Supplementary materials

Ocean warming and acidification may challenge the riverward migration of glass eels

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DETAILED METHODOLOGY

A) ANIMAL COLLECTION AND ACCLIMATION

European glass eels, stages VI A1 – VI A2 [1], were collected at the mouth of the Minho Estuary (41º53' 26.33''N 8º49' 30.98''W, Portugal; salinity 35) in January 2017. Newly arrived animals were captured with the aid of local fishermen using hand-held dip and stow nets, following fishing regulations. The animals were then transported to the Laboratório Marítimo da Guia aquaculture facilities (Cascais, Portugal) in aerated and temperature-controlled tanks filled with seawater from the origin of capture.

Animals were randomly assigned to one of four cross-factored experimental scenarios: i) control (C; 14 ºC, pH 8.0); ii) acidification (A; 14 ºC, pH 7.6); iii) warming (W; 18 ºC, pH 8.0); iv) warming + acidification (WA; 18 ºC, pH 7.6); and then left to acclimate for a total of two weeks. For this purpose, temperature and water pH were gradually adjusted, over the course of a week, to the experimental conditions to which the individuals were to be exposed for 100 days. For glass eels exposed to warming and acidification conditions, temperature and pH were gradually adjusted over a one-week period at a rate of 0.5ºC and 0.05 pH units per day, respectively. Individuals were introduced into four replicate tanks (each with a volume of 15L) per treatment. In total, 90 individuals were placed per replicate in the C and A treatments, and 110 per replicate in the W and WA treatment.

Treatment specifications were set to reflect present-day conditions at the collection site and predicted end-century OW and OA levels [2]. To simulate the different salinities of the riverward migration of this species, a gradual salinity reduction took place over the last two weeks of exposure. The behavioural trials took place at salinity 35, 15 and 0, with one week between each set of trials. Specifically, trials were conducted at the end of the 12th, 13th and 14th weeks (day 85, 90 and 99 respectively). Animals were fed cod roe every other day.

Temperature was kept stable using water chillers (FRIMAR, Fernando Ribeiro Lda., Portugal) and submergible water heaters (Eheim, Germany). The acidification set-up followed standard acidification experimental design guidelines [3]. Levels of pH were permanently monitored in each replicate using a pH probe (GHL, Germany) connected to an automated controler (Profilux 3.1, GHL, Germany) - calibrated with TRIS-HCI (TRIS) and 2-aminopyridineHCl (AMP) (Mare, Belgium) seawater buffers – which adjusted pH values every two seconds. Water pH was downregulated by the injection of a certified CO_2 gas mixture (Air Liquide, Portugal), via solenoid valves (Etopi, Portugal), and upregulated by the injection of atmospheric air to maintain the desired conditions. Daily manual monitoring of water temperature (TFX 430, WTW GmbH, Germany) and pH (SevenGo pro SG8, Mettler Toledo) was performed. Seawater carbonate system speciation (Table S1) was calculated weekly from total alkalinity (TA; λ=595 nm) [4] and other parameter measurements. Total dissolved inorganic carbon (CT), pCO_2, bicarbonate concentration and aragonite saturation levels were calculated using the CO2SYS software [5], with dissociation constants from [6] as refitted by [7]. A flow-through system ensured continuous low-flux seawater renewal to each replicate, maintaining overall water quality and the maintenance of TA and adequate CT speciation. Water quality was further ensured using protein skimmers (Macroaqua, China), wet-dry filters, and 30-W UV-sterilizers (TMC, Chorleywood, UK). Ammonia, nitrite and nitrate levels were monitored regularly by means of colorimetric tests (Profi Test, Salifert, Holland) and kept below detection levels. The rearing tanks were inspected daily and any glass eels that died overnight were removed and recorded for survival analysis.

Salinity reductions took place gradually over 24 hours, through the addition of filtered and dechlorinated tap water, and kept stable over a week before behavioural trials. Salinity was regularly monitored with resource to a refractometer (TMC Iberia, Portugal). To simulate the salinity gradient associated with riverward migration, two gradual reductions took place following 85 (salinity 15) and 90 days (salinity 0). A fixed photoperiod of 12 h: 12 h (light/dark) was maintained by illuminating the tanks from above with white fluorescent lamps.

B) BEHAVIOURAL TRIALS

Eel migratory response and cue preference were assessed using a binary-choice test between two water flows: i) plain treatment water (sham); and ii) test water [with cue; freshwater (FW-test) or geosmin (Geo-test)]. Geosmin concentration (10^{-10} mg L^{-1}; > 97%, Sigma-Aldrich) was chosen to reflect environmentally-relevant levels [8,9]. The experimental device (Fig. S1) was designed according to the behavioural characteristics of glass-eels and based on previous studies [8,10,11]. The trials were performed in an acrylic tank (35 x 25 x 25 cm), with two lateral openings of two glass funnels (6 mm). These were connected to the main chamber by a silicon cork attached to the neck of two 250-mL filtering flasks, placed outside the choice chamber to form an eel-trap. Two peristaltic pumps delivered the testing flows to the traps at a constant rate of 45 mL min^{-1}. Opposite to the traps, an outflow ensured a stable water height of 6 cm, with constant flow and water renewal in the chamber. Prior to the trials, a dye test was performed to ensure that the two cue-plumes did not mix. At the beginning of each experiment, a group of 12 ± 4 individuals was gently introduced in a delimited area inside the choice chamber - filled with the respective treatment water - and left to acclimate for 20 minutes. Following release, the animals were left to choose between both water flows for the following 20 min. After the test, the number of animals trapped in each flask was registered. A total of two trials were performed per replicate, per treatment, changing the flow input position. The FW-test was

conducted at salinities 35 and 15, using water at salinity 15 and 0, respectively. Individuals which responded to the flows were considered active – i.e. exhibited upstream locomotor activity [10] - while the others were considered inactive. Thus, the proportion of animals collected in the traps, regardless of cue preference, was used as a proxy of migratory activity. Moreover, within this group, the proportion of animals collected in the cue side was used to assess cue preference. All trials were conducted in the dark in order to minimize the influence of light. Following each test, the apparatus was carefully cleaned with 96% ethyl alcohol and water, to remove traces of test-cues and conspecific odours.

C) STATISTICAL ANALYSES

Analysis of temperature and pH effects (both with two levels: control and high, control and low respectively) on glass eel survival and behavioural responses was performed via generalized linear and mixed models (GLM and GLMM). Backward step selection was performed to select which factor variables to include as fixed effects in each analysis (pH, temperature and Salinity). The best models were chosen according to the lowest value of Akaike Information Criterion (AIC). Survival was analysed with resource to a Negative binomial model, which was successfully validated resource to simulation studies - for this purpose, 1000 random datasets were generated and modelled for negative binomial data, and the position of the original theta within a frequency of random thetas' distribution was assessed. For easier visualization, the proportion of surviving eels was plotted. Behavioural outputs were analysed with GLM and GLMM with binomial distribution, when necessary. Replicate and cue-side were considered as random factors and included when a better model fitting was rendered. An α level of 0.05 was used to determine statistical significance. All statistical analyses were performed using the R software (version 1.1.453) [12].

D) SUPPLEMENTARY TABLES AND FIGURES

Table S1. Summary of seawater parameters in experimental treatments. Values represent means ± standard deviation.

Salinity 35	**Control**	**Ocean acidification**	**Ocean warming**	**Ocean warming and acidification**
Measured				
Temperature (°C)	14.19±0.41	14.20±0.43	18.16±0.53	18.28±0.54
pH_T (total scale)	8.04±0.06	7.66±0.10	8.08±0.06	7.65±0.09
Salinity	35.09±0.36	35.08±0.34	35.06±0.28	35.04±0.25
TA µmol kg^{-1} SW)	1917.30±179.69	1920.85±196.33	1940.80±155.11	1911.46±146.23
Calculated				
pCO_2 (µatm)	322.26±45.38	859.57±92.17	302.60±47.64	893.17±142.39
CT (µmol kg^{-1} SW)	1722.40±170.32	1857.21±190.06	1705.41±151.83	1835.22±151.56
Ω Arg	2.00±0.24	0.94±0.14	2.43±0.23	1.05±1.03
Salinity 15				
Temperature (°C)	14.39±0.25	14.43±0.07	17.71±0.11	17.83±0.08
pH_T (total scale)	7.95±0.05	7.60±0.11	7.97±0.06	7.56±0.05
Salinity	15.02±0.30	15.03±0.32	15.01±0.40	15.06±0.35
Salinity 0				
Temperature (°C)	14.87±0.22	14.95±0.50	18.03±0.29	18.24±0.29
pH_T (total scale)	7.97±0.03	7.61±0.06	8.00±0.03	7.57±0.07
Salinity	0.00	0.00	0.00	0.00

Temperature, pH (pH_T), and total alkalinity (AT) were used to calculate carbonate system parameters [pCO2 (carbon dioxide partial pressure), CT (total inorganic carbon), and Ω Arg (aragonite saturation state)].

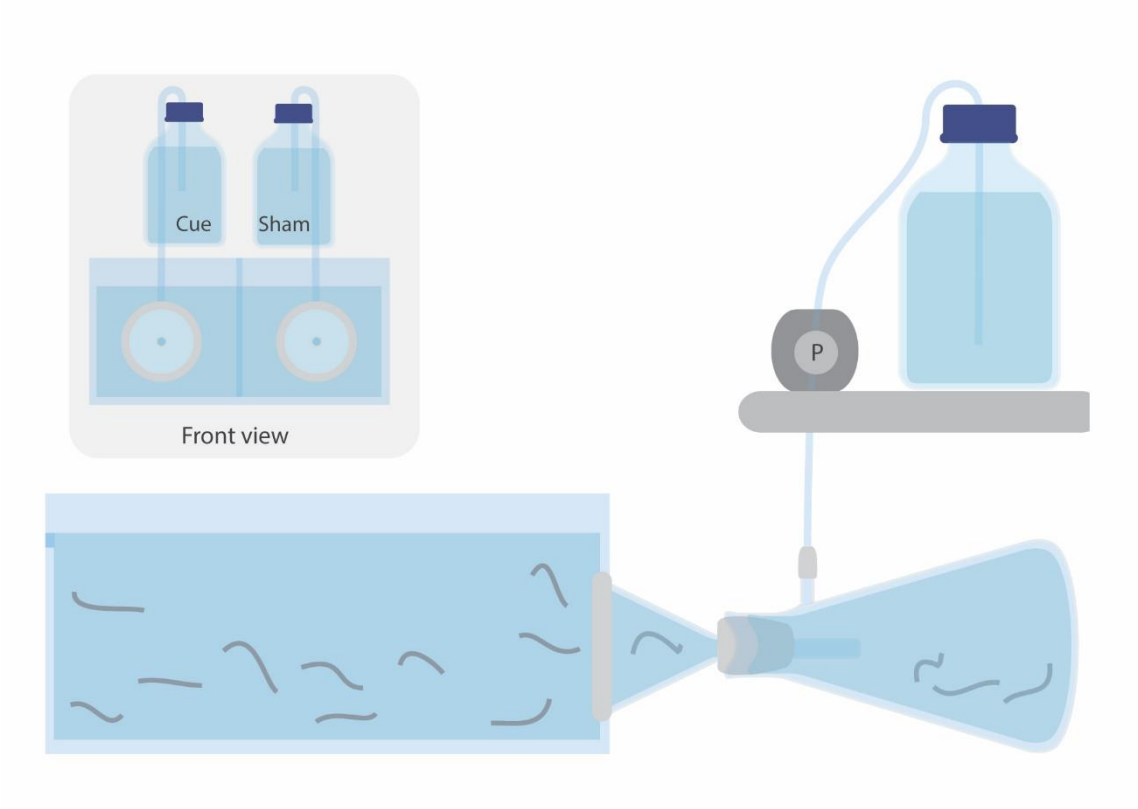

Figure S1. Simplified view of the binary-choice device used in the behavioural trials to assess locomotor response and cue preference. Two header flasks containing a test cue and sham-water fed to the 'eel traps' placed on one end of the choice-chamber. Openings on the opposite side of the traps ensured that the water was kept at a stable height of 6 cm, and two peristaltic pumps (P) delivered water from the header flasks at a constant flow (45 mL min^{-1}), creating the currents to stimulate eel upward movement.

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