Aquaculture Reports 37 (2024) 102256

Contents lists available at ScienceDirect

Aquaculture Reports

journal homepage: www.elsevier.com/locate/aqrep

Environmental enrichment reduces the effects of husbandry stressors in gilthead seabream broodstock

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ARTICLE INFO

Keywords:
Environmental enrichment
Fish welfare
Sparus aurata
Heart rate
Animal care
Rearing procedures
Internal device

ABSTRACT

Husbandry procedures can be stressful for captive animals. Knowledge of the physiological effects of these procedures and the reduction of stress during regular maintenance is of pivotal importance to ensure good welfare. Environmental enrichment (EE) can be an asset to animal keepers as it has many benefits on captive animals, including the reduction of stress indicators in many aquatic species. This is particularly relevant for broodstock animals, who may spend years in captivity and who are essential for successful fish farming. We studied whether structural enrichment had a stress-reducing effect on broodstock of gilthead seabream (*Sparus aurata)* during standard husbandry procedures, monitoring their heart rate with surgically implanted internal bio-loggers (DST milli HRT, Star-Oddi) in 18 fish. These fish were distributed in six tanks, three of which had an EE structure consisting of a 1 m^2 floating structure with nine suspended organic cables, while the other three tanks had no enrichment. We added seven more unmarked fish to each tank to simulate broodstock farming conditions (*i.e.*, ten fish per tank). After five days of post-surgical recovery, we tested feeding, netting, and cleaning every day for three consecutive days, and a formaldehyde bath as a prophylactic procedure on the fourth day on the logger-implanted fish and continued to record their recovery for eight more days. Overall, when subjected to stressful husbandry procedures, fish reared under EE show reduced heart rate and amplitude, and faster recovery to baseline levels than non-enriched animals. Our results show that EE can be used to improve the welfare of farmed fish by reducing their stress and should be employed as a good management practice in finfish production, and is especially relevant for high-value fish that spend long periods in captivity, such as spawners.

1. Introduction

Husbandry procedures are an integral part of animal farming and deeply impact the welfare of captive animals (Webster, 2011). Within aquaculture fish production, animals are regularly exposed to diverse husbandry procedures that can be very stressful for fish, given their aquatic nature and the discordance of their natural environment with that of captive conditions. Furthermore, there are more than 400 species of finfish being farmed (FAO., 2020) and most of them have low domestication levels (Saraiva et al., 2018; Teletchea and Fontaine, 2012), *i.e.*, the species have not fully developed the necessary mechanisms to cope with life in captivity. Moreover, of the species with high domestication level, only a reduced number of them have been artificially selected to have reduced levels of stress in captivity (reviewed in Milla et al., 2021). This means most cultured finfish species are susceptible to stress induced by life in a fish farm (Saraiva et al., 2019). In this sense, environmental enrichment (EE) has been suggested as a tool to improve welfare and reduce stress in captive fish (Arechavala-Lopez et al., 2022; Oliveira et al., 2024). EE can be classified in several types: sensory (*i.e.*, stimulating the senses of captive fish), occupational (*i.e.*, reducing monotony and boredom in fish by introducing challenges), social (*i.e.*, promoting healthy social interactions,

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https://doi.org/10.1016/j.aqrep.2024.102256
Received 17 November 2023; Received in revised form 11 July 2024; Accepted 12 July 2024
Available online 17 July 2024

including not only other individuals but also the necessary space), dietary (*i.e.*, promoting foraging behaviour by improving food type and feeding strategy) and physical (*i.e.,* increasing the complexity of the rearing environment by introducing structures, objects or structural modifications (Arechavala-Lopez et al., 2022; Jones et al., 2021; Näslund and Johnsson, 2016). For example, structural EE has been reported to promote faster recovery of opercular beat rates and reduced the coefficients of variation in plasma cortisol following a stressor in rainbow trout (*Oncorhynchus mykiss*) (Pounder et al., 2016), and attenuate the effects of chronic unpredictable stress in zebrafish (*Danio rerio*) measured in trunk cortisol and brain reactive oxygen species (Marcon et al., 2018). Structural EE also reduced basal stress levels (*i.e.*, stress levels related only to housing conditions) in juvenile black rockfish (*Sebastes schlegelii*), measured in visceral cortisol levels and opercular beat rate (Zhang et al., 2021), and in juvenile Atlantic salmon (*Salmo salar*), measured on plasma cortisol, behaviour and fin deterioration (Näslund et al., 2013). Finally, structural EE improved the welfare of juvenile gilthead seabream (*Sparus aurata*) by reducing the level of aggressions and interactions with the net pen and reducing fin erosion (Arechavala-Lopez et al., 2019), and enhanced exploratory behaviour, spatial orientation, learning abilities, and physiological brain functions (Arechavala-Lopez et al., 2020), which are skills usually neglected in farming conditions and that are key for positive welfare (Fife-Cook and Franks, 2019). In the on-growing phase, structural enrichment can also modify the spatial distribution of seabream within a net-pen, improving the use of space (Muñoz et al., 2020).

The positive effects of EE may be especially relevant for broodstock, as they may be in captivity for several years and the consequences of chronic stress can have negative effects on the animals, such as reductions in growth, decreased food utilization efficiency, suppressed reproductive function, and diminished immune function and disease resistance (reviewed in Ashley, 2007), with serious economic repercussions for aquaculture companies. Despite the importance of broodstock welfare, scarce research has been conducted on the positive effects of the use of EE in their rearing, except for the use of dietary additives (Asturiano et al., 2001). More recently, Oliveira et al., (2024) explored the effects of EE in seabream broodstock and found that structural EE increased the use of space in the tank, enhanced social displays, promoted individual swimming patterns, and stimulated foraging behaviour.

Cortisol is the usual stress indicator in vertebrates. There are however several disadvantages: measurement of plasma cortisol often involves fish handling and exposure to anaesthetic agents, which can influence sample results (Sneddon, 2012); it is a non-specific marker for the activation of the hypothalamus-pituitary-intrarrenal axis upon stimulation, and may therefore present confusing results when the stimuli are positive, as in the case of exposure to EE (*e.g.*. no effect for rainbow trout (Pounder et al., 2016), juvenile Atlantic salmon (Näslund et al., 2013), and zebrafish (Wilkes et al., 2012), but reduction for chinook salmon (*Oncorhynchus tshawytscha*) (Cogliati et al., 2019) and juvenile black rockfish (Zhang et al., 2020, 2019); and the collection procedure can influence the results, since the fish have to be confined, captured, handled, anaesthetized and sampled in a short time to prevent an effect of the manipulation in the result (reviewed in Sadoul and Geffroy, 2019). An alternative way to measure stress physiologically can be by monitoring heart rate (J. Brijs et al., 2019a,2019b; Svendsen et al., 2021), which is considered a robust secondary indicator of stress in vertebrates, including fish (Schreck and Tort, 2016). Heart rate recordings have been performed in fish for several decades already, using a variety of methods, from external electrocardiogram recorders (Frank, 1968) to modern internal bio-loggers (*e.g.*, Hvas et al., 2020; Svendsen et al., 2021), as well as other invasive procedures including canulated arteries (*e.g.*, Davison et al., 1995). The advantage of internal bio-loggers over plasma cortisol samples is that the former do not involve manipulation at the time the samples are taken. Additionally, internal bio-loggers can sample at various time points over long periods, and exactly at the time when stress occurs. Heart rate bio-loggers have already been used to study the stressful effects of husbandry procedures in several species (*e.g.*, rainbow trout (Brijs et al., 2018, 2019a), Atlantic cod (*Gadus morhua*) (Bjarnason et al., 2019), European whitefish (*Coregonus lavaretus*) (Hjelmstedt et al., 2021), and Atlantic salmon (Yousaf et al., 2022)). In seabream, the stressful effects of hypoxia and warming were measured with heart rate bio-loggers, with an interesting increase in heart rate while undisturbed in the respirometers, probably due to the stress of being confined in isolation (Mignucci et al., 2021). This seabream study also found that seabream required 60 h to recover regular heart rate after surgery, which is slightly faster than what was found for other farmed species such as rainbow trout (3 days, J. Brijs et al., 2019a, 2019b) and Atlantic salmon (4 days, Føre et al., 2021).

In the present study, our objective was to evaluate the effects of EE on the welfare of a captive broodstock of gilthead seabream subjected to daily husbandry procedures. We explored these effects during feeding, netting attempts, and tank cleaning, as well as during prophylactic measures such as an antiparasitic bath with formalin, commonly used in fish aquaculture (Leal et al., 2018) in Greece, Portugal and Spain as prophylactic method for external parasitosis in gilthead seabream at aquaculture facilities (https://medicines.health.europa.eu/veterinary/en/600000044026). Specifically, we monitored the individual's heart rate and amplitude before, during, and after the procedures to observe whether EE had any buffering effect on the stress response. To our knowledge, this is the first study in which welfare is monitored in gilthead seabream by using internal bio-loggers that were surgically implanted to collect the heart rate and internal temperature of the fish automatically, without having to manipulate the subjects at the time of physiological recording. Simultaneously collecting internal temperature and heart rate is of upmost importance given the influence of temperature on heart rate in ectothermic animals such as fish, whose heart rate is positively correlated with environmental temperature (Mignucci et al., 2021; Skeeles et al., 2020). We hypothesise that EE would positively influence welfare and, therefore, we expected a reduction in heart rate and amplitude in fish reared under EE.

2. Materials and methods

2.1. Experimental settings

The study was conducted at the Estação Piloto de Piscicultura de Olhão (EPPO) of the Instituto Português do Mar e da Atmosfera (IPMA), Olhão, Portugal, from November 10th-28th, 2021 in fish that had been exposed to EE or a bare environment for six months, as part of a study exploring the behavioural and physiological effects during EE (Oliveira et al., 2024). Sixty adult gilthead seabream (mean weight: 791 ± 90 g) had been randomly distributed in six 3000 L outdoor cylindrical tanks (2 m diameter x 1 m, water level 90 cm), housing 10 fish per tank (~3 kg/m^3) to simulate farming conditions (Ortega, 2008) and avoid potential masking effects due to high densities (Carbonara et al., 2019). Three of the tanks had an environmental enrichment structure (hereafter, fish living under these conditions will be referred to as "EE group") consisting of a 1 m^2 floating structure made of 2.5 cm diameter polyethylene pipes, from which 9 organic ropes (75–80 cm in length and 2.5 cm in diameter) were suspended, placed 50 cm apart from each other in a 3 × 3 format. The other three tanks had no enrichment (hereafter, fish living in these conditions will be referred to as "NE group"). All tanks had air stones and a continued water flow in an open flow-through system anda dark cover to prevent direct sunlight. The fish were fed *ad-libitum* with commercial 8 mm pellets (Standard Orange 8, AquaSoja, Portugal) or 6 mm pellets (Aller Blue Ex Vitamax,Aller Aqua, Denmark).

2.2. Bio-logging and surgery procedure

After living in enriched and bare conditions for six months, and before starting the trials of this study, a total of 18 adult gilthead

seabream (mean weight 785 ± 76 g, with no significant differences in weight between tanks (One-way ANOVA, $F(5,10) = 1.41$; $p = 0.3$), corresponding to three fish in each tank, were surgically implanted bio-loggers (DST milli HRT, 13 mm × 39.5 mm, 12 g, Star-Oddi, Iceland, www.star-oddi.com), following the steps developed by Mignucci et al. (2021). The fish were not fed the day before surgery. On the day of surgery, they were anaesthetized with 2-phenoxyethanol (0.5 ‰, Sigma–Aldrich, USA), and kept anaesthetised during the procedure with a gill bath of 0.25 ‰ 2-phenoxyethanol. After disinfection of the skin with a few drops of 5 % povidone-iodine (Betadine, Viatris/Mylan, Lda., Portugal), a 2 cm abdominal incision was made along the ventral midline to insert the bio-logger in the intraperitoneal space of the thoracic cavity, in close proximity to the pericardium. The bio-logger was fixed to the ventral thorax by one stitch of non-absorbable monofilament nylon suture and another stitch of silk suture. This step was necessary to avoid any movement of the bio-logger inside the fish, which would otherwise disrupt the accuracy of the data recording. The abdominal incision was closed with two interrupted stitches of absorbable glyconate monofilament suture, after including 0.5 mL of a 1:1 mixture of nitrofural (Furacin 2 mg/g ointment, SeidLab, Spain) and Blastoestimulina® (1 % ointment, Almirall, Spain) inside the wound to prevent wound infections and promote a faster wound healing. All the stitches and wounds were then covered with drops of an ointment to reduce pain and promote healing (Aloclair® PLUS Gel, Alliance, Spain). Immediately after, the fish were placed in their experimental tank and monitored until complete recovery. The surgeries took place on two consecutive days, running three tanks on the first day and the other three on the second day, and bio-loggers began recording at the end of surgeries on each day. All fish were left undisturbed (except for feeding them) for five days to ensure a full recovery before undergoing the husbandry procedures (Table 1), which is longer than the 60 h of surgery recovery found in Mignucci et al. (2021) for adult gilthead seabream. This recovery period also allowed the evaluation of the cardiac response of the fish to the implantation of the bio-logger itself. No mortality was found in implanted fish, and only one non-implanted fish died on the first day of the experiment. This individual was not replaced to avoid disruption of social cohesion in the group.

2.3. Husbandry procedures

After the five-day recovery period, the fish were subjected to four different husbandry procedures: feeding, chasing, cleaning and anti-parasite treatment. Feeding, chasing, and cleaning were performed on days 6, 7 and 8 (*i.e.,* repeated on three consecutive days), while an anti-parasite treatment was performed only once on day 9 of the experimental study (Table 1). Cardiac response and internal temperature were recorded during all these phases of the husbandry procedure. Feeding was performed with commercial 8 mm pellets (Standard Orange 8, AquaSoja, Portugal), at 0.5 % of their body mass always at 10:00 AM. Netting consisted of moving a ~ 2000 cm^2 hand-net in each tank for 5 minutes always starting at 11:00 AM. The movement was from side to side and from the front to the back of the tank at steady pace to simulate a catching or sampling event. We did not chase any specific fish with the nets and never caught any fish inside the net. Cleaning was always done at 12:00 AM, and consisted of closing the aerators and scrubbing the sides and bottom of the tank for 8 minutes with a broom (22 cm wide) while lowering the water 8 cm. We then waited 2 minutes for any dirt to sink and opened a purge system at the bottom of the tank for 10 seconds, which lowered the total volume of water by a further 10 cm. We covered the tank and left the water reach its regular height.

After three days of these husbandry procedures, we performed a single antiparasitic treatment on day 9 of the study (Table 1) by lowering the water level to one third of the tank and adding 150 ppm formalin (37 % formaldehyde, and 10 % metanol, Sodacasa, Portugal) into the tank and leaving the fish undisturbed for one hour. This formaline solution is the most widely used in fish aquaculture in Portugal (Leal et al., 2018). After this period, the tanks were thoroughly cleaned with a broom and clean water, and the water containing formalin was drained. Once the water level reached 20 cm, we opened the water inlet and started filling the tank. Two hours later, we lowered the water level back to one third of the tank and filled it with clean water. This step ensured dilution of any remaining formaldehyde in the tank. The fish were not fed on the day of this procedure.

Table 1
Timeline of the 17 study days, with the dataset divisions, the procedures performed on each day, the logger settings, and the divisions of the data within each dataset.

Study day	Dataset	Procedures	Logger settings	Data dDivisions
1	Surgery Recovery	Surgery day	Recording once every 10 min	Data divided into day (5:40–18:50) and night (19:00–5:30)
2		Left undisturbed		
3				
4				
5				
6	Husbandry Procedures	Feeding, Netting, Cleaning	Recording once every 2 min during husbandry procedures (10:00–13:00) and once every 10 min the rest of the time	Data divided into: Morning (5:40–9:50), Feeding (10:00–10:58), Netting (11:00–11:58), Cleaning (12:00–12:58), Afternoon (13:00–18:50), and Night (19:00–5:30)
7				
8				
9		Prophylactic bath	Recording once every 2 min from 10:00–13:00, and once every 10 min the rest of the time	Data divided into: Morning (5:40–9:50), Formaldehyde bath (10:00–12:30), Afternoon (12:30–18:50), and Night (19:00–5:30)
10	Recovery from husbandry procedures	Left undisturbed	Recording once every 2 min from 10:00–13:00, and once every 10 min the rest of the time	Data divided into day (5:40–18:50) and night (19:00–5:30)
11				
12				
13			Recording once every 10 min	
14				
15				
16				
17				

After these four days of treatments, the fish were fed and kept undisturbed in the tanks for the followings eight days (days 10–17 of the study), during which period the bio-loggers kept recording (Table 1). This allowed us to evaluate the recovery of the cardiac response after the period of exposure to stressful husbandry procedures, and the possible influence of structural enrichment on coping. On day 18th of the study the bio-loggers turned off automatically and the fish were left undisturbed for six more months before the recovery of the bio-loggers for data processing. The tagged fish were euthanized with an overdose of 2-phenoxyethanol (1 ‰) before the retrieval of the bio-loggers. The remaining fish remained in the facilities for scientific and reproductive purposes.

2.4. Data retrieval and analysis

We used the Mercury v 6.30 software application and the associated

Communication Box (Star-Oddi, Iceland) to retrieve the data from the bio-loggers. For validation purposes, all logged heart rate measurements were graded with a data verification quality index (QI), ranging from 0 to 3, where QI0=Great, QI1=Good, QI2=Fair and QI3=Poor. To ensure data accuracy and a similar number of data points per individual, we manually assessed the ECGs with the Star-Oddi HRT Analyzer v.1.1.0 application software. Data points with QI2 and QI3 were observed, and the R peak or S peak in the QRS complex were manually selected so that the software could calculate the correct heart rate. Additionally, we randomly checked QI0 and QI1 data points to ensure their accuracy. When we could not differentiate the QRS complex, the data point was left blank. Using this method, of the 50,148 data points, we recalculated 17.4 %, confirmed 1.5 %, and left 10.3 % blank. Additionally, we used the Star-Oddi HRT Analyzer v1.1.2 software to calculate the average amplitude of the heart rate recordings, *i.e.,* the height of the ECG waves, measured by the software in Arbitrary units.

We analysed the heart rate and body temperature of tagged seabream in three different periods of the study (Table 1): 1) recovery from surgery (first 5 days of the study), 2) husbandry events (following 4 days), 3) recovery from husbandry events (last 8 days), and the amplitude of the heart rate recordings during the husbandry events.Throughout the study, the bio-loggers recorded heart rate at 150 Hz, but the interval between sampling points varied depending on the data resolution we needed, as explained in the following paragraphs. During the first five days, the bio-loggers recorded heart rate and body temperature once every 10 minutes. The data were divided into Day (5:40–18:50) and Night (19:00–5:30) periods, based on the time of dusk and dawn during the experimental period, and we calculated the average heart rate and body temperature for each of them. We calculated the baseline HR for day and night as a reference in the graphs for this population of seabream using the 5th day and night data (57.2 and 47.8 bpm, respectively), which occurred at an average body temperature of 16.8 ºC. We calculated two baselines because the heart rate of gilthead seabream varies according to a circadian rhythm (Aissaoui et al., 2000). These baselines were only used in the graphs as reference points for the reader, as these values were only estimates of what could be considered average heart rates for day and night respectively. However, they were not used as control measurements in all statistical analyses because it did not represent the HR of the individuals during the experimental procedures (*i.e.*, using the morning heart rate before stressors as a control measurement for the stressful events, which occurred during the morning, is more representative than using the average of the whole daytime). See Section 2.5 Statistical analysis for further details on the data used as reference for each comparison.

For the following four days, the data was divided into Morning (5:40–9:50), Feeding (10:00–10:58), Netting (11:00–11:58), Cleaning (12:00–12:58), Afternoon (13:00–18:50), and Night (19:00–5:30). The next day, the data were divided into Morning (5:40–9:50), Formaldehyde bath (10:00–12:30), Afternoon (12:30–18:50), and Night (19:00–5:30). Heart rate and body temperature were recorded once every two minutes from 10:00–12:58 (*i.e.*, during the feeding, netting, cleaning, and formaldehyde bath events) to achieve higher data resolution during this period, and every 10 minutes from 13:00–9:50 of the following day). We calculated mean heart rate and body temperature for each time period.

For the following 8 days, data were divided into Days (from 5:40–18:50) and Nights (from 19:00–5:30). Heart rate and body temperature were recorded once every 10 minutes, except on the first day of the recovery, when data were recorded every two minutes from 10:00–12:58, and we calculated the average heart rate and body temperature for each.

2.5. Statistical analysis

Statistical analyses were performed with SPSS Statistics 29 (IBM Corp., Armonk, NY, USA). The three data sections were analysed separately using linear mixed models with a first-order autoregressive (AR1) repeated covariance matrix. In all models we used individuals as the subject variable, time period as repeated measure and fixed factor, treatment as fixed factor, body temperature as a covariate, individuals as random factor, and heart rate as the dependent variable. For the surgery recovery dataset, we used in the model the first day of data as reference event for the comparisons, for the husbandry procedures dataset the morning of day 6, since it was the only period of that dataset not affected by the husbandry procedures, and for the recovery from husbandry procedures dataset we used the last day as reference since it would be the least affected by the husbandry procedures. We repeated the same analyses for amplitude as dependent variable and without temperature as a covariate because it had no effect on the model. We checked the normality of the residuals to meet the assumptions of the model and removed the outliers shown by the software, which improved the fit of the model based on its Akaike's Information Criterion (AIC). We ran pairwise comparisons with Sidak correction for multiple comparisons. When there was an interaction between the treatment and time period, we ran planned comparisons of two-tailed independent samples t-tests for each husbandry event to compare the two treatments at each point, and we calculated the effect size. We checked the model fit using AIC. Fish body temperature had a statistical significant effect on the models for the surgery recovery and husbandry recovery, but it did not have a significant effect on the model during the husbandry procedures ($F(1,303.2) = 0.14$; $p = 0.71$). However, its removal from the model reduced its fitness according to the AIC, so we included it in all our heart rate analyses.

2.6. Ethical note

The experiment was conducted at Estação Piloto de Piscicultura de Olhão (EPPO) facilities from IPMA (Olhão, Portugal), after compliance with internal ethics boards and under ethical permit 2023DGV/000066293 issued by Direção Geral de Alimentação e Veterinária, Ministério da Agricultura, Florestas e Desenvolvimento Rural, Portugal in compliance with the European (Directive 2010/63/EU) and Portuguese (Decreto-Lei no. 113/2013 de 7 de Agosto) legislation for the use of laboratory animals. All procedures were conducted by trained scientists under Group-C licences issued by the Direção Geral de Alimentação e Veterinária, Ministério da Agricultura, Florestas e Desenvolvimento Rural, Portugal, and under the supervision of an in-house veterinarian at EPPO.

3. Results

3.1. Surgery recovery

During the 5 days of surgery recovery, there was a significant effect of the time period on heart rate ($F(9,76.8) = 40.5$; $p < 0.0001$), with all periods having significantly lower HR than the first day of recordings (Fig. 1; $p < 0.001$ for all periods), and day and night 5 having no significant difference with days and nights 2, 3, and 4 respectively (Sidak's pairwise comparisons: day 5 *vs.* day 2: $p = 0.58$; day 5 *vs.* day 3 and 4: $p = 1.0$; night 5 *vs.* night 2, 3 and 4: $p = 1.0$). There was no significant effect of treatment ($F(1,15.9) = 1.39$;$p = 0.25$), and no interaction effect between treatment and time period ($F(9,75.2) = 1.72$;$p = 0.10$). Fish body temperature had a significant effect on the model ($F(1,105.5) = 10.0$; $p = 0.002$).

For amplitude, there was a significant effect of time period (Fig. 2; $F(9,64.3) = 20.8$; $p < 0.0001$), with all periods except Night 1 ($p = 0.69$) and Day 2 ($p = 0.06$) being significantly different than Day 1 (all $p < 0.001$). There was a significant interaction between treatment and event ($F(9,64.3) = 6.0$; $p < 0.0001$), with all time periods being significantly different from Day 1 (Night 1: $p = 0.045$, Day 2: $p = 0.002$, all others $p < 0.001$). There were no significant differences between treatments ($F(1,13.9) = 4.3$; $p = 0.057$).

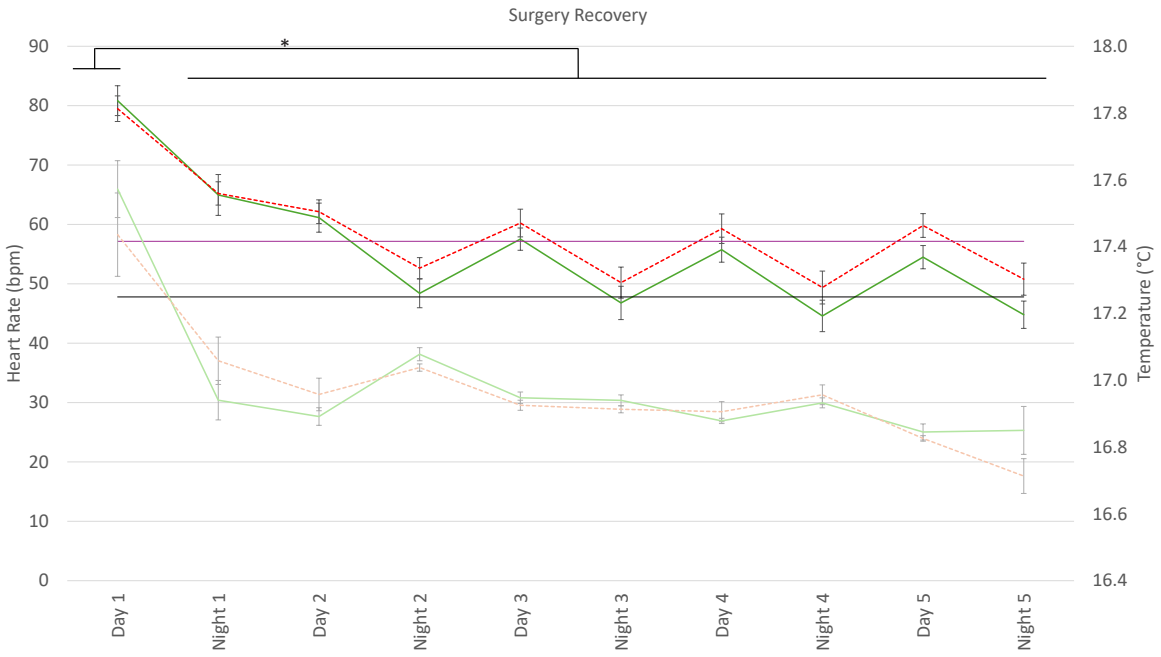

Fig. 1. Mean heart rate (± standard error of mean) in beats per minute (bold colours) and mean internal temperature (± standard error of mean) in ºC (faint colours) for the two treatment groups of gilthead seabream (Sparus aurata): Fish living with environmental enrichment (EE, green straight line), and fish living in bare tanks (NE, red dashed line), during the first five days of data recordings. Baseline heart rate during daytime at 16.8 ºC (blue horizontal line) and during the night at 16.8 ºC (black horizontal line) are placed as reference. Significant differences (p-value < 0.05) are represented with (*).

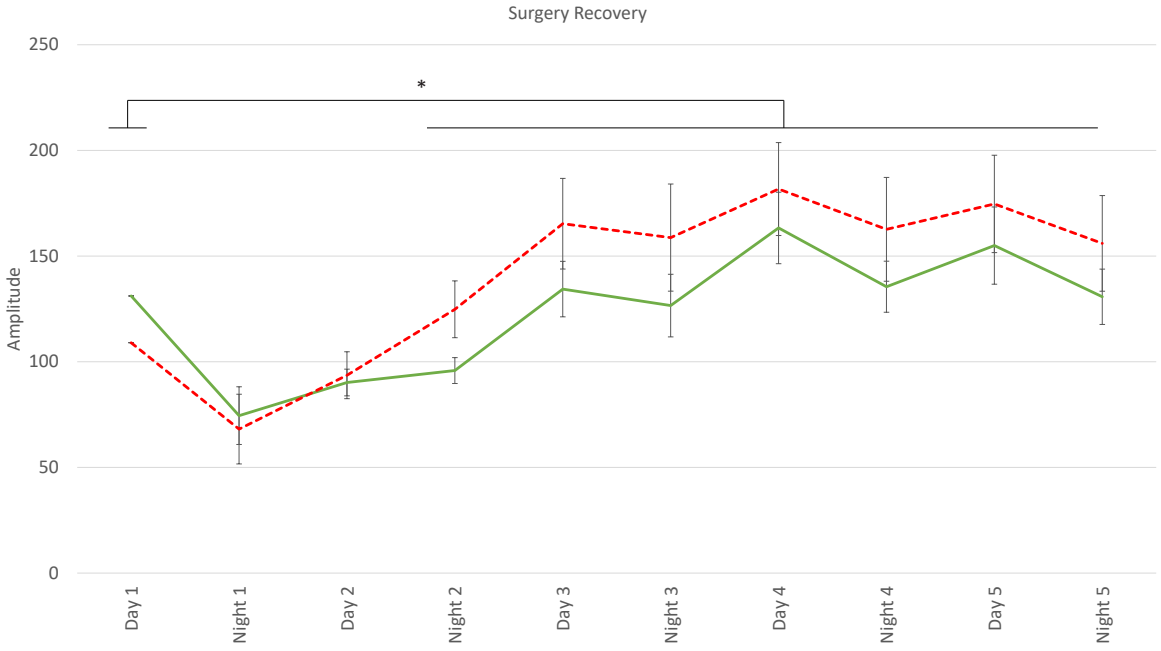

Fig. 2. Mean amplitude (± standard error of mean) (in Arbitrary Unit) for the two treatment groups of gilthead seabream (Sparus aurata): Fish living with environmental enrichment (EE, green straight line), and fish living in bare tanks (NE, red dashed line), during the first five days of data recordings. Significant differences (p-value < 0.05) are represented with (*).

3.2. Husbandry procedures

On the following 4 days, when husbandry procedures took place, there was a significant effect of treatment on heart rate ($F(1,16.1) = 6.14$; $p = 0.025$), with the EE group having a significantly lower HR than the NE group overall. There was also a significant effect of time period ($F(21,186.9) = 41.2$; $p < 0.0001$), with HR being significantly higher in all periods compared to the first morning of this section (morning 6 of the entire study), except on the mornings of days 7 and 9, and the night of day 6, demonstrating that all husbandry procedures increase the heart rate of gilthead seabream. There was also an interaction effect between treatment and time period ($F(21,170.5) = 1.65$;$p = 0.043$), with the EE group having significantly lower HR than the NE group during the netting and cleaning events on days 7 and 8 (Fig. 3; Netting day 7: $t(10.7)$=-3.90, $p = 0.003$, $r = 0.77$; Cleaning day 7: $t(15.6)$=-2.24, $p = 0.040$, $r = 0.49$; Netting day 8: $t(15.4)$=-3.17, $p = 0.006$, $r = 0.63$;

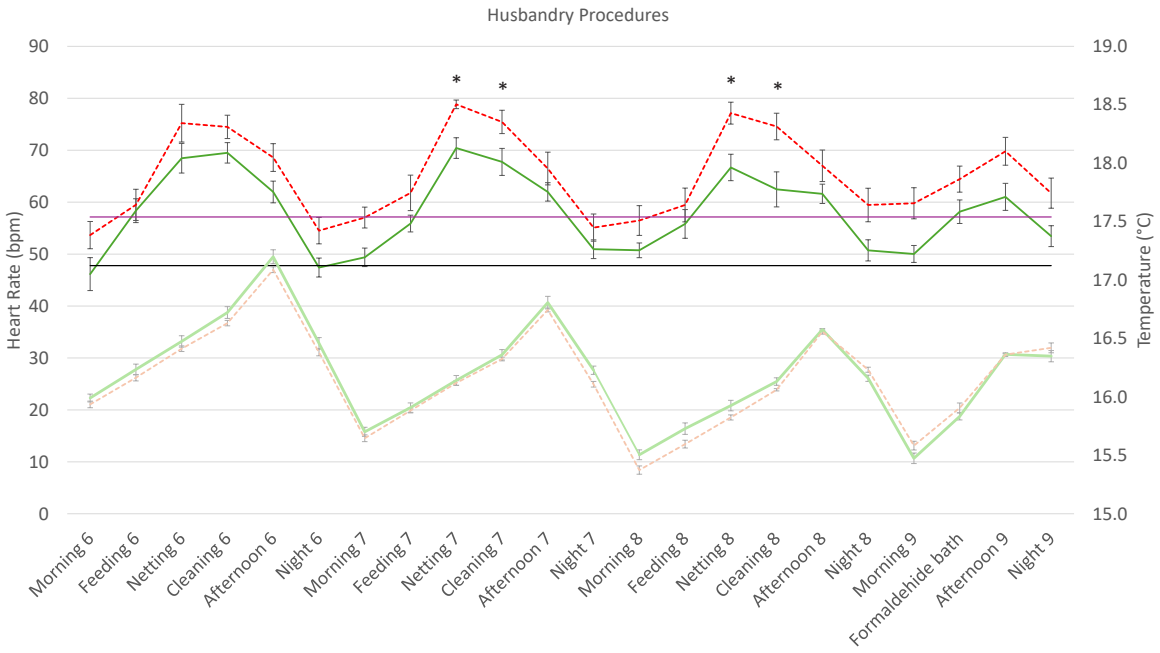

Fig. 3. Mean heart rate (± standard error of mean) in beats per minute (bold colours) and mean internal temperature (± standard error of mean) in ̊C (faint colours) for the two treatment groups of gilthead seabream (Sparus aurata): Fish living with environmental enrichment (EE, green straight line), and fish living in bare tanks (NE, red dashed line), during days 6–9, when husbandry procedures (feeding, netting, cleaning, and a formaldehyde bath) took place. Baseline heart rate during daytime at 16.8 ºC (blue horizontal line) and during the night at 16.8 ºC (black horizontal line) are placed as reference. Significant differences (p-value < 0.05) are represented with (*).

Cleaning day 8: $t(14.9)$=-2.86, p = 0.012, r = 0.60). All other planned comparisons were not statistically significant (p>0.05).

Regarding amplitude, there was a significant effect of time period (Fig. 4; F(21,148.0) = 9.4; p < 0.0001), with all husbandry events having significantly higher amplitude than on Morning 6 (Feeding day 7: p = 0.012; Feeding day 8: p = 0.004; All other husbandry procedures: p < 0.001). Additionally, the amplitude on the afternoons of days 8 and 9 (p = 0.005 and <0.001, respectively) and on the morning and night of day 9 (p = 0.026 and 0.021, respectively) was significantly higher than on Morning 6. There was no significant difference between treatments (F (1,14.0) = 1.6; p = 0.22) and no significant interaction between treatment and event (F(21,148.0) = 1.4; p =0.13).

3.3. Recovery from husbandry procedures

In the 8 days following the husbandry events, we found that HR

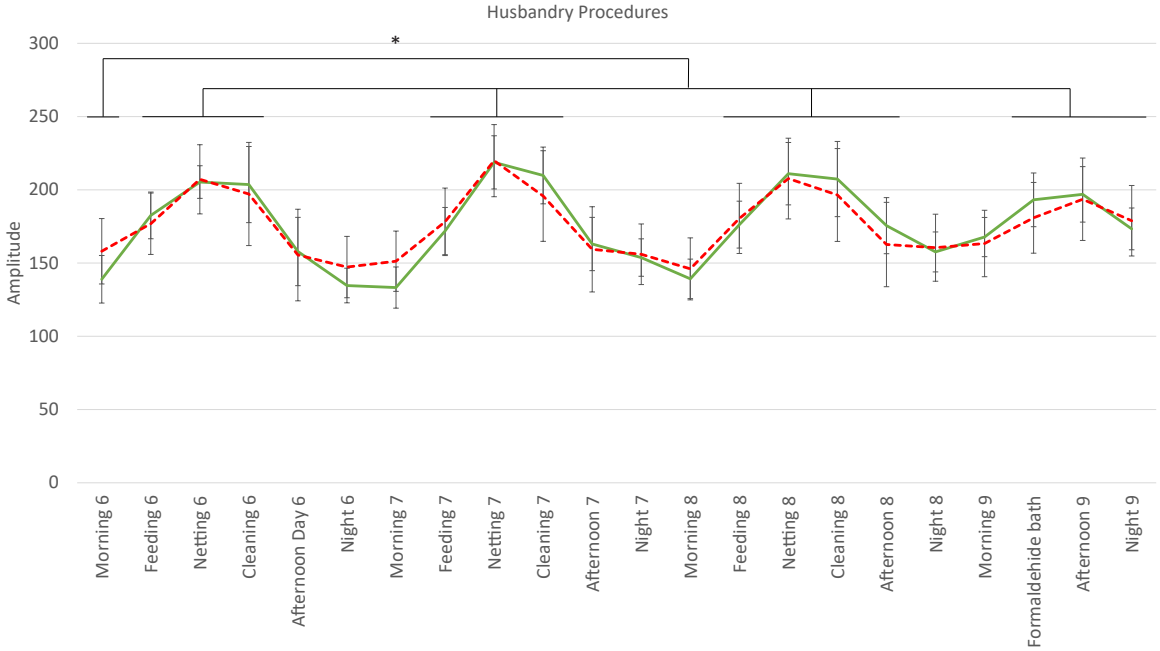

Fig. 4. Mean amplitude (± standard error of mean) (in Arbitrary Unit) for the two treatment groups of gilthead seabream (Sparus aurata): Fish living with environmental enrichment (EE, green straight line), and fish living in bare tanks (NE, red dashed line), during days 6–9, when husbandry procedures (feeding, netting, cleaning, and a formaldehyde bath) took place. Significant differences (p-value < 0.05) are represented with (*).

differed significantly depending on the time period ($F(15,132.7) = 14.3$; $p < 0.0001$), with all periods having a significantly higher HR than the last night of the study, except for day 13 and nights 11–16). There was an interaction effect between treatment and time period ($F(15,123.6) = 2.5$; $p = 0.003$), with the EE group having a significantly lower HR (Fig. 5) on days 10 ($t(144.9) = 2.6$, $p = 0.010$) and 13 ($t(142.4) = 3.0$, $p = 0.004$) and nights 13 ($t(171.4) = 3.5$, $p < 0.001$) and 15 ($t(236.5) = 2.1$, $p = 0.037$). On day 10, (*i.e.*, 24 h after the husbandry procedures) individuals in the NE group had a heart rate above the average day basal level while the EE group had a heart rate below the day basal level (Fig. 3). Heart rate did not fall below the night basal level until night 12 for the EE group, and until night 14 for the NE group (Fig. 5). There was no significant effect of treatment ($F(1,15.9) = 2.9$;$p = 0.11$). Fish body temperature had a significant effect on the model ($F(1,244.8) = 7.0$; $p = 0.008$), which is apparent in Fig. 5, as heart rate decreased as body temperature decreased over time due to lower water temperature.

For amplitude, there was a significant effect of time period (Fig. 6; $F(15,110.9) = 9.4$; $p < 0.0001$), with all husbandry events having significantly greater amplitude than on Night 17 (all $p < 0.016$) except for day 15 onwards (all $p > 0.05$). There was no significant difference between treatments ($F(1,14.0) = 0.3$; $p = 0.61$) and no significant interaction between treatment and event ($F(15,110.9) = 1.2$; $p = 0.25$).

4. Discussion

EE significantly reduced the stressful effect of husbandry procedures on gilthead seabream broodstock. Fish reared with EE had a lower increase of heart rate and amplitude during and after husbandry procedures and the recovery to baseline levels was faster compared to fish living in bare tanks.

Our results also show how much do husbandry procedures increase the heart rate of gilthead seabream. Similarly, adult Atlantic salmon increased their heart rate when water level in their tanks was repeatedly lowered (Svendsen et al., 2021), during a crowding event (Hvas et al., 2020), and during a critical swim speed test (Hvas et al., 2021); adult brown trout (*Salmo trutta*) increased their heart rate when handled with a net (Norling, 2017); and heart rate increases with swimming speed and oxygen consumption in gilthead seabream (Hachim et al., 2021). The mechanism underpinning the increase in heart rate observed during the husbandry procedures in our study can be explained by an increase of both swimming exercise and oxygen demand, especially since netting and cleaning trigger both reactions, and such stressful situations require higher energetic expenditure, as shown in yellowtail kingfish (*Seriola lalandi*) (Palstra et al., 2024). However, husbandry procedures are also known to increase plasma cortisol levels in gilthead seabream 30 minutes after netting stress (Palstra et al., 2020) as a primary stress response. Therefore, the observed increase in heart rate may be a combination of 1) a direct activation of the stress response axis and the ensuing cascade of physiological regulatory mechanisms and 2) possibly a consequence of increased swimming activity and oxygen consumption. Husbandry procedures also increase the amplitude of the heartbeat signal. Fish regulate cardiac output by adjusting stroke volume (*i.e.*, the volume of blood that is pumped out of the heart) to a greater extent than heart rate, unlike amphibians, reptiles, birds, and mammals, that primarily modulate heart rate (Farrell, 1991; Farrell and Jones, 1992). During stressful events such as our experimental procedures, seabream might increase the end-diastolic volume to adjust to the physiological demand of the situation. This increase in volume might have brought the heart closer to the bio-logger, which would have received a stronger signal due to this proximity and therefore recorded a higher amplitude of the signal. However, further research measuring stroke volume in seabream is needed to confirm this explanation.

After the stressors, broodstock gilthead seabream living in EE tanks needed less than 24 hours to reach their baseline heart rate during the day, and two nights to reach their baseline levels at night. In contrast, fish living in non-enriched tanks required at least five nights to reach their basal heart rate levels. The amplitude remained above the control for five days and nights for both treatments. However, the number of days to recover could be longer than we found, given the effect of temperature on seabream heart rate, as heart rate decreases as water temperature drops, which occurred in the latter part of this experiment where water temperature started to drop from the 4th day post husbandry procedures (Fig. 3). Therefore, the reduction in heart rate and amplitude over the course of the days that we see in Figs. 5 and 6 might

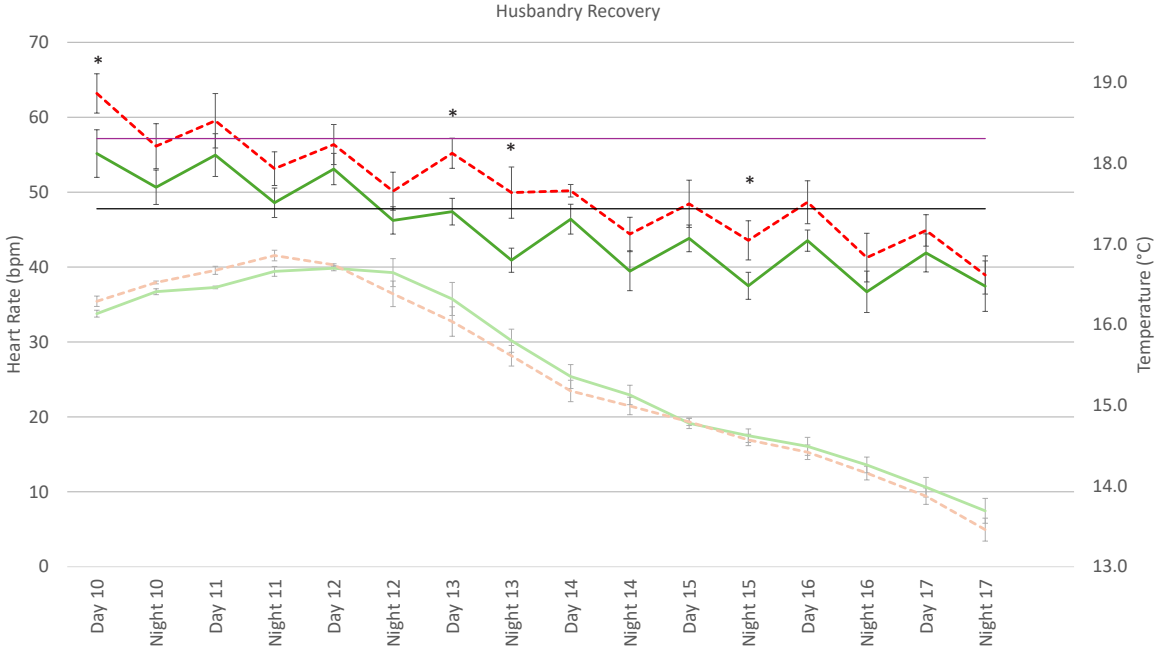

Fig. 5. Mean heart rate (± standard error of mean) in beats per minute (bold colours) and mean internal temperature (± standard error of mean) in ° C (faint colours) for the two treatment groups of gilthead seabream (Sparus aurata): Fish living with environmental enrichment (EE, green straight line), and fish living in bare tanks (NE, red dashed line), during days 10–17, when recovery from husbandry procedures took place. Baseline heart rate during daytime at 16.8 ºC (blue horizontal line) and during the night at 16.8 ºC (black horizontal line) are placed as reference. Significant differences (p-value < 0.05) are represented with (*).

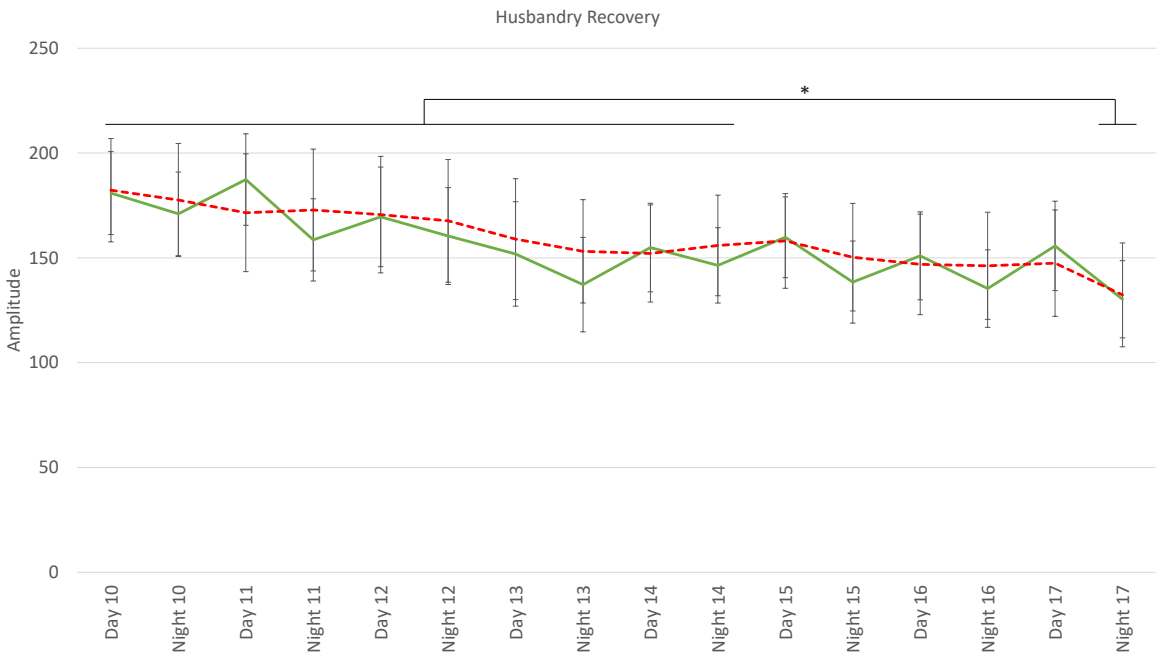

Fig. 6. Mean amplitude (± standard error of mean) (in Arbitrary Unit) for the two treatment groups of gilthead seabream (Sparus aurata): Fish living with environmental enrichment (EE, green straight line), and fish living in bare tanks (NE, red dashed line), during days 10–17, when recovery from husbandry procedures took place. Significant differences (p-value < 0.05) are represented with (*).

have been influenced by the drop in external temperature, and we cannot rule out the possibility that seabream might require a longer period to recover from husbandry procedures, had the ambient temperature remained constant along the whole study. Our results differed from those observed in Atlantic salmon, in which heart rate remained elevated for only 24 h after induction of husbandry stressors (Hvas et al., 2020; Svendsen et al., 2021) and after a critical swim speed test, in which some fish recovered their basal heart rate after 24 h while other fish kept it elevated for four days (Hvas et al., 2021). Heart rate also remained elevated in Atlantic salmon for three days after crowding and vaccination, and for 10 days after grading (Yousaf et al., 2022). These differences in recovery time among species suggest that some species may be better adapted to cope with specific farming stressors than others, regardless of their level of domestication (Huntingford and Kadri, 2008; Saraiva et al., 2019, 2018), but also that different husbandry procedures can cause different levels of stress duration and intensity on fish, depending on how they are carried out.

Regarding biologgers implantation, Atlantic salmon required three weeks to recover from surgery (Yousaf et al., 2022) and four to six days to stabilize heart rate after surgery (Føre et al., 2021). In agreement with our results, Mignucci et al. (2021) found that gilthead seabream stabilized their heart rate three days after surgery. A previous study of heart rate in gilthead seabream using wired electrodes at a similar temperature (16 ºC, *vs.* 16.8 ºC in our study) showed a mean of 91/63 bpm in high/low activity periods respectively in a 12:12 L/D photoperiod (Aissaoui et al., 2000), which was higher than what we recorded in this study (57/48 bpm at high/low activity periods, respectively). Although this difference in basal heart rate could be due to a difference in the body mass of the fish (163 g *vs.* 791 g in our study), it could also be due to different levels of invasiveness of the procedure, with previous methods of ECG recording being more prone to cause incidental stress than the current bio-logger method. Interestingly, Aissaoui et al., (2000) might have misinterpreted the high activity data they observed during the dark period as being due to a normal nocturnal activity of seabream. In fact, what was assumed to be increased activity may well be manifestations of extreme negative valence states (Mendl et al., 2010), which are predicted to occur when animals are unable to cope with environmental challenges, such as the period of darkness during isolation to which the fish in their study were exposed. In general, when interpreting ECG data, it is important to keep in mind that different species cope differently with different types of stressors, so it is necessary to evaluate and take into account the recovery time of each specific species when planning this type of studies, as well as to consider the environmental conditions of the studied fish during the progress of the recordings, as environmental challenges can drastically affect the recording results.

Interestingly, EE had no effect on heart rate during post-surgery recovery of gilthead seabream. This result is similar to that of the study of Pounder et al. (2016) in which EE had no effect on the recovery rate of a group of rainbow trout treated with a painful stimulus. The signal amplitude decreased after surgery, probably as part of a negative "staircase effect" or "force-frequency relationship" in which the force of contraction is compromised after a maintained increase in heart rate (Farrell and Jones, 1992), and then increased reaching the basal level after the second night of the study, which could be related to the wound healing process and the heart rate going back to baseline levels. These results suggest that the intensity of the effects of EE might depend on the intensity of the stimulus. Therefore, additional measures to help fish cope with pain, e.g. administration of analgesic drugs (Sneddon, 2003, 2012; Sneddon et al., 2003), should be explored and considered.

In conclusion, our results show that EE is an effective tool that can be used to improve the welfare of captive fish by reducing their stress, especially considering that some stressors are unavoidable, such as routine husbandry procedures. Therefore, the use of enrichment strategies should be recommended as good management practice in fish experimentation and production, with particular importance for high-value fish that spend long periods in captivity, such as broodstocks. In addition, the use of biologgers on reared fish allows monitoring the physiological response of fish to stress events and can therefore provide relevant information to help improve good practices and management in aquaculture.

CRediT authorship contribution statement

María José Cabrera-Álvarez: Writing – review & editing, Writing – original draft, Visualization, Validation, Software, Resources, Project administration, Methodology, Investigation, Formal analysis, Data

curation, Conceptualization. **Pablo Arechavala-Lopez:** Writing – review & editing, Supervision, Resources, Project administration, Investigation, Funding acquisition, Conceptualization. **Alexandre Mignucci:** Writing – review & editing, Resources, Methodology. **Ana Rita Oliveira:** Resources, Methodology. **Florbela Soares:** Writing – review & editing, Supervision, Resources, Funding acquisition. **João L. Saraiva:** Writing – review & editing, Supervision, Resources, Project administration, Investigation, Funding acquisition, Conceptualization.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgements

This study received Portuguese national funds from the FCT-Foundation for Science and Technology through project UIDB/04326/2020 and doctorate grant UI/BD/151304/2021, and project DIVERSIAQUA II (MAR-02.01.01-FEAMP-0175) co-financed by Opperational Programme Mar 2020 from the Portuguese Government, and the European Union (FEAMP). PA-L was supported by a Ramón y Cajal (Ref. RYC2020-029629-I) postdoctoral grant from the State Research Agency of the Spanish Government. The authors also thank the intensive work of staff and students of the Estação Piloto de Piscicultura de Olhão (EPPO) of Instituto Português do Mar e da Atmosfera (IPMA), in Olhão, Portugal, as well as David J. McKenzie, Jérôme Bourjea, and staff at IFREMER, in Palavas-Les-Flots, France, Asgeir Bjarnason from Star-Oddi for their support, and the anonymous reviewers for their valuable feedback.

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