstrate that food availability played a stronger influence on two-spotted goby behaviour and physiology than temperature itself. Fish under LR showed poor body condition and increased mortality. Moreover, reproduction was completely impaired in fish under LR treatments, regardless of temperature conditions, which indicates that changes in food availability have paramount implications for populations. Contrarily, exposure to HT, per se, did not induce changes in adults' behaviour, metabolism, or reproductive performance, but it did affect egg area, with smaller eggs under higher temperatures.

Individual variation in routine behaviour is important in a species as it allows for populations to solve challenges posed by their complex environments differently [68]. In this study, assessing fish activity after the first month of exposure to the treatments allowed us to understand how fish would be affected by temperature and food availability in routine behaviours like swimming, resting, hiding, as well as assessing their aggressiveness, which is higher during the reproductive season [49]. Swimming and resting activities were not influenced by exposure to high temperature, low food availability, or the interaction of stressors. Different studies suggest that temperature can impact locomotion and activity of fish differently. Elevated temperature was shown to negatively influence swimming activity in juvenile *Salmo trutta*, brown trout [69]. In [26] while testing two species of minnows, *Chrosomus erythrogaste*, demonstrated strikingly opposite trends of activity rates. Although it was hypothesized that food availability would limit fish activity, as a strategy for saving energy, this was not the case. Ref. [70] showed an effect of low food ration on swimming activity of brown trout fry, but the effects were opposite to our hypothesis, as fry tended to be more active. Likewise, aggressiveness in the two-spotted goby was also not influenced by any of the treatments. Exposure to elevated temperatures can increase aggressive behaviours towards conspecifics, as described in *Pomacentrus moluccensis*, lemon damselfish, after 4 days of exposure to the temperatures, but a prolonged exposure to elevated temperature decreased aggressiveness to control levels [71], which the authors justify as an acclimation response. Exposure to food shortage conditions leads to an increase in aggressiveness in brown trout fry [70]. Interestingly, time spent by two-spotted goby hiding in the nests or algae was significantly lower in the LR treatment, which might suggest higher propensity to take risks when seeking for prey options. Hiding can be a response to predators' presence [72] and can be related to the fish willingness to take risks or be averse to them. A study on juvenile *Dicentrarchus labrax*, seabass, showed fish under a food-deprived regime for 7 days increased their risk-taking behaviour [73]. A different study on juveniles of the same species however, showed that after a 3-week period of food deprivation, the seabass, did not show any differences in risk-taking and exploratory patterns [74]. Interestingly, and opposite to what was found under LR, under the combination of HT × LR, two-spotted goby spent more time hiding. Increasing temperatures usually increase energetic demands [75], and since these fish had lower access to food, this might suggest a strategy to conserve energy, particularly because they were in their reproductive season. Contrarily to our results, a study on juveniles of *Pomacentrus chrysurus,* whitetail damselfish, exposed to different temperatures and food availability conditions, showed the interaction of these two factors influence their risk assessment behaviour, making the fish more risk-prone in their foraging behaviour [45]. Risk-prone individuals accept the risk to gain, in return of important information and higher rewards, while risk-averse individuals, avoid risking situation accepting that lower risk will also lower the gains [74].

Metabolic performance of the two-spotted goby was not affected by exposure to elevated temperature or low food availability, which indicates an import degree of plasticity towards thermal challenge. Although we expected higher temperatures to increase metabolic and energetic demands [75,76], the high temperatures tested fell within the two-spotted goby temperature range of variability. It is therefore possible that fish successfully acclimated and can manage their metabolic demands in these higher temperatures. A study on the effects of temperature and food availability on larvae of *Amphiprion percula*, the anemonefish, showed routine oxygen consumption was higher at elevated tempera-

tures [77], however food availability did not impact the oxygen consumption, as the method to calculate metabolic rates accounts for individuals' weight. A different study in the white seabream, *Diplodus sargus*, showed that the interaction of elevated temperatures and low food availability decreases RMR [48]. In our study, the lack of an effect on temperature and food availability in the RMR of the fish can be related with the exposure time. Fish were only exposed to the treatments for one month when they were tested, and this might have not been enough time to induce metabolic changes. More so, as mentioned before, two-spotted goby specimens were tested for elevated temperatures within their temperature range of variability, and it might be possible they are already acclimated to these temperatures. Finally, our experiment was done in fully grown adults, which have wider thermal windows and aerobic scope than early life stages [13,78] and are therefore less likely to be negatively affected by environmental stressors. Future studies should look to the impacts of temperature and food availability on behaviour and metabolism for longer periods of time, and also test different levels of temperature, especially outside this species variability range, like extreme climatic events (marine heat waves).

Temperature is one of the most important cues for the onset of the reproductive season. Changes in temperature can delay or even interrupt the reproductive process, affecting the long-term survival of the species [79]. Reproduction of the two spotted goby was shown to be impaired by high temperature. In a different study fish from the same species (different population) were exposed to ambient and +3 °C for a 3-month period, coincident with the breeding season, and showed reproduction failure in couples under high temperature, as well as decreased egg size [5]. Although temperature in the current study did not affect reproductive performance of the fish, it also led to smaller eggs. We argue that these seemingly contradictory results, on the same species, might reflect a population dependent effect, as the author of the study tested fish from the Swedish population of two-spotted goby, which inhabit a fjord [5]. The Portuguese population of the present study, on the other hand, inhabits the rocky shores of the Atlantic Ocean coast, the different habitats can have a strong influence in the fish plasticity to temperature variability.

In this study it was also investigated how the regulatory molecular mechanisms underlying reproduction were affected by temperature and food availability. It was showed that the pattern of expression of the gene cyp11b1 was influenced by low food availability (LR) and high temperatures (HT). LR is responsible for significantly decreasing the expression of cyp11b1 in males, which can explain why male fish in this condition had lower GSI and did not show courtship behaviour (observation only). In the case of male fish exposed to HT there is a decrease in their GSI in accordance with a previous study [5], and also a decrease in the expression of cyp11b1. However, GSI and cyp11b1 on males exposed to higher temperatures and low food availability (HT + LR) was not affected. This unexpected result might be related to low statistical power of the data on the males GSI and gene expression of cyp11b1 for HT + LR, since at the end of the experiment the sample size of this group was low. Together, these results suggest that low food availability has impaired male reproduction, given the pivotal role of cyp11b1 in the synthesis of the most important fish androgen, 11-ketotestosterone, which displays higher expression during the pre-spawning phase of the testicular cycle [80], consequently affecting the regulation of testis growth and maturation [81–83] and reproductive behaviour (e.g., [84]). Thus, though females were not seen to lay eggs, even if they did males were probably not able to fertilize them. Still, further studies should measure 11-ketotestosterone blood levels and analyse gonads histology to confirm this hypothesis. Interestingly, we detected no differences in females' GSI and ovaries' gene expression, for all the treated groups in comparison to the control group, even though we reported an impairment of the reproductive output in the short fed groups. Thus, it was hypothesized that, in these groups, other mechanisms and/or molecular pathways are being affected, since steroidogenic enzymes are putatively present (because gene expression occurs), but still, females did not lay eggs nor were seen courting. It seems like females continue investing in functionally reproductive ovaries even in food shortage conditions, contrary to males that stop investing in mature testis

in these stressful situations. Moreso, LR treated females may be saving energy through a different mechanism, such as skip spawning. This is a phenomenon that has been reported in many fish species and is linked to deficient feeding conditions and energy accumulation (reviewed in [85,86]). For instance, it has been reported that in fish may occur retaining (i.e., fully ripened eggs are never released) or reabsorbing (vitellogenic oocytes are reabsorbed via follicular atresia) processes (e.g., [87]) leading to spawning omission. In the two-spotted goby, food demanding conditions seem to induce different energy allocation strategies between males and females, as males seem to invest in their physical condition while females continue allocating energy in gonad maturation but skip spawning. Sex differences in energy allocation have already been reported, in a study with *Salmo salar* L., the Atlantic salmon. In this species, researchers showed that during the reproduction season, males spend more energy investing in somatic tissue while females' energetic changes occur preferentially at the gonadal tissue [88].

The FA composition of fish eggs is often used to evaluate their quality, as they must provide the required nutritional needs to endure embryonic and larval development, which will have direct consequences on hatching, survival and growth of fish larvae [89]. In the present study, the FA profile of eggs of the two-spotted goby, showed similar contents to the ones found in eggs of *Solea solea,* common sole, [90] and *Acipenser baerii,* Siberian sturgeon, [91], showing high levels of oleic and vaccenic acids (18:1 n-9/n-7), followed by palmitic acid (16:0), 18:3 n-3, linoleic acid (18:2 n-6), stearic acid (18:0), docosahexaenoic acid (DHA, 22:6 n-3) and eicosapentaenoic acid (EPA, 20:5 n-3). Differences in eggs FA profiles observed between the two different temperature regimes were minimal and mostly underpinned by higher levels of SFA, mainly related to higher levels of palmitic acid (16:0), and eicosapentaenoic acid (EPA, 20:5 n-3) at the higher temperature. Nonetheless it is important to mention that this is the first report on the FA profile of two-spotted goby embryos, and that comparisons between different populations, or even different life stages, were impossible to make.

Temperature is known to affect the FA profile of marine organisms, specifically due to lipids role in maintaining cell membrane integrity maintenance, and both palmitic acid and EPA are important for this task [89,92]. However, in the present study, we obtained different results from those already described in literature [93], as the proportion of unsaturated FA tend to increase at lower temperatures rather than at higher temperatures, in a process called homeoviscous adaptation that helps organisms to maintain the correct membrane fluidity at low temperatures. Nonetheless, the FA analysis in the present study refers to the total FA proportion in the two-spotted goby eggs and not only on the phospholipidic fraction. Therefore, high levels of PUFAs, such as EPA at higher temperatures could be a sign of maternal investment on the success of progeny, as this essential fatty acid (EPA) is not synthetized by the fish and must be provided via diet [94,95]. EPA has been proven essential not only for reproduction but also for embryo/larval development in many fish species [96].

Despite this possible increase in FA content in eggs due to maternal investment, we did observe smaller eggs under HT treatment, as well as an increase in lipid peroxidation levels in larvae from this exposure treatment. Early life stages are known to be particularly sensitive to environmental fluctuations and in this study, it was not an exception. The rise in temperature did not cause changes in the antioxidant capacity (TAC) or HSP70 levels, which concomitantly leads to lipid damage. Previous studies have also showed an increase in MDA levels with temperature in *Solea Senegalensis,* Senegalese sole, larvae after 30 days post-hatch [97].

5. Conclusions

Overall, the findings of the present study suggest a pronounced effect of low food availability on fish reproductive performance, as reproduction was completely cancelled. In a different study, it was found that *Acanthochromis polycanthus,* spiny chromis, reproduction failed completely when fish were under higher temperatures and low food availability [47].

Although lack of breading in some species may represent only a delay, the two-spotted goby life cycle is of 1 to 1.5 years [49], and they have only one breeding season. The success or failure of the breeding season of the two-spotted goby will certainly affect the species' long-term survival. Moreover, the fitness of fish under the low ration treatment after 5 months of exposure decreased significantly. Low condition fish presented a pronounced curvature in the spine that increased with exposure time, which translated in smaller sized fish by the end of the experimental period. Unexpectedly, fish under high temperature and low ration treatment did not present significant differences compared to control conditions, which might be attributed to outliers, and also to the fact that half of the fish under this treatment died before the end of the experiment, which might mean the fish in the worst body condition did not make it and thus the remaining fish had better, even if slightly, conditions. Exposure to warming conditions, per se, did not induce significant changes in reproductive performance; however, there seem to be costs at the offspring level, with smaller eggs, altered FA contents, and increased lipid peroxidation levels in recently hatched larvae. Whether or not these changes at the offspring level will have consequences later in life remains to be tested and should be a priority for future research.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/fishes7040194/s1, Table S1. Temperature (°C) (mean ± s.d.) over the experimental period (April–August 2019) in the four treatments: the two levels of temperature control (CT) and high (HT), and the two levels of food availability, high ration (HR), and low ration (LR); Table S2. Primer sequences and amplicon size for the genes studied; Table S3. Aikake's Information Criteria (AIC) model selection for "glmm" with: (1) negative binomial distribution for Time to Ignore the observer; (2) gaussian distribution for duration of Locomotion behaviours; (3) negative binomial distribution for duration of Rest behaviours; (4) negative binomial distribution for the frequency fish Hide; (5) negative binomial distribution for the frequency fish show Aggression. Temperature refers to Control (CT) or High T°C (HT). Food refers to high ration (HR) or low ration (LR) treatments, day refers to the day of observation (1 to 3). The selected model is shown in bold; Table S4. Effect of temperature (control, CT, and high, HT) and food availability (high ration, HR, and low ration, LR) on the gonadosomatic index of males (♂) and females (♀) of two-spotted gobies, *Pomatochistus flavescens*. Bold text indicates significant treatment effects ($p \leq 0.05$) as compared to CT + HR; Table S5. Effect of temperature (control, CT, and high, HT) and food availability (high ration, HR, and low ration, LR) on two-spotted goby, *Pomatochistus flavescens* size (SL) and weight (W), after 4 months of exposure to the treatments. Bold text indicates significant treatment effects ($p \leq 0.05$) as compared to CT + HR; Table S6. Effect of Control (CT) and High (HT) temperature treatments on fatty acid profile (% of total FA) of two-spotted goby, *Pomatochistus flavescens*. Bold text indicates significant treatment effects ($p \leq 0.05$) as compared to CT + HR treatment.

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