Deliverable 6.3
Beneficial Effects of Physical Enrichment and Training on Key European Species Used in Aquaculture

Version 1

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Deliverable 6.3
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1. Objective

This report addresses one of the overall objectives of WP6 - JRA3.- *« Improvement of fish welfare in experiments and industry »* which was centred on *« Improvement of fish welfare in experiments (Refine), to set up common ways to evaluate and monitor fish welfare and propose ways of improvement. »*

WP6 has developed a comprehensive framework to evaluate, monitor and potentially improve the welfare of fish used in experimental and commercial settings, in relation to a Refinement and Reduction (less losses, less grading necessary) perspective. In WP6 **task 3**, methods to improve welfare through the implementation of enrichment in experimental units, and the development of a training/ exercising protocol for fish early life stages for promoting robust juvenile fish have been tested in key European fish species (Atlantic salmon, rainbow trout, European seabass). This report presents the major results from this task and aims to provide operational recommendations for improving welfare in animal experiments in the field of aquaculture, as well as delivering useful outputs to commercial aquaculture operations.

All the experiments were conducted after ethical approval and following the 3Rs and directive 2010/63, licence information is stated for each study.

Current dissemination:

Prentice P., Rey Planellas S. (2023). The effects of environmental enrichment on behaviour and welfare of juvenile Atlantic Salmon (*Salmo salar*) housed for Aquaculture research – 56th Congress of the International Society for Applied Ethology, ISAE 2023, 1st – 5th August, 2023, Tallinn, Estonia.

Moro-Martínez I., Abbink W., Agbeti W., Arechavala-Lopez P., Bégout M.-L., Lallement S., Lankheet M., Palstra A. P. (2023). Oxygen consumption and locomotory behaviour during a swim fitness test of European seabass (*Dicentrarchus labrax*): relation with origin and early life exercice training . EAS 2023 - Aquaculture Europe 2023. September 18-21, 2023, Vienna, Austria.

Prentice P.M., Chivite M., Cisar P., Rey Planellas S. (2025) Early-life environmental enrichment promotes positive animal welfare for juvenile Atlantic salmon (*Salmo salar*) in aquaculture research. *Submitted* to Scientific reports, Animal Welfare, 15: 5828; https://doi.org/10.1038/s41598-025-88780-0.

Chivite M., Cisar P., Rey Planellas S., Legueun I., Bégout M.L. Effects of structural environmental enrichment upon the welfare of juvenile seabass (*Dicentrarchus labrax*) in aquaculture research. *In prep.*

2. Background

Optimising fish welfare in aquaculture research is fundamental for ensuring scientific quality, its relevance, reproducibility and effectiveness in producing robust, resilient fish. Fish welfare has been addressed in many research and review articles (*e.g.,* Ellis et al., 2002; Huntingford et al., 2006; Martins et al., 2012; Stien et al., 2013), but assessing the welfare of each fish species is still very challenging. There is still need for larger data-sets on welfare and performance to be able to view bigger trends across species in Europe. This requires that experiments are done the same way in different species; measuring the same indicators with the same technology. Fish welfare is increasingly on the agenda for regulatory bodies for all aquaculture species, and since we are facing different operational

challenges resulting in acute and chronic stress, we need to focus **on how to improve welfare**. There are several important variables to take into account when improving welfare, in WP6 – task 3, we have focused on environmental enrichment, and training juveniles for better welfare in later life (Palstra et al., 2010).

Approach and objective

The welfare indicators reviewed in WP6 Task 6.1, and tested in Task 6.2 have been implemented in the experiments for improving welfare conducted in Task 6.3. Protocols and indicators were chosen in common to reach some standardisation and are fully presented in milestone M34 report, here below is a simplified version of M34 focusing on task 6.3.1 shared experimental set up.

	USTIR (Atlantic salmon)	IFREMER (Sea bass)	IMR (Rainbow trout)
Type of facility	RAS	Flowthrough	Flowthrough
Tank volume, size, N	700 L/8 tanks	1500L / 6 tanks	2000 L, 3 m diameter, 6 tanks
Experiment duration	Duration 3 months		
Fish size/age	From ca 1g for 3 mo	50-200 g 12 mo old at start	From ca 5 g
Fish number	375 x 8 tanks = 3000 indiv.	120 x 6 tanks = 720 indiv.	2000 x 6 tanks = 12000 indiv.
Temperature	Controlled temperature 12°C	Controlled temperature 21°C	Controlled temperature 12°C
Light	24 h regime/ artificial light	Artificial light, natural cycle	
Oxygen	> 100%		
Feeding method	self feeder (24h)		automatic feeders
PIT-tagged	No	Yes	No
Experimental plan	Two conditions: CTRL, ENRICH with **false weeds** in quadruplicate tanks (4 per condition)	- Two conditions: CRTL, ENRICH with **false weeds** – triplicate tanks - Tagged fish either trained or untrained from task 6.3.2	- Two conditions: **2D patterns** (50% bottom area) with dark/light bottom, or all light bottom (CRTL)
Stressors	Short-term stressors: - Crowding by water lowering at time of weeds removal for cleaning		Short-term stressors: - Shadow above tank (simulating predatory bird) - Applied daily from Day 30, to compare habituation rate
Stress coping style assessment	-Dropping a GoPro camera with a pole (for novel object: before, during and after ENRICH treatment): latency to resume normal behaviour - Number of applications: 6 times	- pre-screening using Group risk taking test (Sadoul et al. 2021) - Dropping a Go Pro camera with a pole (for novel object: before, during and after ENRICH treatment): latency to resume usual behaviour - Number of applications: 3 times	- Dropping a Go Pro camera in tank to measure latency to approach, distribution change, to resume usual behaviour - Number of applications: 3 times
Morphological OWIs from FISHWELL morphological OWI scoring scheme, on a scale 0-3	- Fin damage: active and healed (dorsal, caudal, pectoral) - Body injuries (scale loss, haemorrhaging, wounds) - Spine – vertebral deformities - Snout damage, upper Jaw deformity, lower jaw deformity, operculum deformity - Eye (e.g., cataract, keratitis pigmentosa, haemorrhaging, exophthalmia		
Behavioural OWIs as listed in the Task 6.1 MS30	- Distribution within the tank (horizontal and vertical if possible) -Frequency of area occupation	- Horizontal distribution -Frequency of area occupation	- Horizontal distribution
Physiological OWIs	-Growth performance (tank FCR, individual SGR)	- Growth performance (tank FCR, individual SGR)	- Growth performance (at tank level)

	- Blood/plasma for cortisol, lactate, glucose on a sub sample of fish - Mucous/ faeces for cortisol - Brain for neuroplasticity analyses	- Blood/plasma for cortisol, lactate, glucose on a sub sample of fish - Tank water for cortisol	- Blood/plasma for cortisol, lactate, glucose on a sub sample of fish

Deliverable 6.3 presents achievements of subtask 6.3.1 (involving partners UoS, IFREMER, IMR) which focused on the approach to improving welfare with environmental enrichment (EE) using Atlantic salmon, European seabass and rainbow trout respectively.

As recently reviewed by Arechavala-Lopez et al (2022), environmental enrichment (EE) can improve the welfare of captive fish "Its objective is to provide new sensorial and motor stimulation in order to help meet their behavioural, physiological, morphological and psychological needs, whilst reducing stress and frequency of abnormal behaviours". The current knowledge, mostly based on small scale experimental approach, requires upscaling to experimental production conditions to determine what we need to know to make it work well in production conditions. Here we have chosen simple structural enrichment to add physical complexity and increase heterogeneity of the rearing environment (Näslund & Johnsson, 2016).

In subtask 6.3.1, the specific objectives were to assess the impact of EE (false plants or 2D contrasted coloured bottom) on a large set of fish welfare indicators. Here we have tested enrichments with physical characteristics that rendered them acceptable in an experimental facility or in professional aquaculture. An additional stress event was occasionally presented to the fish to evaluate their responsiveness and latency to recover a normal behaviour (short term novel object introduction) as well as a final stress challenge common to husbandry practice (i.e., net chasing for 5 min) to evaluate fish stress responses. We measured morphological, behavioural, and physiological welfare indicators throughout the experiment, comparing responses from enriched with non-enriched (NE) treatment groups.

In subtask 6.3.2 (involving partners WU, WR, IFREMER) an early life training procedure was used in European seabass. The specific objectives were to test whether there is an effect of early life training in later life maximum aerobic capacity and swimming efficiency (oxygen consumption, energy balance and swimming speed), whether there is a difference between the three strains of the European seabass, and whether the effect of early life training is strain dependent.

The following text structure outlines the results of these tasks.

3. Assessing the effect of early-life environmental enrichment on the welfare of juvenile Atlantic salmon (*Salmo salar*) in aquaculture research (UoS)

3.1 Animals and experimental set up

Atlantic salmon fry (*Salmo salar*) were hatched and raised at the University of Stirling's Niall Bromage Freshwater Research Unit (NBFRU). After resorption of their yolk sack, fry (n = 3000, 1g in weight), fish were randomly distributed equally among eight identical Recirculatory Aquaculture System (RAS) tanks (n = 375 per tank). RAS tanks were 1m in diameter (0.78 m^2) with a total volume of 700l, and a water renewal rate of 900L/hr. The water temperature was maintained at 12◦C during the experiment and tanks were covered with lids that supported a mounted light (providing 24hr light). A SWAN CCTV camera was mounted onto each of the RAS tank lids for behavioural recording. Each tank was equipped with an automatic Arvo-Tec feeder that provided continuous ad libitum commercial fish feed from BioMar Ltd. Husbandry operations included daily mortality checks and cleaning of the central outlet pipe, further to weekly tank cleaning (including the enrichment structure) and sampling to check weights. All fish were kept under these conditions for the duration of the experiment (13 weeks).

Prior to the start of the experiment, fry were left to acclimate in the RAS tanks for 14 days.
Structural **environmental enrichment** (**EE**) was added to four randomly assigned RAS tanks, resulting in four EE tanks (4 x EE), and four **non enriched** (**NE**), barren tanks (4 x NE).
The enrichment consisted of a smooth circular PVC grid (70 cm diameter) covered with artificial plastic plants.

The enrichment structure was suspended from the rim of the tanks, providing cover throughout the water column created from vertical suspension of plant leaves.

Ethics

This work was conducted under the auspices of the UK Animals (Scientific Procedures) Act (1986) with approval of the University of Stirling research ethics committee (AWERB 2022 7013 5863). Experimental procedures and behavioural assays were developed in accordance with the principles of the three Rs and ASAB guidelines, and written up following ARRIVE guidelines. As such, animals housed in control tanks were returned to the stock population for reuse in later trials. All periods of handling and emersion were kept to a minimum and only fish deemed healthy and exhibiting normal behaviour were used in trials. End points were considered at each stage of the experimental protocol.

3.2 Welfare Indicators

To assess the impact of EE on juvenile salmon, we measured morphological, behavioural, and physiological welfare indicators throughout the experiment, comparing EE with NE treatment groups.

i. Morphological welfare indicators

To assess whether EE affects morphological welfare indicators, we compared morphological scores from samples of fish at the beginning, with scores at the end of the experimental procedure. Fish were sampled (n = 20 fish per tank) at week 0, and again at the end of the experiment (pooled across two sampling points: n=10; week 12, n=10; week 13). During these sampling sessions, fish were weighed, and length measured, and the following welfare indicators (WI) scored; dorsal fin damage, and body condition. The indicators were measured using the FISHWELL scoring scheme (Noble et al. 2018) and scored on a scale of 0-3 (Level 0: Normal condition, Level 1: Minor occurrences, Level 2: Compromised condition, Level 3, clear evidence of the OWI).

ii. Behavioural welfare indicators

Behaviour was recorded throughout the experimental period with an overhead camera attached to the tank lids. The camera was oriented towards the bottom of tanks, with the field of vision capturing half of the whole tank. Video recordings (automated to reduce disturbance) captured footage for 30 minutes at 4 timepoints each day (6am, 12pm, 6pm and 12am). To extract quantitative data of fish behaviour we used inhouse software to analyse the video footage. The software was implemented in MATLAB, and based on statistical modelling using the tank background to represent the monitored scene and background subtraction to detect the fish group, fish individuals and enrichment structure. The software produces the position and size of each fish group, positions of individuals, and area of the enrichment for all consecutive video frames. To compare the behaviour of salmon fry under EE with NE conditions, we analysed the following traits: *group cohesion*, a*ctivity*, and *enrichment occupation,* calculated from the parameters produce by the software (Table 1).

To further assess the effect of EE on behaviour, we exposed each tank to a novel object test, once every 2 weeks (6 trials per tank in total). The test consists in the experimenter suddenly submerging a Go Pro camera attached to the end of metal pole (2 m) into each tank in order to elicit a fright response. The camera recorded the response of fish to the novel object for 30 minutes. From this footage the trait *latency to resume normal activity* was extracted and quantified using the software BORIS (Friard, O., & Gamba, M. 2016).

iii. Physiological welfare indicators

To assess the effects of environmental enrichment (EE) on stress physiology and neuroplasticity, we measured multiple physiological welfare indicators across the 3 sampling sessions. To measure the effect of enrichment on the stress response, we analysed the cortisol data for each treatment, before and after exposure to the stressor event (chasing with a net). To evaluate neurogenesis, the following target genes were used *ndf1* and *bdnf,* and hsp90 for its role in signal transduction and the maturation and affinity of glucocorticoid receptors. Firstly, we measured a population base line cortisol level by sampling fish from the stock population (n=20; week 0). The fish were culled using a Schedule 1 approved method (overdose of MS-222 at 0.05% (w/v) followed by confirmation of death), and frozen on ice, transported back to laboratory (Institute of Aquaculture, University of Stirling) and stored at -80 C for whole body cortisol analysis (protocol described below).

At the end of the experiment, we sampled fish from each tank prior to, and following an acute stress event. Firstly, we sampled fish from each tank (n=10 per tank; week 12), following an overdose with MS222, blood samples were taken for plasma cortisol analysis. By week 12, fish had grown in size (mean 30.82 ± 4.88g), facilitating the extraction of blood samples, allowing for a more robust and clean measure of cortisol than whole body cortisol.

Table 1: Ethogram of behavioural welfare indicators

Behaviour	Description
Overhead camera	
Group cohesion	The ratio between area occupied by the fish group (minus any outliers) and area of the tank monitored by the camera Categorical score: 0 = Tight; ratio < 0.25 1 = Loose; 0.25 > ratio < 0.75 2 = Dispersed; ratio > 0.75
Activity	Mean distance swum by all fish (cm) Calculated as the mean distance for all fish in one frame, divided by the number of frames
Enrichment Occupation	Percentage of fish under the enrichment (%) Calculated as the ratio between area occupied by fish under the enrichment, and the total area occupied by all fish
Novel Object Test	
Latency to resume normal activity	Time taken for fish to resume "normal swimming" (seconds). Normal swimming resumed when the occurrence of darting behaviour (sudden, rapid, high velocity movements) was less than 2 fish per 10 seconds

A heparinised syringe withdrew approx. 2 ml of blood (dependant on the size of individual fish) from the caudal vein, which was then chilled on ice. Blood samples were centrifuged for 10 min at 3500xg, following which plasma was transferred and frozen on dry ice. At this stage, brain tissue was also extracted for molecular analysis of RNA gene expression. Samples were immediately frozen on dry ice and stored at -80C° until analysis (details below). One week later (week 13), fish were exposed to an acute stress event (mild stressor: chasing with a net for 5 minutes). 30 minutes following the acute stress event, fish were sampled again (n=10 per tank) and blood and brain tissue was extracted.

A. Blood plasma and whole-body cortisol analysis

Plasma cortisol was extracted by iquid-liquid phase extraction (LLE). Briefly, 2 ml of ethyl acetate was added to both samples and calibrants, then 500 µl potassium chloride solution (0.88 % w/v). Samples were mixed and centrifuged at 14,000 rpm for 5 minutes. The supernatant was separated, with a second ethyl acetate extraction (500 µl) performed on the remaining lower phase. Pooled supernatant was dried under a stream of nitrogen and resuspended in 100 µl of cold 1:1 (v/v) methanol to water solution. After mixing, samples were centrifuged at 14,000 rpm for 2 minutes.

Whole body cortisol samples were homogenised in 15 mL water using an Ultra Turrax. 500 µl of methyl-tert-butyl ether (MTBE) and 500 µl of 1% (w/v) potassium chloride solution added to 250 µl of homogenate, then mixed, centrifuged at 14,000 rpm for 2 minutes, and supernatant isolated. A second MTBE extraction (500 µl) was performed, pooled with the first, and the extracts dried under nitrogen. Samples were resuspended in 60 µl of 1:1 (v/v) methanol/water solution and centrifuged at 14,000 rpm for 2 minutes. d4-cortisol was used as the internal standard in both approaches.

Cortisol was analysed using LC-MS/MS (Acquity I-Class UPLC coupled to a Waters Xevo TQS mass spectrometer). Chromatographic separation was achieved with an Acquity HSS T3 (Waters) 2.1 x 50 mm column (1.8 µm) with a 5 mm guard column. The mobile phase comprised a binary solvent system:

MilliQ water (solvent A) and methanol (solvent B), both containing 0.1% (w/v) ammonium formate with 0.1% (v/v) formic acid. 12 µl of sample were injected into the column. The instrument was operated in positive electrospray ionization (ESI) mode using MassLynx V4.1 software (Waters). Limits of detection and quantification of 0.02 and 0.06 ng/ml, respectively, were achieved with sample inter-assay CV (n=5) = 7.1% and intra-assay average %CV (n=9) =1.1%.

B. Molecular analysis

Primer design and cloning

Primers were searched from literature where available or designed with a primer design tool (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). All primers were found specific for all target genes after the blasting process (http://www.ncbi.nlm.nih.gov/BLAST/). Touchdown PCR protocol was run to amplify the cDNA product in each of the target genes from a pool of brain samples. The amplified PCR product was verified in a 2% agarose gel and an aliquot was collected and purified by a commercial kit (Qiagen). Fragments were cloned using pGEM-T easy vector and JM109 competent cells (Promega). Plasmid DNA was obtained using QIAprep Spin Miniprep Kit (Qiagen) and sequenced to verify their identity (Eurofins genomics Europe). The concentration of the product was measured with a spectrophotometer (Nanodrop ND 2000C). The number of copies/µg RNA was calculated by using the molecular weight of the product and Avogadro's constant. The plasmid solution was serially diluted to obtain a standard series from 10^7 to 10 copies/µl.

RNA isolation and absolute qPCR quantification

Total RNA was extracted from whole brains by using the TRIZOL-reagent method (EE and NE pre-stressor n=12, EE and NE post-stressor n=24). 2µg of total RNA was used to obtain cDNA, using (RT2 First Strand Kit, Qiagen). Transcript copies of target genes were determined by real-time quantitative PCR (qPCR) using a Biometra TOptical thermocycler. Analyses were performed on 1 µL of diluted cDNA (1/20) using the Luminaris HiGreen qPCR Master Mix (ThermoFisher Scientific), containing 100 nM of each primer. Transcript levels were quantified by using standard curves which were run under the same conditions.

C. Brain 5HT and 5HIAA analysis

The whole brains (n=24 for each treatment) were homogenized using ultrasonic disruption in 300µL of pre-cooled mobile phase buffer (63.9 mM NaH2PO4, 0.1 mM Na2EDTA, 0.80 mM sodium 1-octane sulphonate, and 13% (v/v) methanol, pH adjusted to 2.95). After homogenization the samples were centrifuged (16000g, 10 min) and the supernatants were diluted (1:5) with the mobile phase. 20 µl were injected into the HPLC system, which consists of a Jasco PU-2080 pump, a 5 µm analytical column (Phenomenex, Nucleosil C18, 150 mm length × 4,6 mm diameter) and an ESA Coulochem II detector. The oxidative potential was set with a double analytical cell (M5011) at + 40 mV in the first electrode and + 340 mV in the second one. The concentrations of 5-hydroxytryptamine (5HT) and 5-hydroxyindoleacetic acid (5HIAA) were quantified. Data were integrated using ChromNAV version 1.12 software (Jasco Corp.) and relativized against total protein concentration, which was estimated using the bicinchoninic acid method.

3.3 Statistical analysis

Data analysis of morphological and behavioural traits was carried out using R software (version 4.3.0) (R Development Core Team, 2019). Model assumptions were visualised with the “ggplot2” package. Physiological traits were analysed, and all graphs were created with GraphPad Prism 8.0.1. Mixed model structure and selection was based on either Akaike Information Criterion (AIC) or Bayesian Information Criterion (BIC), dependant on model frameworks. All models assumed Gaussian error structures, deemed acceptable based on visual inspection of the model residuals. In statistical tests, levels of significance were set at $p \leq 0.05$ for all statistical tests.

i. *Morphological welfare indicators*

A linear mixed effect model (LMM) (lme4 package], was used to analyse the effect of enrichment on fish growth by measuring the length and weight of fish, comparing these traits at the beginning to the end of the experimental treatment. The standard assumption being that random effects and residuals are normally distributed. Both response variables (*length* and *weight*) were modelled independently and used identical fixed effect structures of *treatment* (factor with 2 levels: EE or NE treatment groups), and *session* (factor with 2 levels: week 0 and week 12). We also included *tank* as a random effect to control for repeated measures on each tank.

To analyse the effect of the enrichment on the morphological traits *dorsal fin damage* and *body condition* (both measured on a 0 – 3 scale), we used a generalised linear mixed model (GLMM), fit with MCMCglmm. Both response variables (*dorsal fin damage* and *body condition*) were modelled independently with ordinal distributions, with fixed effects of *treatment* and *session*, and a random effect of *tank.* GLMM models implemented with the MCMCglmm package function under Bayesian frameworks, contrasting with frequentist models. In this Bayesian context, model significance is determined by whether credible intervals straddle zero or not, rather than relying on p-values. Nonetheless, it is possible to derive p-values using the MCMCglmm package, which represent the likelihood of observing the given data assuming the null hypothesis (that there is no effect) holds true.

ii. *Behavioural welfare indicators*

To measure the change in enrichment occupation across the experimental procedure, we firstly transformed the data as the variable *enrichment occupation* was measured on a percentage scale. The data was converted to proportions, then square-root transformed to conform to normality. We used a LMM with a fixed effect structure of *week* (a continuous variable, from 0 – 12) and *time* (factor with 4 levels; 12am, 6am, 12pm, 6pm). We also included *tank* as a random effect to control for repeated measures from each tank, allowing individual slopes for each tank across weeks (1+*week:tank*).

To analyse the effect of enrichment on the behavioural trait *activity,* the variable was firstly mean centred and scaled to standard deviation units to ease interpretation of results. We used a LMM with a fixed effect of *treatment* (a factor with 2 levels; EE and NE), with further fixed effects of *week* and *time,* and *tank* as a random effect. For *group cohesion* (measured on a 0 –2 scale, 0=tight cohesion, 1=loose cohesion, 2=dispersed), we used a GLMM, fit with MCMCglmm with an ordinal distribution. We included fixed effects of *treatment* and *session*, with an interaction term (*treatment:session*) to measure any relationship between the two, and a random effect of *tank.* To analyse the effect of the enrichment on the behavioural response during the novel object tests, firstly we transformed the variable *latency* (*to resume normal activity*) using square root transformation, so that the raw data was more normally distributed. We then fit a LMM with *latency* as the response variable, fixed effects of *treatment* and *trial* (a factor with 6 levels, representing each novel object trial), and a random effect of *tank.*

iii. *Physiological welfare indicators*

Cortisol analysis

Firstly, the population base line measure of whole-body cortisol was compared between both treatment groups using a t-test. Second, to measure any effect of the enrichment treatment on cortisol expression pre and post stressor at the end of the experiment, we used an LMM. The variable *cortisol* (blood plasma cortisol) was square root transformed to better fit normality, then a LMM was fit with *cortisol* as the response variable, fixed effects of *treatment* and *session* (a factor with 2 levels: pre- and post-stressor), and a random effect of *tank.*

Monoamines and brain gene expression

To measure the effect of the enrichment on monoamines and brain gene expression, we analysed the monoamine concentration and absolute expression of the RNA transcripts for each treatment (treatment groups pooled together for analysis), before and after exposure to the stressor event. A

two-way ANOVA followed by multiple variable Tukey's t-test was performed to evaluate any effect of the enrichment treatment on monoamines and gene expression, using categorical variables *treatment* and *session.* Furthermore, a Pearson correlation analysis between the levels of the *ndf1* and *bdnf* and between *ndf1* and *hsp90* was conducted within treatment groups (EE and NE).

3.4 Results and Discussion

Results

i. *Morphological welfare indicators*

No effect of the enrichment on any morphological trait was observed. No difference was found in length between the NE group (mean ± SE = 14.07 ± 0.167 cm), and the EE group (13.96 ± 0.167 cm; t=0.554, df=8, p=0.595). Similarly, no difference in weight was found between treatment groups (NE = 31.03 ± 0.742 g; EE = 30.62 ± 0.742 g; t = 0.462, df = 8, p = 0.656) meaning all animals grow under a similar pattern and were homogenously distributed in weight and length at the end of the trial.

The GLMMs did not provide support for an effect of the enrichment on *dorsal fin damage* or *body condition.* For *dorsal fin damage,* the estimated effect of the enrichment treatment was non-significant, with confidence intervals (CI) straddling zero (posterior mean = -9.099, 95% CI: -23.761 to 5.056, pMCMC = 0.092). A similar non-significant effect of treatment is observed for *body condition* (-1.146, 95% CI: -8.71 to 3.00, pMCMC = 0.922). This WI did not show any detrimental effect of the experimental conditions on the animals related to fin damage or body condition (indicative of aggression or disease for example).

ii.*Behavioural welfare indicators*

A high percentage of fish were observed under the enrichment in the first 2 weeks of the experiment (week 1; mean ± SE = 47.9% ± 0.767, week 2; 41.8% ± 0.738). Enrichment occupation declined each week, and a significant effect of time was observed (-0.133 ± 0.021, t = -6.425, df = 4 p = 0.003l of note, a strong significant decline in enrichment occupation was observed at 12pm (-0.247 ± 0.057, t = -4.334, df = 1.144, p < 0.001), corresponding to the timing of daily husbandry routines. These coefficients are on the square root scale, and caution is advised with their interpretation. However, the sign and strength of the relationship is meaningful and relevant here.

Activity declined significantly over time across both treatments (-0.056 ± 0.004, t = -13.954, df = 4787, p < 0.001), but no effect of treatment was observed (-0.005 ± 0.003, t = -1.743, df = 4787, p = 0.081). Following exposure to the novel object test (Fig. 1 A), fish in the EE group were faster to resume normal swimming compared to NE fish (4.304 ± 1.124, t = 3.830, df = 18.182, p < 0.005), and this relationship was also observed over time (3.069 ± 0.392, t = 7.830, df = 9.232, p < 0.001) showing higher resilience to novelty.

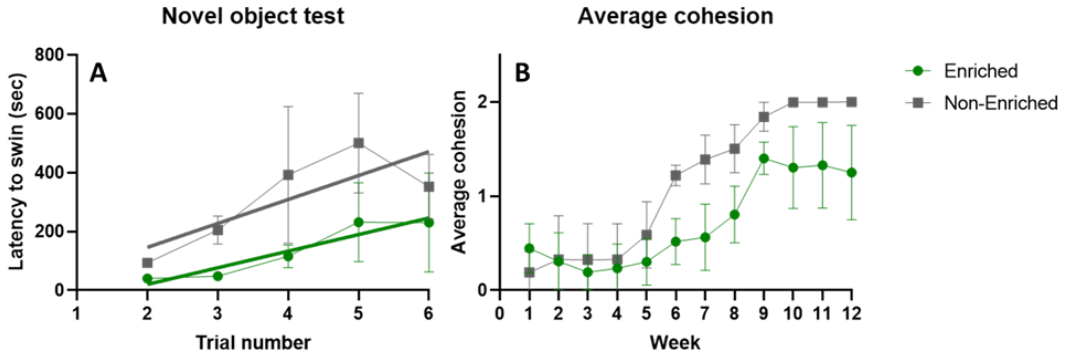


Fig. 1: (A) Latency to resume normal swimming following presentation of novel object (camera). Data are expressed as means (± SD), fitted with regression lines, showing EE tanks (green points) and NE tanks (grey points). (B) Average cohesion profiles (0=tight cohesion, 1=loose, 2=dispersed) measured across 12 weeks for EE and NE tanks

A significant interaction between treatment and time on group cohesion was observed (treatment:week = 4.190, 95% CIs: 0.873 to 7.957, pMCMC <0.001, Fig. 1 B), indicating the relationship between time and cohesion differs between treatment groups, with EE fish displaying more cohesion over time (and NE fish more 'dispersed'). Although there was a non-significant effect of enrichment on group cohesion (-12.061, 95% CI: -39.187 to 4.727, pMCMC = 0.170), this estimate relates to group cohesion at week 1 only (the intercept), and interpretation of the interaction term is more relevant here. Of note, there was a significant positive effect of time on group cohesion (week = 3.767, 95% CI: 0.917 to 6.939, pMCMC < 0.001). This relationship can be visualised in Fig 1B for clarity, fish display 'tight' cohesion at the beginning of the experiment, becoming more dispersed over time in both treatment groups.

iii) Physiological welfare indicators

No difference was observed in baseline cortisol expression between NE (636.578 ± 346.762 pg.g) and EE treatments (854.262 ± 1623.882 pg.g; t = 0.828, df = 42.73, p-value = 0.412). A significant effect of acute stress was observed with plasma cortisol increasing across both treatments following acute stress (2.623 ± 0.571, t = 4.597, df = 168, p < 0.001). No effect of the EE treatment was observed (-0.453 ± 0.469, t = -0.965, df = 8, p = 0.363).

Figure 2, shows the brain concentration of 5HT, 5HIAA, the ratio (5HIAA/5HT) and mRNA abundance of serotonin synthesis rate-limiting enzymes *tph1* and *tph2*. The highest levels of 5HT and 5HIAA were found in EE fish ($F_{1,93}$ = 4.981; p < 0.05 and $F_{1,93}$ = 5.274; p < 0.05, respectively). In addition, our results indicate that the acute stressor induced significant increases in 5HIAA levels ($F_{1,93}$ = 18.14; p < 0.0001), and in the 5-HIAA/5-HT ratio ($F_{1,93}$ = 77.34; p < 0.0001), showing a significant interaction between the enrichment condition ($F_{1,93}$ = 4.493; p < 0.05). However, the stressor only significantly impacted NE fish in 5HT (q=3.779, df=93 p=0.0434), which shows lower amine levels. Results of gene expression related to serotonin synthesis show no significant change in *tph1a* transcript levels (Fig 2A). However, expression of *tph2* (Fig 2B) was significantly modulated by the stressor ($F_{1,68}$ =4.037; p < 0.05) independent of treatment groups.

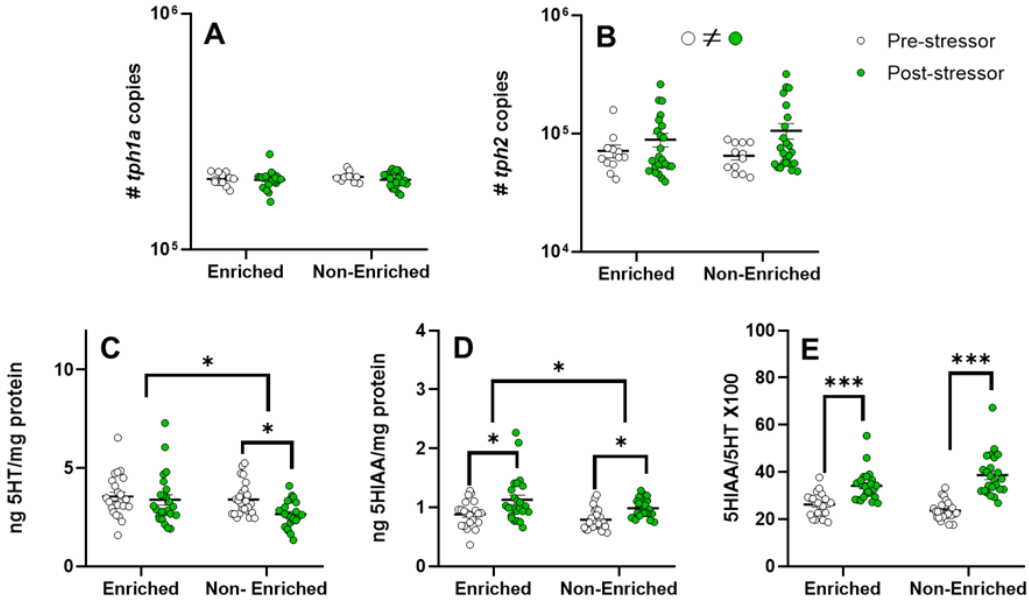


*Fig. 2: (A). Expression levels of tph1a (tryptophan hydroxylase 1a), (B) tph2 (tryptophan hydroxylase 2), (C) tissue content of 5HT (5Hydroxytriptamine, serotonin), (D) 5HIAA (5hydroxyindolacetic acid) and (E) ratio between 5HIAA and 5HT in the brain of S. Salar reared during 12 weeks with or without structural environmental enrichment (enriched vs non-enriched) before stress, white dots, and after exposure to a stress event (5min net chasing), green dots. Data are expressed as mean (± SEM). Significant differences among groups are indicated with * or ≠ (p<0.05), ** (p<0.01) and *** (p<0.001)*

In terms of the expression of genes related to neurogenesis and stress response (Fig. 3), a significant increase was found in *bndf* and *ndf1* transcript levels in EE fish (*bndf*: $F_{1,68} = 5.379$, $p < 0.05$, *ndf1*: $F_{1,68} = 4.250$, $p < 0.05$), meanwhile there was no significant effect of acute stress on these genes ($F_{1,68} = 0.6689$, $p = 0.416$ and $F_{1,68} = 1.211$ $p = 0.275$). Conversely, acute stress promotes a significant increase in expression of *hsp90* ($F_{1,68} = 4.556$, $p < 0.05$) and *cfos* ($F_{1,68} = 4.300$, $p < 0.05$), without detected enrichment effect ($F_{1,68} = 0.512$, $p = 0.477$, and $F_{1,68} = 0.024$, $p = 0.877$ respectively). No significant difference was found between treatment groups in terms of transcript levels of genes related to stress axis (*crfb*, *gr1*, and *gr2*, data not shown).

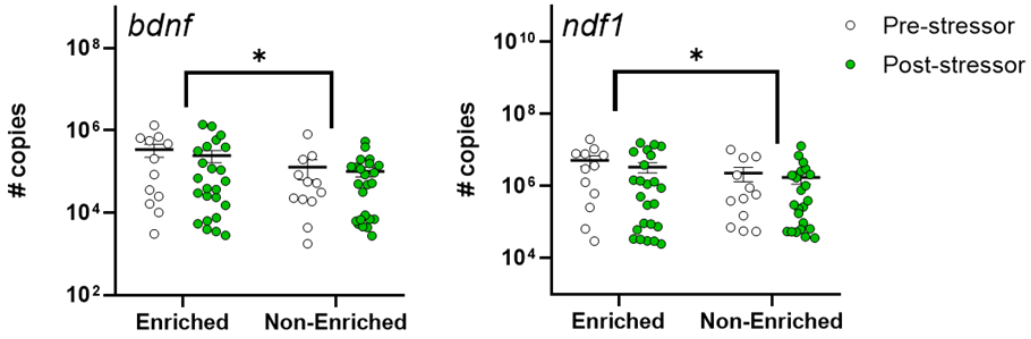


*Fig. 3: Changes in mRNA abundance of bdnf (brain-derived neurotrophic factor), ndf1 (Neurogenic Differentiation Factor 1) in brain of S. Salar reared during 12 weeks with or without structural environmental enrichment (enriched vs non-enriched) before stress, white dots, and after exposure to a stress event (5min net chasing), green dots. Data are expressed as means (± SEM). Significant differences among groups are indicated with * (p<0.05)*

Correlation analysis indicates positive relationship between the levels of the *ndf1* and *bdnf* ($p < 0.001$, Pearson index = 0.708), and between *ndf1* vs *hsp90* ($p < 0.05$, Pearson index = 0.257), and *bdnf* vs *hsp90* ($p < 0.05$, Pearson index = 0.308). In particular, in these last two pairs, there was a high correlation between fish reared in the enriched treatment ($p < 0.001$, Pearson Index = 0.609 and 0.728 respectively), which was not present in the non-enriched treatment group ($p = 0.270$, Pearson Index = 0.189 and $p = 0.120$, Pearson Index = 0.264, respectively).

Discussion

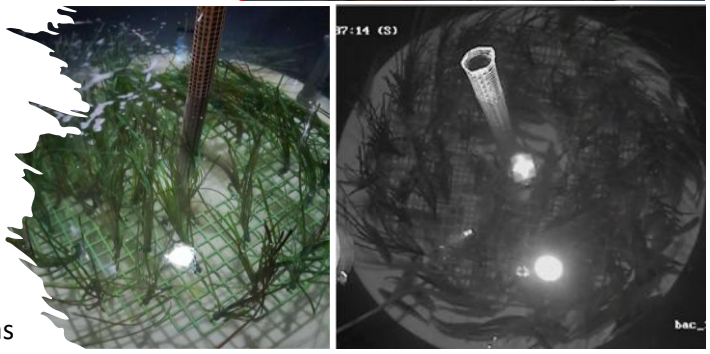
In this study we to examined whether rearing early life *S. salar* in enriched housing conditions, compared to barren environments, can improve the welfare of this species when housed in captivity for research purposes. The results obtained here demonstrate some beneficial effects of enriched rearing environments on welfare in this species. Firstly, and of considerable importance, the provision of EE did not negatively affect growth or result in a higher degree of fin damage, compared to NE fish, but neither did it improve growth or reduce fin damage amongst the fish. EE did significantly affect fish behaviour, specifically social behaviour, strengthening group cohesion over time. Furthermore, EE accelerates recovery from exposure to novel objects, producing less neophobic (fearful) behavioural profiles. Concurrently, EE promotes neurogenesis at the physiological level, as evidenced by the increased expression of genes related to neurogenesis. This potential augmentation in associated cognitive function correlates with an enhanced ability to respond to stress, reflected in higher serotonergic activity. Taken together, these findings suggests that EE improves the well-being of early life stage salmon parr held in captivity, enhancing stress-coping behavioural profiles, neurogenesis and possibly cognitive abilities in line with an improved mental state.

4 Effects of structural environmental enrichment upon the welfare of juvenile seabass (*Dicentrarchus labrax*) in aquaculture research (IFREMER)

4.1 Animals and experimental set up

The fish used in this project were 1-year old juvenile seabass. Born in Ifremer Palavas-les-Flots in February 2022, they were from 3 distinct genetic origins: the North Atlantic (ATL), the Western Mediterranean (WM) and the Eastern Mediterranean (EM). The individuals were first reared in circular tanks (500 L) separated according to population origin. Each tank contained 400 fish, and two tanks per population were set up. All fish were tagged with PIT Tags (1.2 mm x 7 mm, Biolog-ID, Tiny tag) at 7 months of age, on 22/09/2022, when their weight was between 5 and 10g, to allow individual monitoring of their growth and behaviour.

Six tanks were used in this experiment (see picture, 1.5 m^3) and separated from the rest of the area by a tarpaulin in order to limit disturbances. After an initial biometry in February 2023, the fish were installed into the 6 tanks, 120 fish per tank, with 1/3 of the fish per population, for a total of 720 fish. The water temperature was kept at 21 °C and circulated in a semi-open circuit, with a supply of new water of 500L/h (~33% per day).



The fish were allowed 2 weeks of acclimatization in their tank before starting the experiment.

To harmonize protocols as much as possible, the exact same EE material was used as for salmon fry. Structural EE was added to three randomly assigned RAS tanks, resulting in three EE tanks (3 x EE), and three NE barren tanks (3 x NE). The enrichment consisted of a smooth circular PVC grid covering 50% of the surface area of each tank covered with artificial plastic plants (PANGEA, Danemark). The enrichment structure was suspended from the rim of the tanks (20 cm off the bottom), providing cover throughout the water column created from vertical suspension of plant leaves. The enrichment was added on 28/03/2023, marking the start of the experiment.

Ethics

This experiment was conducted at Ifremer facilities in Palavas-les-Flots, which is authorized for animal experimentation by the French Ministry (facility ID E-34-192-6), and in accordance with regulations for the use of animals in experimentation (permit APAFIS #41448-2023030812485854 v4).

4.2 Welfare indicators

Two weeks before the start of the experiment, a risk-taking test was carried out in order to assign a boldness rank to each individual. This test, developed and validated by Millot et al. (2009) and Ferrari et al. (2016), consists of separating the tank into two equal parts by a partition opened by a circular passage equipped with a PIT tag reader. The fish were all grouped together in the same half tank, left in the dark, while the PIT tag reader recorded each fish passage to the other area of the tank, lit according to the experimental photoperiod. The test was carried out in a single 1.5 m^3 tank (same as experimental tanks), equipped to accommodate the partition that would separate it into two, thus involving transfers of fish under light sedations to undergo the test. The test lasted 24 hours for each tank and all tests were carried out between 19/03/2023 and 24/03/2023 for the 6 tanks. As a result, each individual was assigned a boldness score (Timid = zero passage, Bold = more than two passages).

i. Morphological welfare indicators

To assess whether environmental enrichment (EE) affects morphological welfare indicators, we compared individual morphological scores at the beginning (28/02/2023), with scores obtained every on two other occasions (19/04, 15/05) until the end of the experimental procedure (26/06/2023, 12 weeks). During these scoring sessions, fish were weighed, and length measured, and the following welfare indicators (WI) scored: dorsal fin damage and body condition. The indicators were measured using the existing scoring scheme for seabass (Person & Le Bayon, 2009) modified to be scored on a scale of 0-2 (Level 0: Normal condition, Level 1: Minor occurrences, Level 2: Compromised condition).

ii. Behavioural welfare indicators

A camera (HDTVI 2.9" Sony 2.12Mp Object M 42L) was installed above each tank and connected to a recorder (ABUS HDCC90012) to film all the tank simultaneously twice a week (Tuesdays, Thursdays), for 24 hours. Unfortunately, night-time videos were difficult to read despite the use of an infrared camera, so only the hours of light (10am-8pm) were retained for analysis. In a similar manner to the experiment on the salmon fry, to further assess the effect of EE on behaviour, we exposed each tank to a novel object test, once every 2 weeks (6 trials per tank in total). Every 15 days, the experimenter introduced a pole used to support a GO PRO camera (02.51), enabling a one-hour video to be obtained for this analysis, while the cameras above the tanks recorded continuously. The time it took for the fish to adapt to the introduction of the novel object was measured across two parameters: distance from the novel object and area occupied by the fish. From this, the trait latency to resume normal activity was extracted and quantified.

iii. Physiological welfare indicators

At regular intervals during 12 weeks of rearing, 7 500 mL water samples were taken from each tank, for a total of 42 samples, to determine the levels of cortisol present in the water (Sadoul & Geffroy, 2019). These samples were filtered at 0.45 µm (Millipore filter) to remove suspended particles and excreted mucus. The samples were then filtered on a Sep-Pak C18 silica cartridge (WATERS, Netherlands), previously activated with 10 mL methanol, to concentrate and retain cortisol. Cortisol was then desorbed from the cartridge using 6mL of a mixture of cyclohexane and ethylacetate (50-50) for elution, then the remaining water was frozen on dry ice to remove all of it. An evaporation step under nitrogen flow was performed to leave only the cortisol in the samples. Finally, after recovering the cortisol from the walls with ethanol and resuspending it in 400 µL of 1X PBS, the cortisol was ready for analysis using the SALIVA ELISA kit in 96-well plates (TECAN, France) and following its protocol.

Cortisol quantification was performed with a spectrofluorometer at 450 nm within 15 min of stopping incubation.

During the last sampling on 26/06/23, a sampling protocol for blood and plasma was set up. The aim was to compare plasma cortisol levels before and after a stressful event. To this end, 10 fish taken at random from each tank were caught (without anaesthetic) and immediately euthanized with an overdose of anaesthetic (1000 ppm, Benzocaine, SIGMA) in order to take blood samples (2 mL) using heparinized syringes to prevent clotting. Each tank was then confined by reducing the water level by half, and a 2-minute dip net chase was carried out to stress the individuals. Finally, a second set of 10 individuals was sampled 60 minutes after this stress event in each basin, and blood sampling conducted. A total of 120 fish were sampled.

Cortisol was analysed on all plasma samples using the SALIVA CORTISOL ELISA kits from TECAN and for half of the blood samples (5 fish per tank, before and after stress), we analysed additional blood characteristics using Abbott's I-Stat CG4+ and CG8+ cartridges. Various physiological parameters were measured for comparison between treatment (pH, PCO_2, PO_2, HCO_3, TCO_2, Na, K, iCa, Lactate, Glucose, Haematocrit, Haemoglobin).

4.3 Statistical analysis

Data analysis of morphological, behavioural and physiological welfare indicators was carried out using R software (version 4.3.0) (R Development Core Team, 2019). In statistical tests, levels of significance were set at $p<0.05$ for all statistical tests. For morphological data (namely specific growth rate, condition factor and fin damages), 3-way ANOVA was used with enrichment, origin and personality as fixed factors. For behavioural data, the time (minutes) of fish adaptation after the introduction of novel object for minimal distance between the fish and the novel object was compared using an ANOVA and enrichment as fixed factor. For physiological data, water cortisol was also compared using an ANOVA and enrichment as fixed factor. Finally, for plasma cortisol and other blood measures, 3-way ANOVA and/or GLMM was used with enrichment, origin and personality as fixed factors.

4.4 Results and Discussion

Results

i. Morphological welfare indicators

Influence of structural enrichment on growth

Figure 4 shows the SGR of European seabass over the 12 weeks of the trial. ANOVA analysis revealed a significant effect of fish origin on SGR, with ATL fish showing the highest growth rates. Significant differences were observed between ATL and WM/EM fish throughout the trial, with ATL fish showing always the highest SGR levels. Differences between ATL and WM fish evident at the beginning of the experiment, decreased over time, disappearing earlier in fish reared with EE.

Initially, fish reared with EE showed lower SGR levels than non-enriched fish. However, between weeks 4 and 8, this trend was significantly reversed and, by the end of the trial, there were no significant differences in SGR. As expected, all fish increased their SGR over time. In addition, the analysis indicated a significant effect on personality, with shy fish showing significantly higher SGR values than bold fish in WM and EM fish.

The condition factor remained stable throughout the experiment (Fig. 5), with no significant differences between fish reared with enrichment and those without, except at the 4-week sampling point, where EE fish showed significantly lower levels. Analyses of variance showed no significant

differences among the different personalities of the individuals. However, there was a significant effect of the origin of the animals, with the highest values shown by fish from the ATL population.

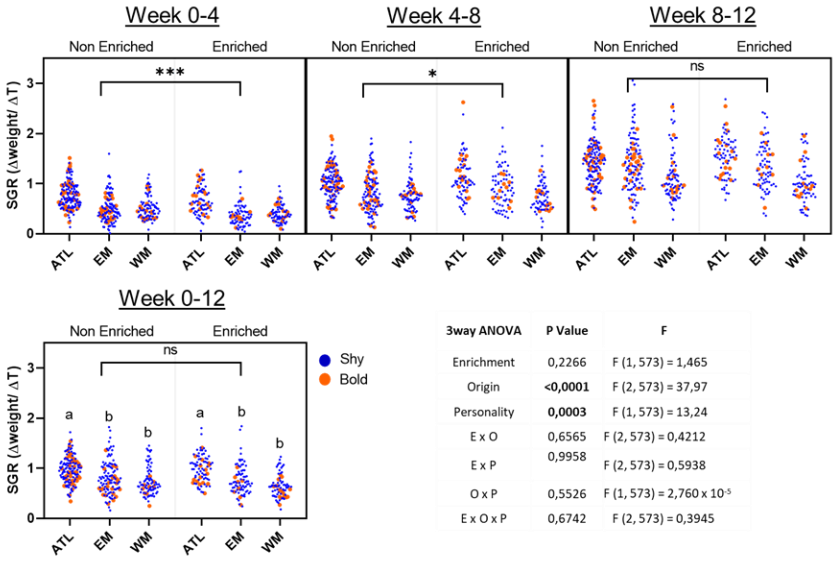


*Fig. 4: Specific growth rate of Seabass reared with or without structural environmental enrichment for 12 weeks. Data are showed split by 4-week intervals and total period (3-way ANOVA table). The scatter plot shows shy (blue spots) and bold (orange spots) individuals. Different superscript letter indicates significant differences (p < 0.05) among Origin, North Atlantic (ATL), East Mediterranean (EM) and West Mediterranean (WM). Brackets indicate significant differences between enrichment conditions where * indicates p-value<0.05, ** p-value<0.01 and *** p-value < 0.001.*

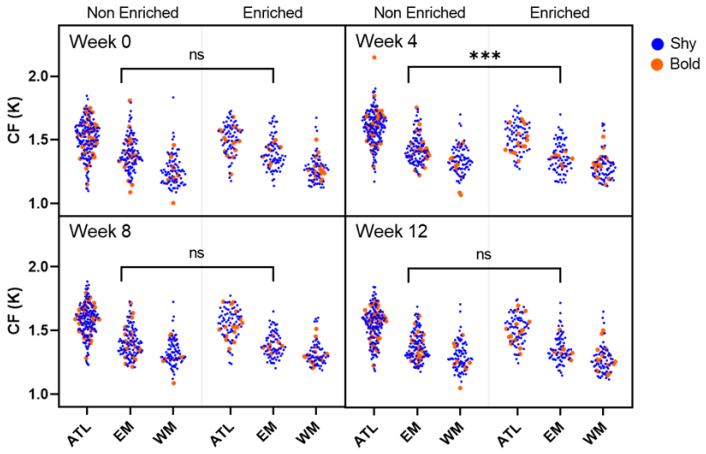


*Fig. 5: Condition factor of Seabass reared with or without structural environmental enrichment for 12 weeks. The scatter plot shows shy (blue spots) and bold (orange spots) individuals. Data are shown split by 4-week intervals, corresponding with sampling points. Brackets indicate significant differences between enrichment condition were * indicates p-value<0.05, ** p-value<0.01 and *** p-value < 0.001*

Influence of structural enrichment on fin damage

Figure 6 shown the results regarding fin damage scores. No significant differences were observed between fish reared in environments with structural enrichment and those in barren environments, nor were there significant differences related to different personalities. However, significant differences were found based on the geographical origins of the animals (p<0.001), with lower values found in Atlantic-origin fish compared to those from the eastern and western Mediterranean.

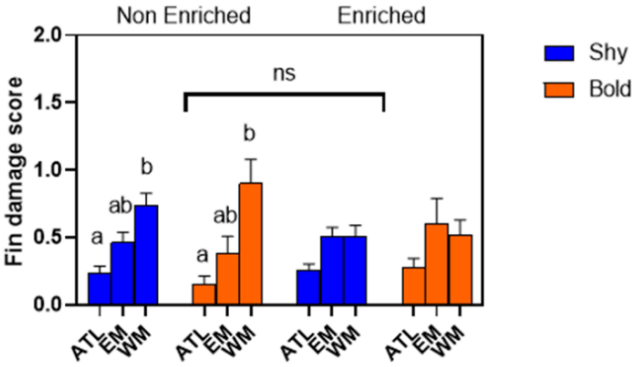


Fig. 6: Fin damage score of Seabass reared with or without structural environmental enrichment for 12 weeks. Bar plots show shy (blue) and bold (orange) individuals. Data are shown as mean ± s.e.m. Different superscript letter indicates significant differences ($p < 0.05$) among Origin, North Atlantic (ATL), East Mediterranean (EM) and West Mediterranean (WM)

ii. Behavioural welfare indicators

The minimal distance between the fish group and the novel object is presented in Table 2. Regarding fish speed, the only statistically significant change occurred during the installation of the novel object, when the fish exhibited a marked increase in speed due to stress from the disturbance. However, no significant differences in speed were observed before and after the introduction of the novel object, suggesting that speed was not a reliable indicator of adaptation to the novel object. In contrast, both the minimal distance from the object and the area occupied by the fish changed following its introduction. The fish initially avoided the area near the novel object for a period of time after its placement.

Table 2: Time (minutes) of fish adaptation after the introduction of novel object for minimal distance between the fish and the novel object.

	tank number	**6.4.2023**	**20.4.2023**	**4.5.2023**	**1.6.2023**	**15.6.2023**
non-enriched	11	6.5	21.1	5.9	4.9	3.5
	14	*no video*	*no video*	*no video*	14.0	3.1
	15	3.1	4.3	2.0	3.9	2.0
enriched	12	22.4	*no video*	*no video*	11.2	2.0
	13	6.5	70.4	6.0	10.0	7.4
	16	2.0	9.2	36.1	5.4	6.0

Statistical analysis comparing adaptation times between non-enriched and enriched tanks revealed no significant differences. However, the variance in adaptation times was significantly greater in the enriched tanks, indicating a wider range of responses to the novel object in these environments.

iii. Physiological welfare indicators

Influence of structural enrichment on water and plasma cortisol levels

No effect of enrichment on water cortisol levels was observed throughout the trial (Fig 7). On the other hand, significant stress-related effects were found for cortisol levels in water, with higher values measured after exposure to stress compared to previously detected levels.

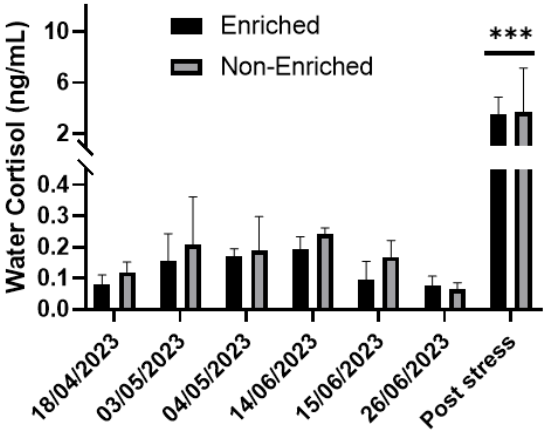
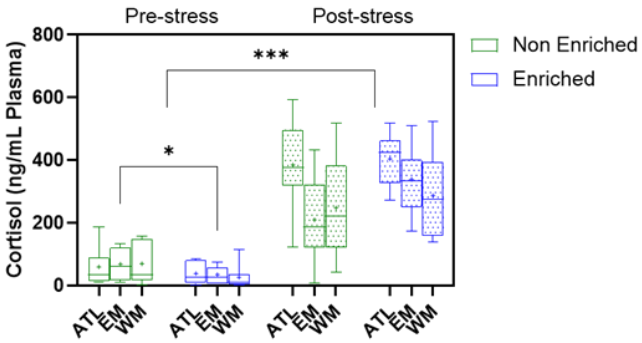


*Fig. 7: Water cortisol levels in experimental tanks housing seabass with or without environmental enrichment (enriched vs non-enriched). Values are mean ± SEM (n=3). *** indicates significant differences between post-stress levels and previous records (p<0.001).*



3way ANOVA	P Value	F
Enrichment	0,4530	F (1, 89) = 0,5682
Origin	**0,0180**	F (2, 89) = 4,202
Stress	**<0,0001**	F (1, 89) = 170,9
E x O	**0,0200**	F (2, 89) = 0,6600
E x S	0,5194	F (1, 89) = 5,611
S x O	**0,0169**	F (2, 89) = 4,274
E x O x S	0,4624	F (2, 89) = 0,7780

*Fig. 8: Plasma cortisol levels of seabass reared during 13 weeks with or without environmental enrichment (enriched vs non-enriched) pre-stress data plotted at left and post-stress exposure (5 min net chasing), at right. Data are expressed in box plots extending from the 25th to 75th percentile, the line in the middle of the box indicates the median, max and min values are shown with whiskers, and "+" indicates the geometrical mean. Significant differences among groups are indicated with * (p<0.05) ** (p<0.01) *** (p<0.001*). Table legend on the right: E: enrichment, O: origin, S: stress.

Cortisol levels were determined in the plasma of sea bass (Fig. 8). After 13 weeks from the start of the experiment, fish raised with environmental enrichment show significantly lower baseline cortisol levels. As expected, after exposure to the stress event, cortisol levels increased significantly, with no significant differences between the levels determined in fish raised under environmental enrichment and those raised in barren environments. As the results of the 3-way ANOVA analysis show, there is a significant effect regarding the origin of the fish evidencing that ATL fish show higher plasma cortisol levels. This analysis of variance also highlights the interaction between the variables stress and origin, showing that ATL fish have higher increases when subjected to stress compared to EM and WM fish. It is also noted that the variability found in fish reared with environmental enrichment is lower in terms of cortisol levels, enriched fish displayed a coefficient of variability of 107.98% meanwhile in non-enriched environment the coefficient raised to 137.97%. There is a significant interaction between enrichment and fish origin underlining a differential sensitivity of the populations, results that will need further investigation.

Influence of structural enrichment and stress on haematological parameters

Table 3 shows the means and standard deviations of each parameter analysed from the blood samples, sorted by enrichment and stress factors. The data indicate that the stressful event significantly altered many parameters, increasing values for PCO_2, BE, HCO_3, TCO_2, and Lactate, while decreasing values for pH, sO_2, and K (ANOVA, $p < 0.05$). Furthermore, blood parameters related to the pH buffering process, such as HCO_3 and CO_2, were affected by the enrichment variable, with enriched fish displaying significantly lower levels compared to non-enriched fish. For all other physiological parameters measured, the GLMM and subsequent ANOVA revealed no significant differences for each factor studied.

*Table 3: Haematological parameters evaluated during the pre- and post-stress periods in both treatments. Data is shown as mean ± standard deviation. Significant differences are indicated between each factor (p-value * < 0.05, ** < 0.01, ***<0.001).*

	Non- Enriched			vs	Enriched		
	Pre stress	vs	Post stress		Pre stress	vs	Post stress
pH	7.31±0.08	***	7.20±0.13		7.29±0.12	***	7.17±0.05
pCO2 (mm Hg)	21.56±4.46	***	36.44±8.72		20.42±5.85	***	31.51±4.01
PO2 (mm Hg)	9.18 ±3.11		6.25±0.96		10.39±5.64		7.17±1.70
sO2 (%)	24.73 ±18.14	***	9.87±5.30		27.80±20.95	***	14.40±9.70
HCO3 (mmol·L^{-1})	13.35±1.04	***	17.71±1.77	*	11.68±1.25	***	15.00±1.5
TCO2 (mmol·L^{-1})	14.57±1.24	***	19.87±1.72	**	12.83±1.23	***	16.87±1.72
BE (mmol·L^{-1})	-15.60 ±1.57	***	-13.27±3.36		-17.50±2.13	***	-16.23±2.02
Lactate (mmol·L^{-1})	3.29 ±0.85	***	4.37±1.80		3.57±0.94	***	4.57±0.95
Glucose mg·dL^{-1}	60.67±16.96		65.87±30.48		57.50±21.18		61.8 ±24.31
Hct (% PCV)	21.20±6.18		26.33±3.77		24.87±11.56		25.80±6.71
Hb (mmol·L^{-1})	7.82±1.21		8.95±1.29		8.43±3.82		8.78±2.28
Na (mmol·L^{-1})	159.47±4.81		159.27±7.76		155.87±8.03		157.13±5.42
K (mmol·L^{-1})	4.47±0.64	***	3.40±0.80		4.50±0.82	***	3.79±0.80
iCa (mmol·L^{-1})	1.44±0.08		1.44±0.15		1.39±0.14		1.40±0.10
Ca^{+}	2.54±0.24		2.67±0.31		2.60±0.28	*	2.79±0.27
Cl^{-}	148.80±16.0		147.51±19.24	**	163.07±17.88		164.92±20.11
PO	383.69±17.3		379.14±15.08	*	387.53±20.74		385.49±20.28

Discussion

The results of this study indicate that rearing juvenile sea bass in environments enriched with plastic plant has a time-dependent effect on their welfare. Initially, this enrichment seems to compromise some aspects of the welfare of the fish, but it may offer potential long-term benefits. In the short term, the addition of structural environmental enrichment (EE) reduces the growth rate of the fish and negatively impacts their condition factor possibly because food access was more restricted because of the presence of the structure. However, in the medium term, these effects are reversed, with a significant improvement in the growth rate, as evidenced by notably higher SGR. By the long term, growth levels in the enriched groups are comparable to those of the control fish, and no differences in condition factor or total growth were observed. Regarding behavioural responses to

novelty, a wider range of responses was observed for fish in the enriched environments, evoking a wider repertoire of behavioural performances. The physiological welfare indicators suggest lower basal stress level in enriched environment as well as higher circulatory system buffering potential. Overall, the data suggest that the addition of EE in the form of plastic plant does not have a detrimental effect on juvenile sea bass welfare. In fact, the presence of this enrichment may even lead to positive responses in the long term, though these effects appear to be mild. Despite these potential benefits, the study highlights that such enrichment does not necessarily guarantee an overall improvement in the animals' quality of life. Therefore, further research is needed to explore alternative strategies for enhancing welfare in juvenile sea bass.

5 Effects of environmental enrichment in welfare of juvenile rainbow trout (*Oncorhynchus mykiss*) in aquaculture research (IMR)

5.1 Animal and experimental set up

All-female rainbow trout eyed eggs from the Aquagen strain arrived at the Institute of Marine Research, Matre research station, on 15 December 2021. The eggs were incubated at 8°C and hatched 8–10 days after arrival. First feeding was done in 3 m tanks at 13°C and continuous light started on 01 February 2022. On 23–25 March 2022, when the fish were 7.5 ± 1.4 g, they were distributed between six 3 m tanks (5.6 m^3) with 2000 fish per tank. The tanks were supplied with 13°C freshwater and at continuous light. In 3 of the tanks the bottom had in advance been covered with black or white foil stickers, creating a pattern with 4 black and 4 white areas of equal size (enriched, Fig. 1). In the remaining 3 tanks the standard light grey colour was kept on the bottoms (control). The fish were kept at continuous light, and the fish were fed from noon to midnight. A video camera connected to a computer was attached above each tank. Video recordings of the tanks from above were made 15 min daily, starting 7 min before start of feeding to include the feeding response. One month (31 days) after the fish had been introduced to the tanks the first stress test and sampling was carried out. A hundred individuals in each tank were sampled for length, weight, and welfare scoring. Sampled individuals were euthanized in an anaesthesia bath. Blood for plasma cortisol analysis were taken from the first 15 of these (baseline), and 15 individuals sampled 1 h after initiation of netting out fish from the tank (1 h post stress). When the fish had been in the tanks for 8 weeks, light was switched off during night and switched on again every morning until the end of the experiment, in order to analyse habituation of the startle response when the light was switched on. Switching on the light was used as a more practical and farm relevant mild stressor than applying a daily brief (~2 s) shadow, representing a bird predator, as was originally planned. Calculation of oxygen hyper consumption as a measure of habituation rate (Folkedal et al. 2010) was planned but could not be done due to technical limitations, i.e., oxygenated water was fed to the tanks when oxygen saturation dropped below 90%, and open respirometry could not be applied. A stress test and sampling were again carried out on Day 73, i.e., 7 weeks after the first sampling, for length, weight, welfare scoring, and baseline and 1 h post stress blood sampling as described above. The experiment was then terminated.

Bonus material: Some of the fish sampled for plasma in the present experiment were also examined for vertebral deformities using radiology, although not with the intension to evaluate effects of enrichment. In order to follow this up, the fish were vaccinated and later transferred to a sea cage after the end of the enrichment experiment and included in a project on fish welfare and development of vertebral deformities (Fjelldal et al. 2025).

Video analysis

The distance from the tanks to the ceiling was too low to allow the camera angle to cover the entire tank area. Furthermore, the tanks were partly covered by a feeding pipe, and the video resolution was relatively low, making it difficult to distinguish individuals from each other at high stocking density and/or dark background (Fig. 9). Reliable and standardized analysis of fish distribution from day to day was therefore not possible, neither automated nor manual analysis. As an alternative an estimation of how large proportion (10% intervals) of the fish that occupied the inner versus outer tank area and for the enriched tanks, the black versus the white fields, was made on 10 different days throughout the experiment, on a single frame from 12:00 (as in Fig. 1) on that day.

Ethics
This experiment was conducted at the Institute of Marine Research's facilities in Matre, which is authorized for animal experimentation by the Norwegian Food Safety Authority (facility ID 110), and in accordance with regulations for the use of animals in experimentation (permit ID: 29406).

5.2 Welfare indicators

Morphological welfare indicators
Morphological welfare indicators were scored using the Laksvel protocol (Nilsson et al. 2022), which is an operational adaption of the Fishwell scoring scheme (Noble et al. 2018), with levels 0 (Level 0: Normal condition, Level 1: Minor occurrences, Level 2: Compromised condition, Level 3, clear evidence of the OWI). The indicators scored were vertebral deformities, emaciation, sexual maturation, scale loss, skin haemorrhaging, wounds, snout damage, jaw deformities, eye opacity, eye injuries, opercular damage, gill damage, and fin damage. At least 300 (range 301-320) individuals per treatment were scored on each sampling occasion.

Physiological welfare indicators
Blood was withdrawn from the caudal vein with heparinized syringes as soon as the fish had become motionless in the anaesthetic bath, and centrifuged at 13000 rpm for 3 min and stored at -80°C. At cortisol analysis the plasma samples were thawed on ice and the concentration of plasma cortisol were measured by an enzyme-linked immunosorbent assay (ELISA) and a Sunrise microplate reader (Tecan). For each sample 20 µl subsamples were used for the cortisol assay (standard range: 10 to 800 ng/ml, RE52061 IBL-International, Hamburg, Germany).

5.3 Statistical analysis

Effects of enrichment on growth (mean weight) and condition factor were tested with Linear mixed-effects model (lme), with treatment (2D enrichment and plain control) and days (Days 31 and 73), and the interaction of these, as factorial predictor variables, and post hoc testing was done with Tukey. Plasma cortisol levels on Day 31 and on Day 73 were also tested with lme, with treatment and sampling time (baseline or 1 h post stress), and the interaction of these, as factorial predictor variables, and post hoc testing was done with Tukey. Effects on welfare indicator scoring were tested with Wilcoxon rank sum test with continuity correction (wilcox.test) with treatment as predictor variable on both Day 31 and on Day 73, for indicators with at least 2% of the fish affected (score ≥1).

5.4 Results and discussion

Behaviour
Based on the estimates of fish distribution there did not seem to be any difference between enriched and control tanks in distribution in relation to the outer versus inner area of the tank, with 42 and 58% fish, respectively, in both treatments. On average 57.5% of the fish in the enriched groups were estimated to occupy the area corresponding to the black tank base substrate, not suggesting a strong preference for black over white under the current conditions.

Growth

Weight increased between Day 31 and Day 73 ($p<0.001$) but did not differ between fish reared in control or 2D enriched tanks, neither on Day 31 (lme, $p=0.999$) nor Day 73 ($p=0.177$) (Fig. 10A). Similarly, condition factor increased between Day 31 and Day 73 ($p<0.001$) but did not differ between treatments neither on Day 31 (lme, $p=0.981$) nor Day 73 ($p=0.997$) (Fig. 10B).

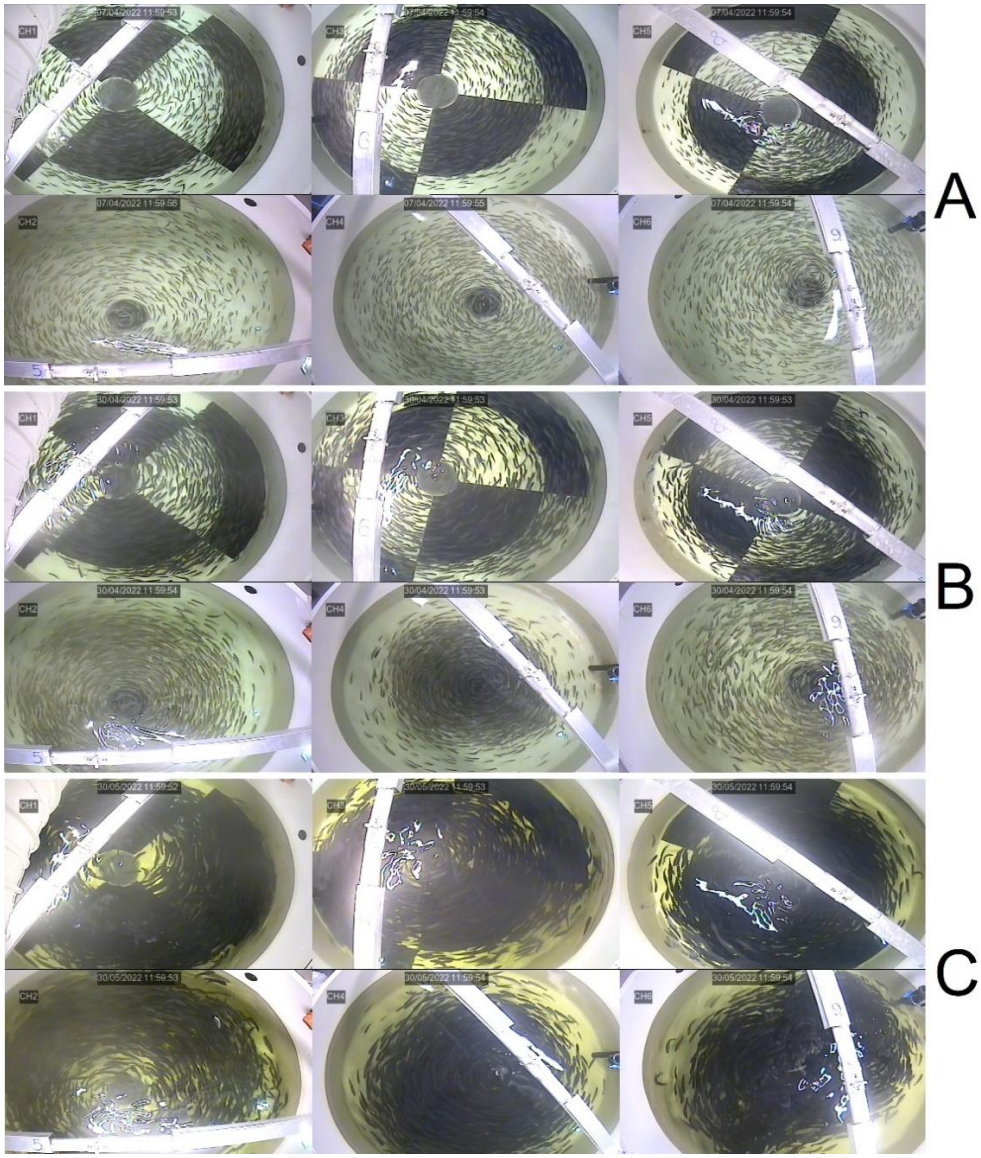


Fig. 9. The experimental tanks. 2D enriched tanks with black and white fields, and plain control tanks. Fish distribution in the six experimental tanks on A) Day 11, B) Day 34, and C) Day 64.

Morphological welfare indicators

The level of morphological defect scores observed macroscopically during scoring was generally low, with only the welfare indicators eye opacity and fin damage affecting more than 2% of the fish (level 2 or higher) on one (eye opacity; Day 73) or both (fin damage) sampling days (Fig. 11). While eye opacity was not detected on any fish on Day 31, the frequency of eye opacity on Day 73 was higher in the 2D enriched group than in the control group (Wilcoxon rank sum test, $p<0.001$, Fig. 3A). The frequency of fin damage did not differ between treatments on Day 31 ($p=0.108$), while it was higher in the 2D enriched group than in the control group on Day 73 ($p=0.04$, Fig. 11B).

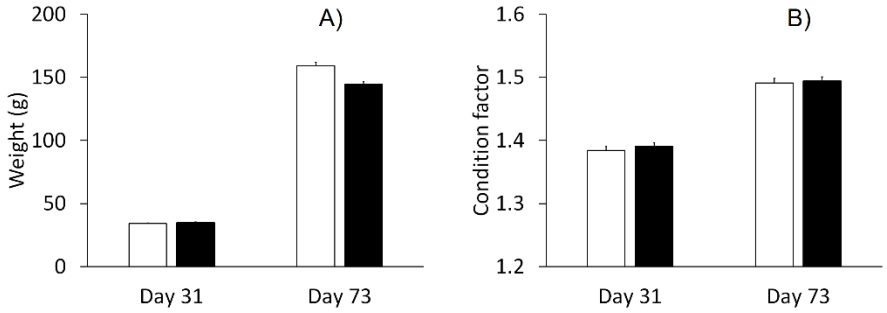


Fig. 10. Mean A) weight and B) condition factor of rainbow trout reared in plain control (white) or 2D enriched (black) tanks for 31 and 73 days.

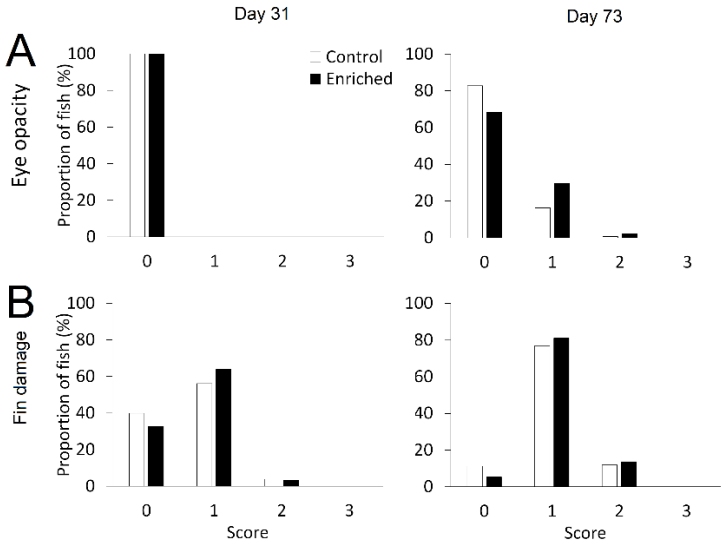


Fig. 11. Frequency of the levels of the morphological welfare indicators A) eye opacity and B) fin damage in rainbow trout reared in plain control (white) or 2D enriched (black) tanks for 31 and 73 days.

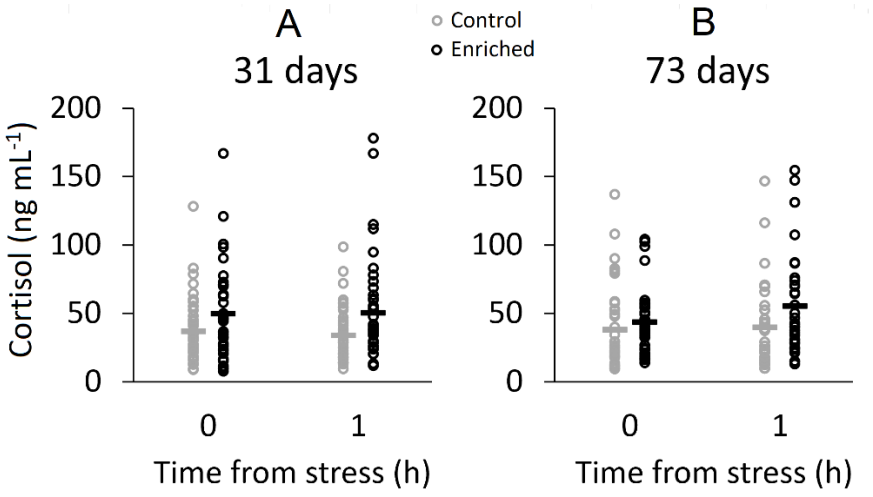


Fig. 12. Baseline (0 h) and 1 h post stress (1 h) plasma cortisol concentration of rainbow trout reared in plain (grey) or 2D enriched (black) tanks for A) 31 days and B) 73 days. Circles indicate individual fish and horizontal lines indicate mean.

Plasma cortisol

Plasma cortisol concentration did not change from baseline to 1 h post stress neither on Day 31 (p=635) nor Day 73 (p=0.827). Levels did not differ significantly between 2D enriched and control groups (Day 31: p=0.0745; Day 73: p=0.210), although mean levels were somewhat higher in the 2D enriched groups on both dates (Fig. 12).

Discussion

Although behaviour could not be automatically analysed due to recording quality, 2D enrichment pattern did not seem to affect fish distribution to a large degree. We could not detect any positive effects of 2D enrichment on growth or condition factor, while 2D enrichment had a negative impact on the welfare indicators eye opacity and fin damage, and also seemed to elevate plasma cortisol although this effect was not significant. With only negative welfare effects documented, 2D enrichment as applied here does not appear to be beneficial for juvenile rainbow trout. Alternative 2D patterns such as other colours or size of the fields could be tested in future studies.

6 Effects of early life training on the European seabass (*Dicentrarchus labrax*) in later life: maximum aerobic capacity (WU)

The objective of this research was to see whether there is an effect of early life training on the European seabass (*Dicentrarchus labrax*) in later life, whether there is a difference between the three types of strains of the European seabass, and whether the effect of early life training is strain dependent. Here the main focus was on improving feed intake and to evaluate the effect of training in early live on the maximum aerobic capacity and thus the robustness of fish in later life. Heat production and the feed intake (indirect measure) are used as indicators for the maximum aerobic capacity. Dissolved oxygen (DO) can be limiting for feed intake as fish will reduce their feed intake in order to prevent exceeding their maximum aerobic capacity (Saravanan et al. 2013). However, no information is available on whether the effect on maximum feed intake (maximum aerobic capacity) will be similar for trained fish (swimming against a current) in early life when compared to fish at normal conditions (not exposed to training). It was hypothesized that trained fish have an increased aerobic capacity, resulting in a higher feed intake when fed a high oxygen demanding diet (indicator for robustness). The potential effect of early life training on the aerobic capacity may be dependent on the genetic line, i.e., in rainbow trout a strong genetic effect on residual feed intake is observed (Grima et al. 2008).

6.1 Animal and experimental set up

For this research, over 960 European seabass were provided from the French Research Institute for Exploitation of the Sea (Ifremer, Palavas-les-Flots, France). To test the effect on strains, each strain had a little over 320 individuals. The available strains were the Atlantic, West Mediterranean, and East Mediterranean strains. To test the effect of training, a subgroup of the fish were exposed to a current ranging from 0.3 to 0.5 m s^{-1} (referred to as the trained fish), and a subgroup of the fish had no deliberate current (referred to as the untrained fish), see Fig. 13. This training phase was from 94 days post hatching (dph) until 164dph (total of 70 days). This resulted in six experimental groups; 3 strains x 2 training history. After the training phase in IFREMER, all individuals were tagged (October 22, 2022) with a PIT tag for later identification of strain and training history which enabled rearing the fish in a common garden. All fish were transported to the Carus facility of Wageningen University and Research (Wageningen, The Netherlands) for this experiment at 220dph (2022-10-13).

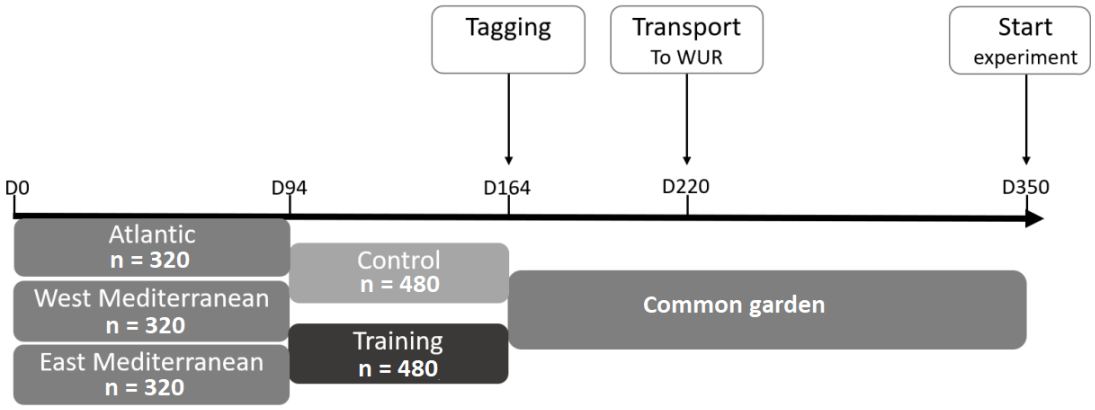


Fig. 13: Timeline of the fish before conducting the experiment. D = days post hatching; n = number of European seabass.

The experiment consisted of two periods. The design and procedures are identical across the two periods. Each period lasted 5 weeks (2 weeks of acclimatization before the first period, and a total of

10 weeks of experimental period). During each period, two dietary treatments are tested, with differing dietary oxygen requirements, to determine which factor (i.e., maximum aerobic capacity) drives feed intake (Table 4). A diet that requires high oxygen: high oxygen demanding (HOD, *i.e.* high protein and low-fat levels) and a diet that requires low oxygen demanding (LOD, i.e. low protein high fat diet) were used. The goal was to maintain the same protein-to-energy ratio by adding comparable amounts of energy in the form of starch in the HOD versus energy in the form of fat in the LOD. The diet was made by extrusion resulting in a 3.5 mm sinking pellet.

Table 4. Ingredient and analysed nutrient composition experimental diets.

	HOD diet	**LOD diet**
Ingredient (g)		
Gelatinized wheat starch	250	-
Rapeseed oil	-	50
Soy oil	-	50
Soya bean meal	110	110
Wheat	127.8	127.8
Fishmeal	300	300
Fish oil	30	30
Soy protein concentrate	50	50
Rapeseed meal	110	110
Premix	10	10
Mono calcium phosphate	10	10
DL-Methionine	2	2
Yttrium oxide	0.2	0.2
Total	**1000**	**850**
Analysed nutrient composition (g/kg DM)		
Dry matter (DM, g/kg)	930.0	957.1
Crude protein	378.7	444.7
Crude fat	72.1	214.3
Carbohydrates	462.2	238.7
Starch + sugars	372.9	120.9
Energy (kJ/g DM)	19.7	23.1
Ash	87.1	102.3
Phosphorus	13.5	15.7

Notes. HOD, high oxygen demanding diet; LOD, low oxygen demanding diet.

The diets were fed to trained and untrained fish, using seabass from three genetic strains (Atlantic, West Mediterranean, and East Mediterranean strain) according to a 2x2x3 design resulting in 12 treatments (see Table 5). For the experiment 24 tanks were used, so each treatment was tested with two replicates per period. The experiment had a crossover design, with each tank (experimental unit) receiving the LOD and HOD diets during one of the two experimental periods, resulting in four replicate treatments across the entire experiment.

Table 5. Experimental design.

Strain	Atlantic (AT)				West Mediterranean (WM)				East Mediterranean (EM)			
Training history	Trained		Not trained		Trained		Not trained		Trained		Not trained	
Diet	LOD	HOD	LOD	HOD	LOD	HOD	LOD	HOD	LOD	HOD	LOD	HOD
Period 1	2	2	2	2	2	2	2	2	2	2	2	2
Diet	HOD	LOD	HOD	LOD	HOD	LOD	HOD	LOD	HOD	LOD	HOD	LOD
Period 2	2	2	2	2	2	2	2	2	2	2	2	2

Notes. LOD; low oxygen demanding diet, HOD; high oxygen demanding diet. In both period 1 and 2: 2 replicate tanks are used (all number 2 in the table refer to this).

Experimental procedures

Before the experiment began, the fish were fasted for 24 hours to completely empty their guts. At the start of the experiment (period 1), 20 fish from each training history and genetic strain (2x3x20=120 total) were euthanised with an overdose of 2-phenoxyethanol to determine initial body composition. Following that, 35 fish were weighted individually and randomly assigned to tanks (based on training history and strain). The faeces from each tank were collected in bottles beneath the swirl separator connected to each tank. Faeces were collected for digestibility and started once the satiation feeding began. Over four weeks per period, faeces were collected four times per tank (Monday, Tuesday, Wednesday, and Thursday). Collection occurred overnight, with the bottles submerged in a Styrofoam box with ice to reduce bacterial degradation. Faeces from each tank were collected in separate aluminium trays and frozen (below -20 °C) for further analysis. Samples from the same tank, week, and period (1 and 2) were pooled. Fish were sedated with 2-phenoxyethanol at the end of period 1 (after 5 weeks from start) and individually weighed. Prior to this, fish were not fed for 24 hours. Then, 10 sedated fish from each tank were killed with an overdose of 2-phenoxyethanol for body composition analysis. The remaining 25 fish were returned to their respective tanks (start period 2). These fish were fed using the same procedures as described for period 1, but with the diet they did not receive during the first period. The fish sampled at the end of period 1 to determine the final body composition for each tank, served as the initial sample for the fish used in period 2. Fish were sedated and individually weighed at the end of period 2 (after 10 weeks from the start); previously, fish had not been fed for 24 hours. Then, 10 sedated fish from each tank were killed with an overdose of 2-phenoxyethanol for final body composition analysis.

During the experiment, the fish were hand fed twice a day (9:00 and 15:00 hours). The first five days of feeding were restricted to allow the fish to adjust to their environment and diet. The feeding level increased during these days to ensure a smooth transition to satiation feeding. Fish were fed until they appeared satiated in order to study the impact of strain, training history, and diet on feed intake and oxygen consumption. Satiation feeding was used for the rest of the experiment, with a maximum of one hour per feeding moment. The weight of the contents of each feed cup per tank was recorded before each feeding, to determine how much feed was fed at each feeding moment. In addition, fifteen minutes after feeding, the pellets spilled in the swirl separator connected to each individual tank were counted to account for uneaten feed. Faecal matter was also used to determine nutrient digestibility.

Housing

The experimental groups were divided into 24 circular tanks (360 L, 98 cm diameter, 35 fish per tank, stocking density < 30 kg/m3). All tanks were connected to a single recirculating aquaculture system (RAS). The RAS included a sump, settling tank, trickling filter, and drum filter. The outflow from each individual tank was connected to a swirl separator tank (AquaOptima AS, column height 44 cm; diameter 24.5 cm), which collected faeces and uneaten pellets. Each tank's flow was kept between

6.9 and 7.1 L/min and checked at least four times per day (before and after both feedings), with adjustments made as needed. The experiment followed a photoperiod of 12L:12D (7:00h lights on, 19:00h lights off), with a light intensity of 200 LUX when the lights were turned on.

Temperature, oxygen, salinity, and pH were measured daily before the afternoon feeding. The temperature was kept between 22.5 and 23.5 °C (WTW Multi 3630 IDS - FDO 925), and the oxygen level was set to be above 6.4 mg/L (WTW Multi 3630 IDS - FDO 925) in order to achieve normoxia conditions (>90% saturation), ensuring that feed intake was not limited by water oxygen levels. All other water quality parameters were measured in the common outflow. The system used artificial seawater with a salinity of 33 to 35 ppt (WTW Multi 3630 IDS - TetraCon 925). The pH ranged between 7.2 and 7.8 (WTW Multi 3630 IDS - SenTix 940), and it was adjusted by adding sodium bicarbonate when it became too low. Ammonium (Merck Aquamerck Colorimetric Ammonium test), nitrite (Merck Aquamerck Colorimetric Nitrite test), and nitrate (Merck MQuant Nitrate test strips) were measured three times per week (Monday, Wednesday, and Friday) prior to afternoon feeding. Throughout the experiment, ammonium remained below 0.5, nitrite below 0.3, and nitrate below 100.

Ethics
Experimental protocols complied with the current laws of The Netherlands and were approved by the Dutch Central Committee for Animal Experiments (CCD), project number AVD10400202216, and by the Animal Experiments Committee (DEC) and Authority for Animal Welfare (IvD) of Wageningen University and Research, experiment number 2022.W-0004.001.

6.2 Calculations and statistical analysis

Growth performance. At the start, end period 1 (after 35 days) and end period 2 (after 70 days), fish were weighed (and the tag was read) to determine the mean growth per tank. Hereby the weight of the fish after period one returned to the tank was used as the start weight for period 2. From these measurements metabolic growth rate (GR_{MBW}, g $kg^{-0.8}$ day^{-1}), feed conversion ratio (FCR) and feed intake (FI_{MBW}, g $kg^{-0.8}$ day-1) and survival (%) was calculated per tank per period, using the formula's presented in table 6.

Nutrient digestibility. During the growth period, faeces were collected, starting from day 10 using the method as described by Amirkolaie et al. (2006). Before analyses, faeces samples were grinded (Retsch ZM 200). The collected faeces were pooled per tank and per available oxygen level (100%; 90% and 80%; 70% and 60%). A weighted sample (according to the number of days at each available oxygen level) was made (after grinding) and analysed. Faeces samples and feed samples (100 g $diet^{-1}$ $week^{-1}$) were analysed for dry matter (DM), ash, crude protein (CP), crude fat (CF), energy and yttrium (Y) content, as described by Maas et al. (2018). Carbohydrate content of the feed and faeces was calculated as 1000 – CP – CF – ash. From these data apparent digestibility coefficients (ADC, %) and digestible nutrient intake (g $kg^{-0.8}$ day^{-1}) were calculated, using the formula's presented in table 6.

Nitrogen and energy mass balance measurements. Fish samples for initial and final body composition were analysed for dry DM, ash, CP, CF and energy, as described by Maas et al. (2018). Frozen fish samples (-20˚) were ground twice using a meat mincer with a 4.5 mm die and homogenised. The energy and nitrogen (N) balance parameters were calculated per tank and expressed as $kJ/kg^{0.8}$/d and mg $N/kg^{0.8}$/d using the formula's presented in Table 6.

Statistics. Data were analysed for the effect of diet, training, strain, sequence of feeding the experimental diets and their interaction effects using a mixed ANOVA model. In this model a random effect of tank nested within training, strain and sequence was included. For the effects of training, strain, sequence and their interactions analyses, tank was used as the experimental unit. These effects

were tested against the random effect of tank. The effect of diet and interactions effects with diets were tested against the final error term of the model (the variation within tanks). In the case of a significant effect of strain and for significant interaction effects ($p<0.05$), a Tukey HSD test (honest significant difference; 95% significance level) was performed to compare treatment means. Statistical analyses were performed using the statistical program SAS 9.4, SAS Institute, North Carolina, USA.

6.3 Results and Discussion

The strain affected both feed intake (g/kg$^{0.8}$/d) and growth (g/kg$^{0.8}$/d) (Table 7), with the Atlantic strain having a higher feed intake and 30% higher growth, whereas the East and West Mediterranean strains had comparable growth and feed intake. This increased feed intake for the Atlantic strain did not result in a higher FCR, which was highly comparable between strains (2.12-2.14) and thus unaffected by strain. The Atlantic strain's higher feed intake resulted in higher energy balance values, with the exception of energy retained as protein, which was comparable across strains. Because protein retention was comparable between the strains, the Atlantic strain's increase in growth and energy retention was attributed to increased fat retention.

Table 6. Calculations.

Fish performance parameters	Symbol	Unit	Equation
Average initial body weight	W_i	g/fish	= B_i / N_i
Average final body weight	W_f	g/fish	= B_f / N_f
Geometric mean body weight	W_g	g	= $e^{((\ln Wf + \ln Wi)/2)}$
Mean metabolic body weight	MBW	kg$^{0.8}$	= (W_g /1000)$^{0.8}$
Absolute growth	GR	g/d	= (W_f - W_i)/t
Growth expressed per metabolic body weight	GR_{MBW}	g/kg$^{0.8}$/d	= (W_f - W_i)/MBW/t
Feed conversion ratio	FCR	g DM/g fish	= FI_{ABS} / (W_f - W_i)
Feed intake as fed	FI as fed	g/fish/d	= FI_{Total}/N/t
Absolute feed intake	FI_{ABS}	g DM/fish	= FI_{Total} * DM% /N/t
Feed intake expressed in metabolic body weight	FI_{MBW}	g DM/kg$^{0.8}$/d	= FI_{ABS}/MBW/t
Apparent digestibility			
Apparent digestibility coefficient	ADCx	%	= (1 (AIA_{Diet}/AIA_{Feaces}*X_{Feaces}/X_{Diet})) *100%
Energy balance parameters *(Saravanan et al., 2012)*			
Gross energy intake	GE	kJ/kg$^{0.8}$/d	= FI_{MBW}* GE_{Diet}
Digestible energy intake	DE	kJ/kg$^{0.8}$/d	= GE* ADC_{GE}
Faecal energy loss	FE	kJ/kg$^{0.8}$/d	= GE - DE
Branchial and urinary energy loss	BUE	kJ/kg$^{0.8}$/d	= (BUN * 24.9)/1000
Metabolizable energy intake	ME	kJ/kg$^{0.8}$/d	= DE - BUE
Retained energy	RE	kJ/kg$^{0.8}$/d	= (BW_f * E_f – BW_i * E_i)/ MBW_m*(100/t)
Retained energy as protein	REp	kJ/kg$^{0.8}$/d	= (RN * 6.25 * 23.6)/1000
Retained energy as fat	REf	kJ/kg$^{0.8}$/d	= RE - REp
Heat production	H	kJ/kg$^{0.8}$/d	= ME - RE
Metabolizable energy for maintenance	MEm	kJ/kg$^{0.8}$/d	= ME-(REp/0.5)+ (REf/0.9))

Notes. ADC_{GE}, apparent digestibility of gross energy diet; B_i, initial biomass (g/tank); B_f, final biomass (g/tank); d, day; FI_{Total}, total feed intake per tank (g feed DM); i, is the initial day of the experiment; N_f, final number of fish (number/tank); N_i, initial number of fish (number/tank); GE_{Diet}, energy content diet (kJ/g feed DM); t, number of experimental days; X= dry matter, ash, protein, fat, total carbohydrates, or energy (in g/kg feed DM or kJ/kg feed DM); RN, retained nitrogen.

This is evident in the Atlantic strain's high retained energy as fat and protein ratio of 2.28, as opposed to 1.18 and 1.36 for the East and West Mediterranean strains, respectively. Heat energy losses, which can be used as an indicator of oxygen intake, were 15% higher for the Atlantic strain than for the East and West Mediterranean strains with the same heat production. The Atlantic strain had the highest maintenance energy requirement, being different from the West Mediterranean strain. The maintenance requirement of the East Mediterranean strain was intermediate between the other

strains. Overall, E efficiency was low across strains, with the Atlantic strain having the highest efficiency (35.6%), followed by the West Mediterranean (30.4%) and East Mediterranean, which corresponds to the relatively high FCR.

Table 7. The effect of Atlantic seabass strain on feed intake, growth, and energy balance during the 70-day trial.

Strain	Atlantic (AT)	East Mediterranean (EM)	West Mediterranean (WM)	SEM	*P*-value
Feed intake (g/kg$^{0.8}$/d)	7.8^{a}	6.4^{b}	6.8^{b}	0.16	***
Growth (g/kg$^{0.8}$/d)	4.2^{a}	3.2^{b}	3.2^{b}	0.20	**
FCR	2.13	2.14	2.12	0.10	ns
Energy (E) Balance (kJ/kg$^{0.8}$/d)					
Gross E intake	186.4^{a}	136.8^{b}	144.1^{b}	3.38	***
Digestible E intake (DE)	160.3^{a}	117.7^{b}	124.3^{b}	2.86	***
Branchial and urinary E loss	9.6^{a}	6.6^{b}	7.0^{b}	0.46	***
Metabolizable E	150.7^{a}	111.2^{b}	117.3^{b}	2.69	***
Heat E	93.7^{a}	79.3^{b}	79.3^{b}	1.84	***
Retained E (RE)	57.1^{a}	31.9^{b}	37.9^{b}	2.95	***
Retained E as protein	17.4	14.6	16.1	0.92	ns
Retained E as fat	39.7^{a}	17.3^{b}	21.9^{b}	2.19	***
E maintenance (kJ/kg$^{0.8}$/d)	71.9^{a}	62.8^{ab}	60.8^{b}	2.53	*
E efficiency (RE/DE, %)	35.6^{a}	26.5^{b}	30.4^{ab}	1.67	**

Notes. FCR, feed conversion ratio; ns, not significant P > 0.1; * P < 0.5; ** P < 0.01; *** P < 0.001.Values are means (n=8) and stand error of the mean (SEM). Means within the same row not sharing a common letter are significantly different (P < 0.05).

Fish were not affected by early training in terms of feed intake, growth, or energy balance later in life (Table 8). The fish were fed two diets with different non-protein energy sources: a starch-rich diet (HOD) and a fat-rich diet (LOD). To keep the dietary protein-to-energy ratio constant, the HOD diet's protein content was reduced. The two contrasting diets were used to help pinpoint which factor is limiting feed intake. The HOD diet led to a 19% increase in feed intake (P < 0.001) over the LOD diet (8.0 vs 6.7 g/kg$^{0.8}$/d), but had no effect on growth (g/kg$^{0.8}$/d) (Table 9).

The higher feed intake on the HOD diet, combined with comparable growth, resulted in a higher FCR (2.36 vs 1.90). The HOD diet's increased feed intake combined with its lower energy content resulted in comparable gross energy, metabolizable energy, and digestible energy intake. As expected, the HOD diet (high in starch) increased heat production and maintenance requirements, resulting in lower energy retention and efficiency because digestible energy intake and metabolizable energy were comparable. The higher energy retention in the LOD diet (high in fat) was attributed to increased fat retention, while protein retention remained similar. Despite the increased energy retention in fish fed the HOD diet (51.2 vs. 33.3 kJ/kg$^{0.8}$/d), growth was unaffected.

Table 8: The effect of early life training of seabass on feed intake, growth, and energy balance during the 70-day trial.

Training history	Trained	Untrained	SEM	*P*-value
Feed intake (g/kg$^{0.8}$/d)	7.4	7.4	0.13	ns
Growth (g/kg$^{0.8}$/d)	3.6	3.5	0.16	ns
FCR	2.10	2.17	0.08	ns
Energy (E) Balance (kJ/kg$^{0.8}$/d)				
Gross E intake	157.7	153.9	2.8	ns
Digestible E intake (DE)	135.8	132.5	2.3	ns
Branchial and urinary E loss	7.7	7.7	0.2	ns
Metabolizable E	128.0	124.8	2.2	ns
Heat E	84.1	84.2	1.5	ns
Retained E (RE)	44.0	40.6	2.4	ns
Retained E as protein	16.9	15.1	0.8	ns
Retained E as fat	27.1	25.5	1.8	ns
E maintenance (kJ/kg$^{0.8}$/d)	64.2	66.2	2.1	ns
E efficiency (RE/DE, %)	31.7	30.0	1.4	ns

Notes. FCR, feed conversion ratio; ns, not significant, P > 0.1. Values are means (n=8) and stand error of the mean (SEM).

Table 9. The effect of diet on feed intake, growth, and energy balance in seabass during the 70-day trial.

Diet	HOD	LOD	SEM	*P*-value
Feed intake (g/kg$^{0.8}$/d)	8.0	6.7	0.09	***
Growth (g/kg$^{0.8}$/d)	3.5	3.6	0.07	ns
FCR	2.36	1.90	0.06	***
Energy (E) Balance (kJ/kg$^{0.8}$/d)				
Gross E intake	157.3	154.3	1.88	ns
Digestible E intake (DE)	133.4	134.9	1.59	ns
Branchial and urinary E loss	8.2	7.3	0.17	**
Metabolizable E	125.2	127.6	0.21	ns
Heat E	91.9	76.4	1.97	***
Retained E (RE)	33.3	51.2	1.63	***
Retained E as protein	16.1	15.9	0.68	ns
Retained E as fat	17.2	35.3	1.70	***
E maintenance (kJ/kg$^{0.8}$/d)	73.8	56.5	2.22	***
E efficiency (RE/DE, %)	24.3	37.3	1.17	***

Notes. HOD, high oxygen demanding diet; LOD, low oxygen demanding diet; FCR, feed conversion ratio; ns, not significant, P > 0.1; **, P < 0.01; ***, P < 0.001. Values are means (n=8) and stand error of the mean (SEM).

During the experimental period, all fish (tanks; experimental unit) were fed both diets; half of the tanks received the LOD diet during the first period, while the other half received the HOD diet. During the second period, the fish were fed the opposite diet as the first (cross-over). The order and period in which the diets were given influenced its effect (interaction effect); thus, results per period were also reported (Table 10). During the second period, feed intake increased regardless of diet, but

growth was almost identical for the HOD diet and numerically lower for the LOD diet, resulting in an increase in FCR for both diets. There was an interaction between diet, sequence, and diet for gross E intake, digestible E intake, branchial and urine E loss, metabolizable E, and heat E production, with higher values in the second period, indicating a time/fish size effect. The time period had no effect on the energy retained, which is consistent with the higher FCR.

Table 10. The effect of diet on feed intake, growth, and energy balance in seabass during the 70-day trial.

Diet	HOD		LOD			*P*-value
Period	P1	P2	P1	P2	SEM	Diet*Seq.
Feed intake (g/kg$^{0.8}$/d)	7.6^{b}	8.4^{a}	6.3^{c}	7.0^{b}	0.13	***
Growth (g/kg$^{0.8}$/d)	3.5	3.4	3.8	3.4	0.10	*
FCR	2.16^{b}	2.56^{a}	1.69^{c}	2.12^{b}	0.08	***
Energy (E) Balance (kJ/kg$^{0.8}$/d)						
Gross E intake	149.0^{b}	165.6^{a}	146.2^{b}	162.3^{a}	2.66	***
Digestible E intake (DE)	127.4^{a}	139.4^{a}	128.4^{b}	141.3^{a}	2.25	**
Branchial and urinary E loss	6.6^{b}	9.7^{a}	7.6^{b}	6.9^{b}	0.23	***
Metabolizable E	120.7^{b}	129.6^{ac}	120.9^{ab}	134.4^{c}	2.11	**
Heat E	87.5^{ab}	96.3^{a}	71.5^{c}	81.2^{bc}	2.79	**
Retained E (RE)	33.3	33.4	49.3	53.1	2.30	ns
Retained E as protein	15.8	16.4	15.8	16.1	0.96	ns
Retained E as fat	17.4	17.0	33.6	37.1	2.40	ns
E maintenance (kJ/kg$^{0.8}$/d)	69.7^{ab}	78.0^{a}	52.0^{c}	61.0^{bc}	3.14	*
E efficiency (RE/DE, %)	23.3	25.3	37.3	37.4	1.66	ns

Notes. HOD, high oxygen demanding diet; LOD, low oxygen demanding diet; P1, period 1 (first 5 weeks); P2, period 2 (last 4 weeks) FCR, feed conversion ratio; ns, not significant, P > 0.1; **, P < 0.01; ***, P < 0.001. Values are means (n=8) and stand error of the mean (SEM).

Over the whole dataset, training had no significant effect on growth, feed intake, or energy balance. However, an interaction effect was observed between early life training and strain in terms of heat energy production and maintenance energy requirements. Heat energy (Fig. 14) and maintenance requirements (Fig. 15) were higher for untrained fish in the Atlantic strain, lower in the East Mediterranean strain, and comparable in the West Mediterranean strain (though not statistically different within strain).

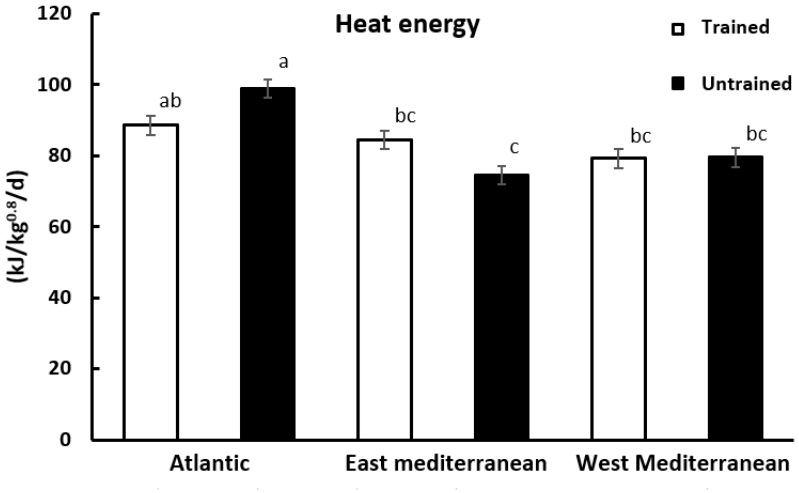


Figure 14. The impact of trained versus untrained fish in early life on three European seabass strains (*Dicentrarchus labrax*; Atlantic, East Mediterranean, and West Mediterranean strains) and their interaction on heat energy (kJ/kg$^{0.8}$/d). Means that do not share a common letter differ significantly; values represent means (n=4), and error bars are standard error of mean.

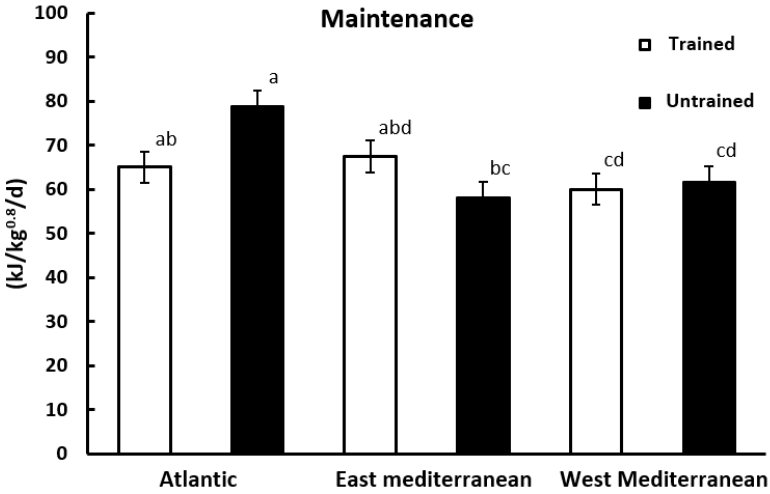


Figure 15. The impact of trained versus untrained fish in early life on three European seabass strains (*Dicentrarchus labrax*; Atlantic, East Mediterranean, and West Mediterranean strains) and their interaction on maintenance requirements (kJ/kg$^{0.8}$/d). Means that do not share a common letter differ significantly; values represent means (n=4), and error bars are standard error of mean.

Discussion

It is well known and well-studied that fish feed intake is reduced in low dissolved oxygen (DO) conditions (hypoxia). The reduction in feed intake caused by the DO level has a negative effect on growth (Pichavanta et al. 2000; Brett & Blackburn 1981). Increasing DO levels above the incipient DO (cut-off point) has no effect on feed intake. DO conditions above the incipient point are referred to as normoxic conditions, or "normoxia" (Brett & Blackburn 1981). Under normoxic conditions, increasing the water oxygen level does not affect feed intake. At normoxia, the dissolved oxygen in the water is sufficient to meet the fish's oxygen requirements. However, under normoxia, other factors such as the ability to absorb oxygen from water (aerobic capacity), stomach capacity, gastrointestinal emptying rate, and energy intake can influence fish feed intake (Jobling, 1981; Grove et al., 1985; Vahl, 1979). Several factors can limit oxygen uptake from water (gill surface, partial pressure of oxygen in the water and blood, ventilation rate) (Owen 2001). Saravanan et al. (2013) found that oxygen

consumption constraints may limit maximum feed intake. In the current study, the fish ate more of the HOD diet, which resulted in significantly higher heat production (used as an indicator of oxygen consumption), indicating that heat production and thus oxygen consumption did not influence feed intake. Furthermore, in the current study there was a clear difference (19%) in feed intake, ruling out stomach fullness as a possible reason for regulating feed intake. The higher intake of the HOD diet, which had a lower energy content due to lower fat levels and higher starch levels, resulted in comparable gross and digestible energy intake. The LOD diet contains more energy from fat, which is more efficiently retained as energy (as fat) than storing energy from starch as fat, resulting in higher heat losses and thus oxygen requirements. Energy intake is regarded as one of the primary factors influencing feed intake. Studies have shown that diluting the feed with an indigestible bulk increases feed intake to compensate for the diet's low energy content (Kestemont & Baras 2001; Bromley & Adkins 1984). The comparable gross energy and digestible energy intake, while the absolute amount of feed intake and heat production (indicator of oxygen consumption) differed significantly, strongly suggest that the energy/digestible energy intake was steering feed intake in the current study. Although the current study does not show evidence that early life training increased the fish's aerobic capacity, the results do not rule out the possibility that the aerobic capacity was increased, but rather that feed intake was not limited in this study by aerobic capacity. The interaction effect of training and strain resulted in numerically higher heat energy production for untrained fish of the Atlantic strain, implying a higher oxygen consumption. Although this contradicts our hypothesis of higher oxygen consumption in trained fish, the Atlantic strain's numerically lower heat production and maintenance indicate a more energy efficient fish, whereas this was absent in the Mediterranean strains. In general, the results for the Atlantic strain were more distinct than the two Mediterranean strains, which was expected given the larger genetic differences due to geographic location. Vandeputte et al. (2019) were the only paper to look at strain differences, and they observed that Atlantic strains had a numerically higher body weight than West Mediterranean strains, though this difference was not statistically significant.

7 Effects of early life training on the European seabass (*Dicentrarchus labrax*) in later life: swimming characterization (WR)

The experimental approach envisioned that exercise trained fish are more efficient swimmers, in relation to oxygen consumption, energy balance and swimming speed, compared to non-trained fish (Fig. 16). The fish used in this experiment also came from Ifremer Palavas-les-Flots as in the previous experiment, underwent the same training protocol and arrived at WU in October 2023 (Fig. 13).

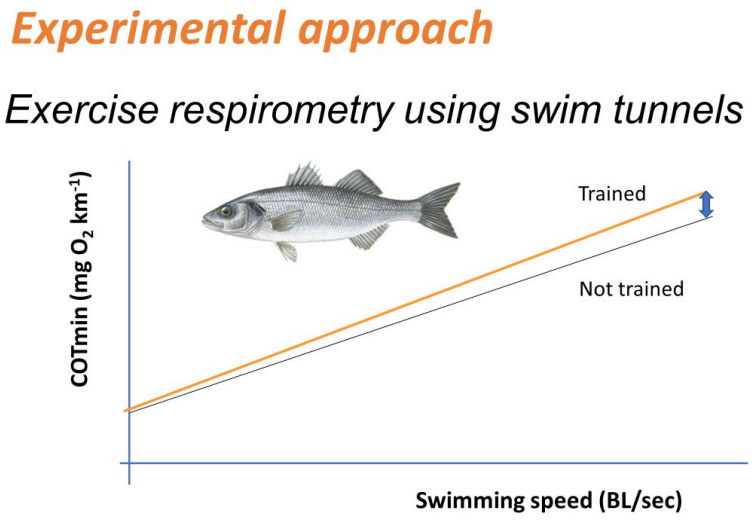


Figure 16: experimental approach and hypothesis.

7.1 Animal and experimental set up

The experimental approach was tested by subjecting the fish from the three populations, trained or untrained, to a swimming exercise experiment. The following protocol was used:

The fish (N= 36, with 3 origins x 2 training conditions is N= 6 fish per group, average 60 g body weight) were subjected to a swimming experiment using a 30-L Loligo swim tunnel (Loligo systems, Viborg, Denmark). The flow in the swim-tunnel was set at five different speeds during the experiment, starting at the lowest speed of 0.1 $m.s^{-1}$ and then increasing stepwise with 0.1 ms^{-1} per hour up to a maximum speed of 0.5 $m.s^{-1}$. Oxygen consumption and locomotory behaviour were assessed at each interval using a galvanic oxygen probe and a Basler 2040-90um NIR USB3 high-speed camera (see also Arechavala-Lopez et al., 2021). From the experiment we obtained the following data: oxygen consumption rates at each of the swimming speeds (MO_2 in $mg.kg^{-1}.h^{-1}$), optimal swimming speed (Uopt in $m.s^{-1}$), Cost of Transport at the optimal speed (CoTopt in $mg.kg^{-1}.km^{-1}$) and critical swimming speed (Ucrit in $m.s^{-1}$). In addition, locomotory behaviour parameters, tail beat amplitude and frequency, and head width amplitude and frequency, were analysed (Fig. 17).

Ethics

Experimental protocols complied with the current laws of The Netherlands and were approved by the Dutch Central Committee for Animal Experiments (CCD), project number AVD10400202216, and by the Animal Experiments Committee (DEC) and Authority for Animal Welfare (IvD) of Wageningen University and Research, experiment number 2022.W-0004.001.

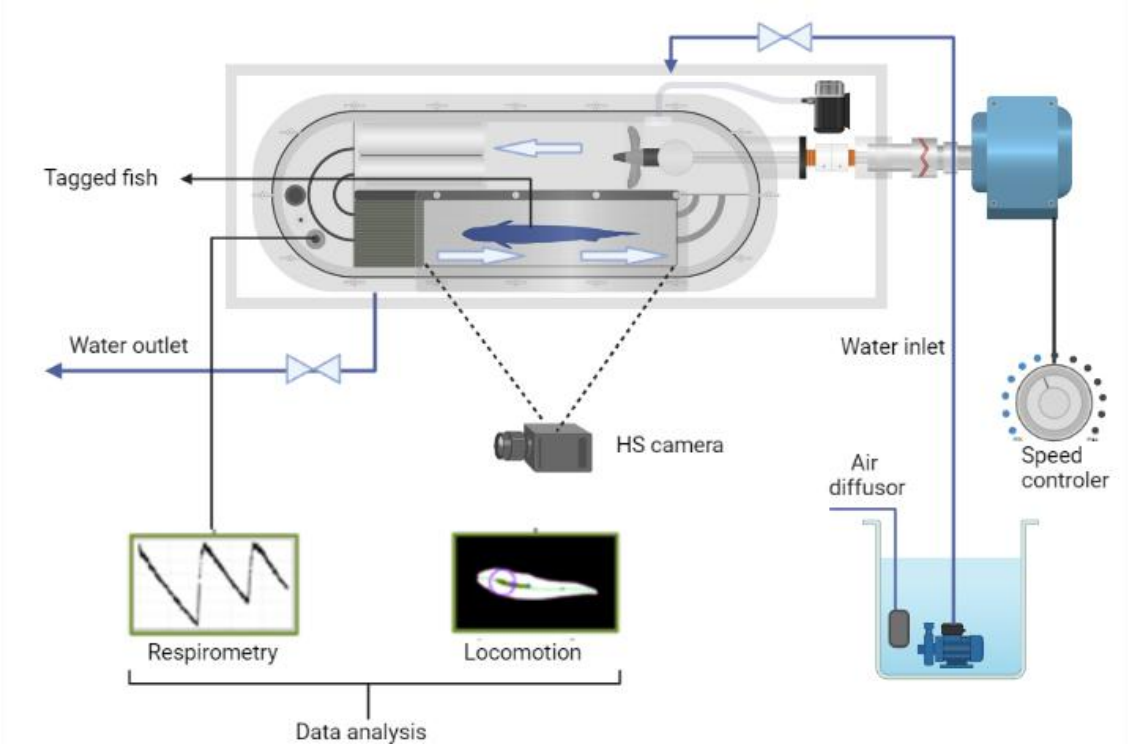


Figure 17: schematic overview of the experimental set-up

7.2 Calculations and statistical analysis

Oxygen Consumption (MO_2) and Body Motion: Head Orientation and Tail Beats
In order to determine the influence of flow speed, body size, and age of the fish on MO_2, a linear mixed model was fitted, accounting for variations both between individuals and between groups. This approach was chosen due to its ability to accommodate the non-independence of observations within groups and individuals, thus capturing the inherent heterogeneity in the dataset. The model included fixed effects for flow speed (including a quadratic term to capture the non-linear relationship), fish length, and age, with random effects for individual fish and groups (population origin, trained *vs.* untrained), to account for variation within these classifications. To investigate the influence of flow speed, fish length, and age on the characteristics of tail beats, specifically amplitude and frequency, a second Linear Mixed Model was fitted to the data.

7.3 Results and discussion

Respirometry
No differences were observed between the trained vs. untrained fish for any variable and the grouping variable was thus not further considered in the data analysis.

No significant positive relationship existed between the studied parameters: Body Weight (BW), Body Length (BL), Critical swimming speed (Ucrit), Optimal swimming speed (Uopt) and Optimal Cost of Transport (CoTopt) for fish from the Atlantic (n=12), the West Mediterranean (n=12) and the East Mediterranean (n=12). Atlantic seabass showed highest Uopt and CoTopt values (186 ± 29 mg/kg/km), but differences were not significant (Table 11).

Table 11 Mean values (±SE) and variation coefficients (%) of body length (BL), body weight (BW), optimal swimming speed (Uopt), critical swimming speed (Ucrit), and minimum oxygen cost of transport (COTmin) and significance of differences for fish from the Atlantic (N = 12), East Mediterranean (N=12) and West Mediterranean (N=12; (ANOVA, significance level: p < 0.05, indicated with an asterisk:).*

	Atlantic			Mediterranean East			Mediterranean West			All individuals				
	μ	σ	CV (%)	μ	σ	CV (%)	μ	σ	CV (%)	μ	σ	CV (%)	P-value	Test
Body Length	182.167	12.770	7.010	184.167	14.572	7.912	187.667	20.353	10.845	184.667	15.291	8.621	0.705	a
Body Weight	69.500	16.229	23.351	73.575	20.830	28.312	74.529	29.460	39.528	72.535	22.289	30.729	0.928	b
Uopt m.s^{-1}	0.310	0.024	7.836	0.289	0.019	6.740	0.308	0.046	15.030	0.302	0.033	10.815	0.131	b
Uopt BL.s^{-1}	1.712	0.202	11.806	1.576	0.144	9.158	1.644	0.186	11.330	1.644	0.183	11.125	0.194	a
Ucrit m.s^{-1}	0.404	0.002	0.532	0.412	0.028	6.901	0.414	0.054	13.119	0.41	0.035	8.463	0.966	b
Ucrit BL.s^{-1}	2.226	0.149	6.673	2.247	0.221	9.851	2.244	0.486	21.675	2.239	0.311	13.894	0.537	b
COT mg. $kg^{-1}.km^{-1}$	185.805	28.818	15.510	151.972	50.744	33.390	157.142	60.960	38.793	164.973	49.658	30.101	0.251	b

The linear mixed model revealed that the relationship between CoTopt and swimming speed is complex and non-linear (Fig. 18). Specifically, the quadratic term for swimming speed had a significant and positive impact on CoTopt (β2=8500.5460, SE = 1248.3275, t = 6.810, p<0.001), indicating that CoTopt increased with increasing swimming speeds. Conversely, the linear term of swimming speed had a negative impact (β1=−4116.1052, SE = 753.3703, t = -5.464, p<0.001), showing a decrease in CoTopt at initial speed increments. Age also showed a negative effect (β4=−4.1257, SE = 0.9843, t = -4.192, p<0.001), indicating a decrease in metabolism with increasing age. Additionally, variability among fish and groups was captured through random effects, with a standard deviation of 39.59 for individual fish and 12.28 for groups, revealing higher variability among individual fish than between groups.

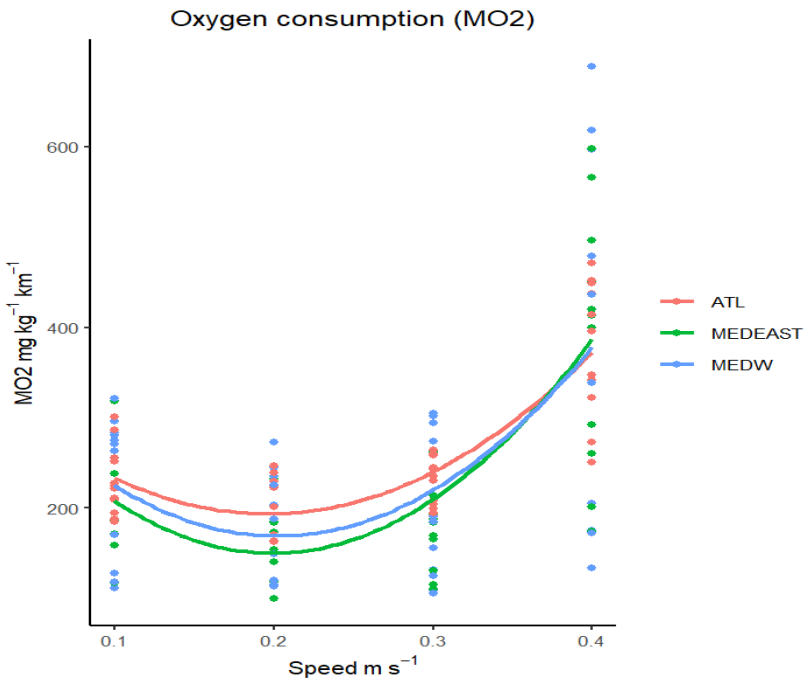


Figure 18: Relationships between swimming speed and oxygen consumption for the three studied populations: Atlantic (n=12), West Mediterranean (n=12) and East Mediterranean (n=12).

Body Motion: Head Orientation and Tail Beats

For tail beat amplitude, swimming speed demonstrated a highly significant effect ($p < 2.2e^{-16}$), indicating a pronounced increase in amplitude with higher speeds (Fig. 19). Fish length also significantly influenced amplitude, with larger fish exhibiting reduced amplitude ($p = 0.00765$), whereas age showed no significant effect. The results from the reanalysis revealed substantial influences of swimming speed on tail beat frequency, both linear and quadratic terms (p=8.76×10^{-6} and p=1.59×10^{-6}, respectively), highlighting a complex relationship where frequency first decreases and then increases with speed. The effects of fish length and age on frequency were not significant.

Significant random effects variance components were observed for individual fish (uj), underlining variability within individual fish, whereas the variance for fish origin was negligible, suggesting minor differences at the group level.

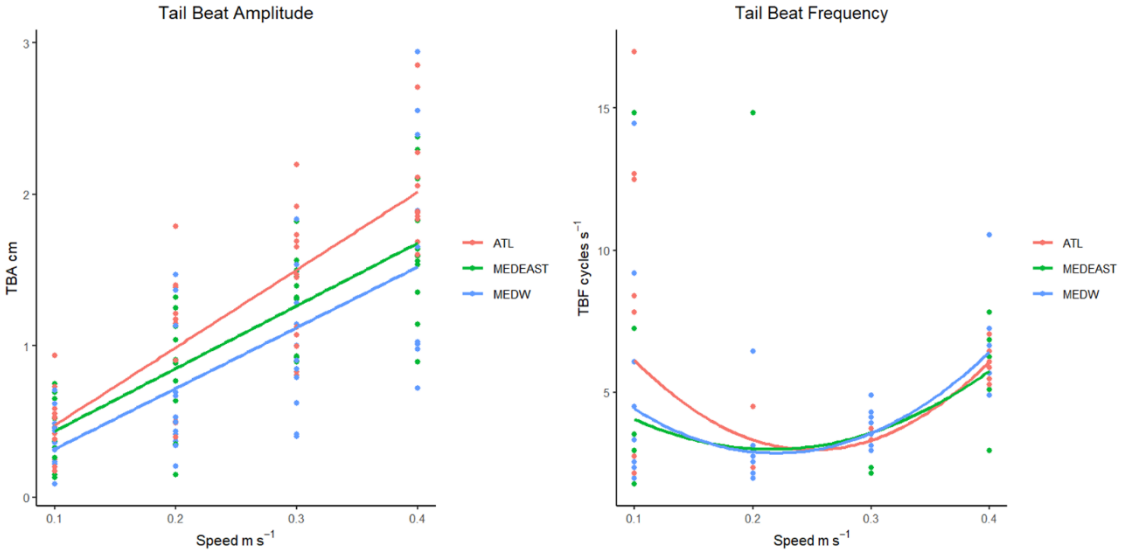


Figure 19: A) relationship between tail beat amplitude and swimming speed, and B) tail beat frequency across the same speed range. Different colours represent fish from different origins: ATL (red), MEDEAST (green), and MEDW (blue). Linear and quadratic models were fitted to describe the relationship between the tail beat characteristics and swimming speed.

Discussion

This study aimed to investigate the differences in energy metabolism between three strains of sea bass (*Dicentrarchus labrax*) from three geographic origins: Atlantic, East Mediterranean, and West Mediterranean with or without an early training. This study successfully characterized the swimming activity of seabass through a multidisciplinary approach in swim-tunnels. A model including tail beat amplitude, among other variables, suggests that tail amplitude is a crucial factor in modelling metabolic rates in seabass, capturing variability that directly correlates with metabolic processes.

Oxygen Consumption and Cost of Transport

The analysis of oxygen consumption (MO_2) revealed a complex, nonlinear relationship with flow speed, where the quadratic term for flow speed was significant and positive, while the linear term was negative. This indicates that metabolism initially decreases with increasing speed but then increases at higher speeds. The initial higher oxygen consumption and cost of transport that drives to the quadratic fit can be explained by the freedom to exhibit spontaneous behaviours at lower swimming speeds, which reduced energy and locomotive efficiencies requiring higher locomotion and oxygen consumption. As swimming flow increases, fish adopted a steady-state swimming behaviour. At highest speeds, burst-and-coast swimming occurs at highest oxygen consumption rates while the non-aerobic

metabolic component of the performance increases. This pattern is in line with earlier experimentation on seabass (Claireaux et al., 2006; Alfonso et al., 2022). Alfonso *et al.* (2022) reported a Standard Metabolic Rate (SMR) of 89.77 ± 20.35 mg O_2 kg^{-1} h^{-1} and a Maximum Metabolic Rate (MMR) of 579.21 ± 94.89 mg O_2 kg^{-1} h^{-1}. Our results ranged from 218.77 ± 62.73 mg O_2 kg^{-1} h^{-1} at 0.1 m s^{-1} to 389.09 ± 139.45 mg O_2 kg^{-1} h^{-1} at 0.4 m s^{-1}. These authors reported a minimum cost of transport (COTmin) of 113.91 ± 17.54 mg O_2 kg^{-1} km^{-1}, while our study resulted in a higher mean COTmin of 164.97 ± 49.66 mg O_2 kg^{-1} km^{-1}.

Swimming Performance: Ucrit and Uopt

Ucrit is generally defined as the speed at which fish fatigue, can no longer remain swimming and are stuck to the posterior grid of the swim chamber (Brett, 1964). The Uopt was determined by setting the first derivative of a polynomial trend line through CoTopt values versus swimming speed to zero, corresponding to the point with the lowest CoT (CoTmin) (Palstra *et al.,* 2008). Measurements of critical swimming speed (Ucrit) and optimal swimming speed (Uopt) showed no significant differences between populations. Ucrit, an indicator of sustained swimming capacity, and Uopt, reflecting speed-specific energy efficiency during swimming, are crucial parameters for understanding fish endurance and performance.

The average Uopt value of 0.30 ± 0.03 $m.s^{-1}$ observed in this study was lower compared to previous studies that reported values of 0.54, 0.51, and 0.68 $m.s^{-1}$ for seabass weighing between 200 and 1000 g (Alfonso *et al.,* 2022; Zupa *et al.,* 2015). Also, the average Ucrit value of 0.41 ± 0.04 $m.s^{-1}$ and 2.24 ± 0.31 $BL.s^{-1}$ observed in this study was lower in absolute terms, but consistent with values reported in the literature for European seabass when expressed in relative terms (Alfonso *et al.,* 2022).

Body Movements: Tail Beats and Head Orientation

The observed swimming movements at lower speeds, specifically at 0.1 $m.s^{-1}$, resulted in a wide range of values for tail and head frequency and amplitude. This variability is attributed to the limitations of the tracking methods employed, which were not optimized for capturing non-steady-state swimming behaviour.

The analysis of body movements showed that both tail beat amplitude and frequency increased with flow speed, while head orientation amplitude also increased. The quadratic relationship observed in head orientation frequency, with an initial decrease followed by an increase, highlights the complex biomechanical adjustments fish make to optimize swimming efficiency at varying speeds. Videler and Wardle (1991) discussed similar adaptive mechanisms in fish, where changes in body movement patterns are crucial for maintaining hydrodynamic efficiency.

No differences were observed between the three origins of seabass related to any of the parameters measured for body movement but incorporating body movement parameters into a linear mixed model for predicting oxygen consumption significantly improved the model's explanatory power. The tail beat amplitude, in particular, was a critical factor, indicating that tail movement is a powerful predictor of energy consumption during swimming. Arechavala-López *et al.,* (2022) also highlighted the importance of tail beat mechanics in determining the metabolic cost of swimming, suggesting that fish optimize their tail movements to balance energy expenditure with swimming efficiency.

8 Concluding remarks

In the first objectives of this deliverable, we sought to examine whether rearing early life Atlantic salmon, European seabass and rainbow trout in enriched (EE) housing conditions, compared to barren environments (NE), can improve the welfare or the performances of these species when housed in captivity for research purposes.

First, for physical enrichment using false plants or 2D patterns, the targeted OWIs were standardised as much as possible but nonetheless dependencies on rearing system representative of large-scale rearing were high and complex and we were not able to analyse all the dataset acquired for technical reasons. In particular, filming large or very large groups of fish produces challenging videos; the full data set was only analysed for Atlantic salmon, it was partially analysed for seabass and not manageable for rainbow trout but 2D enrichment pattern did not seem to affect fish distribution to a large degree. For all three species, the provision of EE did not negatively affect growth or condition factor in the long run. For Atlantic salmon and seabass, differences in fin damage between EE and NE were not observed while in rainbow trout enrichment had a negative impact on the welfare indicators eye opacity and fin damage. With only negative welfare effects documented, 2D enrichment as applied here does not appear to be beneficial for juvenile rainbow trout. On the contrary, for Atlantic salmon and seabass, the behavioural and physiological results obtained here provide support for beneficial effects of enriched rearing environments. For both Atlantic salmon and seabass, EE accelerates recovery from exposure to novel objects, producing less neophobic (fearful) behavioural profiles. Further, in A. salmon, EE promotes cognitive capacity and neurogenesis as evidenced by the increased expression of related genes and an enhanced ability to respond to stress was also reflected in higher serotonergic activity. Taken together, these findings suggests that the presence of EE improves the well-being of salmon parr in captivity when exposed at the early life stages, enhancing stress-coping behavioural profiles and cognitive abilities in line with an improved mental state. In seabass, the presence of this enrichment lead to positive responses in the long term (quicker recovery from stress event, overall lower cortisol and a better pH balance), though these effects appear to be mild.

In the later objectives of this deliverable, seabass juveniles from three populations were subjected to a swim training protocol (70 days) and later in life their metabolic performances examined. To summarize, training fish early in life did not improve feed intake or heat production (an indicator of oxygen consumption) later in life. The interaction effect of training and strain suggests that the Atlantic strain benefits from early life training, which reduces energy requirements for maintenance and heat production. The Atlantic strain stood out among the three strains used, with higher feed intake and growth than the East and West Mediterranean strains, which was driven by increased energy retention as fat. Considering swimming characteristics, the results revealed no significant differences between fish from the three different origins, nor between trained fish and their controls. These findings suggest that origin and early life training do not have any relation with the swimming performance during later life. Further, no differences were observed between origins in body motion characteristics but interindividual differences were significant and incorporating body movement parameters into a linear mixed model for predicting oxygen consumption significantly improved the model's explanatory power.

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Work Participants	Marie-Laure Bégout (Task 3)			

Lead Beneficiary	IFREMER, 7
Authors	Marie-Laure Bégout, IFREMER, mlbegout@ifremer.fr Sonia Rey, Mauro Chivite Alcalde, UoS, sonia.reyplanellas@stir.ac.uk Video analyses contribution: Petr Cisar, JU, cisar@frov.jcu.cz Jonatan Nilsson Lars Helge Stien, HI, jonatan.nilsson@hi.no Maas Roel, WU, roel.maas@wur.nl Wout Abbink, WR, wout.abbink@wur.nl
Reviewers	Felicity Huntingford, Univ. Glascow, Felicity.Huntingford@glasgow.ac.uk Chris Noble, NOFIMA, Chris.Noble@Nofima.no

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