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Fish behaviour and welfare

Edited by
Pablo Almazan Rueda, Rosario Martínez-Yáñez
and Ana C. Puello Cruz

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Fish behaviour and welfare

Topic editors
Pablo Almazan Rueda — Center for Research in Food and Development, National Council of Science and Technology (CONACYT), Mexico
Rosario Martínez-Yáñez — University of Guanajuato, Mexico
Ana C. Puello Cruz — Center for Research in Food and Development, National Council of Science and Technology (CONACYT), Mexico

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EDITED AND REVIEWED BY
Laura Ann Boyle,
Teagasc Food Research Centre, Ireland

*CORRESPONDENCE
Pablo Almazán-Rueda
almazan@ciad.mx
Ana C. Puello-Cruz
puello@ciad.mx

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Editorial: Fish behaviour and welfare

Pablo Almazán-Rueda[1]*, Ana C. Puello-Cruz[1]* and Rosario Martínez-Yáñez[2]

[1]Centro de Investigación en Alimentación y Desarrollo, AC, Mazatlan, Mexico, [2]Departamento de Veterinaria y Zootecnia, Universidad de Guanajuato, Irapuato, Mexico

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Editorial on the Research Topic
Fish behaviour and welfare

World fish production from both aquaculture and fisheries increased in 2022, with a record number of around 223.2 million tons, from which 185.4 million tons consist of aquatic animals and 37.8 million tons are algae (1). Fish culture is an important source for many households in different parts of the world. One of its principal objectives is to effectively apply dry feed (pelleted feed) for promoting fish growth. In 2022, global aquaculture surpassed the 130.9 million tons, of which 94.4 million tons were aquatic animals, 51 percent of the total aquatic animal production. The principal source of fish, as one could see, is aquaculture. Number of fish farms has increased considerably so that the demand of higher population and the need for water related products are met. This has resulted in public concern on how to produce aquaculture products for human consumption, principally on the area of fish welfare. Fish (food) can be produced in an environmentally correct way without causing undue suffering to cultivated organisms. Captivity conditions must contribute to the health and welfare of each animal. Fish must be cared for by trained and experienced personnel, including providing veterinary care, and researchers must be strictly qualified and have sufficient experience and training before undertaking animal research (2). Different approach to different welfare topics of different species are presented in this book. All the different topics have in common the wellbeing of the fish that is being cultured for our benefit: Fish life cycle; from egg to slaughter: monitoring the welfare of Nile tilapia, Oreochromis niloticus, throughout its entire life cycle in aquaculture (Pedrazzani et al.). Welfare indicators under farm conditions: welfare indicators in Tilapia: an Epidemiological Approach (Flores-García et al.), and Qualitative Behavioral Assessments, a welfare indicator for farmed Atlantic Salmon (Salmo salar) in response to a stressful challenge (Wiese et al.). Reproduction: Use of male-to-female sex reversal as a welfare scoring system in the protandrous farmed gilthead sea bream (Spaurus aurata) (Holhorea et al.). Farm conditions: Experimental study on the effect of sound stimulation on hearing and behavior of juvenile black rockfish (*Sebastes schleglii*) (Wang et al.); The effects of aerator noise on the swimming, feeding, and growth of *Micropterus salmoides* (Zhang et al.); and Behavior analysis of juvenile steelhead trout under blue and red light color conditions based on multiple object tracking (Li et al.). Sedation: Does sedation with AQUI-S® mitigate transport stress and post transport mortality in ballan wrasse (*Labrus bergyltae*)? (Calabrese et al.). Killing methods: Farmed fish welfare during slaughter in Italy: survey on stunning and killing methods and indicators of unconsciousness (Clemente et al.); and Humane slaughter in Mediterranean Sea bass and bream aquaculture: farm characteristics, stakeholder views, and policy implications (van Pelt et al.).

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Welfare Indicators in Tilapia: An Epidemiological Approach

*Luis Flores-García[1], Juan C. Camargo-Castellanos[1], Cristina Pascual-Jímenez[2], Pablo Almazán-Rueda[3], Jorge Francisco Monroy-López[4], Pedro J. Albertos-Alpuche[5] and Rosario Martínez-Yáñez[5]**

[1] Biosciences Doctoral Program, Universidad de Guanajuato, Irapuato, Mexico, [2] Unidad Multidisciplinaria de Docencia e Investigación, Facultad de Ciencias, Universidad Nacional Autónoma de México, Hunucmá, Mexico, [3] Centro de Investigación en Alimentación y Desarrollo, Mazatlán, Mexico, [4] Departamento de Medicina Preventiva y Salud Pública, Facultad de Medicina Veterinaria y Zootecnia, Universidad Nacional Autónoma de México, Mexico City, Mexico, [5] Aquaculture Laboratory, Universidad de Guanajuato, Irapuato, Mexico

Interest and concern about rearing methods and their impact on animal welfare have increased. Production evaluation is population-based, and animal welfare analysis should be similar. In fish, the most common welfare indicators are gill state, fin damage, and body condition. The objective of this study was to evaluate the feeding rate effect on the welfare indicators of *Oreochromis niloticus* using an epidemiological approach. Five growth stages (from 1.2 to 360 g) were studied using four feeding rates as treatments: underfeeding (80%), recommended feeding (100%), and two levels of overfeeding (120% and 140%). The evaluated welfare indicators include the presence of lesions in different body areas and fins, the decrease in body condition index, and their impact on biomass production. Incidence and relative risk were determined for each indicator. Statistically significant associations were found in the indicators of mortality, weight, body condition (K), and presence of evident damage in the caudal and anal fin in all stages. The results showed that the feed rate directly affects the welfare indicators and production. Mortality, weight reduction, K reduction, and caudal and anal fin damage incidence showed to be relevant indicators in all *O. niloticus* growing stages. As a result of this study, the epidemiological approach seems to be a valuable tool for production. A risk traffic light method is a proposal that could have great potential, with the suggested limits for WI's concerning the individuals present in the culture pond, allowing progressive evaluation and decision-making to correct risky situations.

Keywords: welfare indicator, *Oreochromis niloticus*, populational risk, incidence, epidemiological approach

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Fernando O. Mardones, Pontificia Universidad Católica de Chile, Chile

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Hernan A. Cañon-Jones, Universidad de Las Américas, Chile
Sunil Kadri, Universidad Austral de Chile, Chile

***Correspondence:**
Rosario Martínez-Yáñez
ar.martinez@ugto.mx

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INTRODUCTION

The main goal of animal production is to obtain protein for human consumption. As in terrestrial animals, in fish, the diet must consider various factors such as age and growth stage, since incorrect management will cause individuals to have few opportunities to develop correctly, regardless of the species (1). The demand for food is growing as a response to the increase in the human population, as it is estimated that by the year 2050 it will reach around nine billion people (2); this implies an enormous challenge for the primary production sectors, which are increasingly under pressure to satisfy this need. Consumption of aquatic animal protein has increased 15% in the last 10 years; in 2018, aquaculture contributed 82.1 million tons of biomass for human consumption, 54.3 million of these came from fish, tilapia (*Oreochromis spp*.) being one of the most important species for freshwater aquaculture (2). On the other hand, the decrease in water availability means that animal production systems must be substantially modified, to make effective and efficient use of water resources and carry out sustainable production. Recirculating aquaculture systems (RAS) are frequent in semi-intensive and intensive aquaculture, based on a cyclical water movement. They consist of taking the water from a pond, passing it through filters, and returning it to the pond already clean. Such systems mean an enormous advantage in saving water, especially in regions where it is scarce. Physicochemical parameters do not have significant variations and prevent diseases from spreading to the entire production unit (3). This technique has been proposed as a sustainable alternative for the efficient use of water and the environmental impact reduction associated with aquaculture.

The public interest and concern about the raising methods and their impact on the welfare of production animals has increased globally, and fish production is no exception. Welfare is defined as the dynamic state of an individual concerning the biological mechanisms used to adapt positively and successfully to changes in the environment, involving health (4), comfort (5), and the emotional state of the animals (4–6). The farmer must be responsible to provide calmness, comfort, protection, and safety to the farmed animals, during their breeding, maintenance, production, transport, and slaughter (7). Like terrestrial production species, aquatic animals require specific management and growth conditions according to the species and life stage. To provide adequate welfare levels, fish farmers must observe, measure, and control various variables such as water quality, population density, and feeding practices (8). According to Mellor et al. (9), the five domains model for the evaluation of animal welfare describes the sum and interaction of the variables related to survival (1: nutrition, 2: environment, and 3: health) and the situational variables (4: behavior), it directly infers on the mental state of the individuals (domain no. 5), which, in turn, allows qualifying the welfare state of the animals at a given moment.

The behavior and welfare of fish have been the subject of debate for years. In 2002, the United Kingdom implemented laws on the management of salmon farm production to improve the living conditions of the animals, resulting in better-quality products (10). Norway in 2005 started regulating aquaculture production with guidelines like those implemented by the UK (11). Recent research has shown that fish welfare is strongly related to fish physiology, which impacts production and considers animal welfare as a key element for the expression of the full genetic production potential of farmed fish (8, 12, 13). Fish possess homeostatic mechanisms that allow individuals to adapt to their environment, through physiological changes both internal and external (14). To know the welfare state in a fish, welfare indicators (WI's) can be used. These can be determined directly on the animals, such as fins condition body deformations, or indirectly, which are mainly environmental conditions. Once WIs are used as standard on laboratories or farms, they become laboratory welfare indicators or operational welfare indicators (10). Most studies coincide that the best and most widely used WIs are individual-based (or direct) welfare indicators, which can be determined both at the farm and laboratory level (10). The most common of these indicators are operculum beating rate, reflex behavior, gill status, condition factor, fin damage, and body integrity (13, 14). Group based welfare indicators most used are the mortality rate (15), swimming behavior (16), appetite (17), growth rate (18), presence of diseases (10), presence of scales or blood in the water (10), the state of the fins, the integrity of the body, and the body condition (19). Fins are anatomical structures that help in the mobility of the fish, therefore, the integrity of these – mainly the dorsal, lateral, and caudal fins – are indicators of health and welfare (10, 16, 19). In fish, the condition factor (K) is a well-accepted tool for assessing the nutritional status (18), overall quality (20), and feeding management (16, 18). Body condition is variable throughout the lives of fish; thus, it is difficult to define exact values that are indicative of reduced welfare. However, <0.9 is usually indicative of emaciation (21). Feed management in aquaculture farms requires a significant amount of resources, and labor, consequently, represents an important production cost. The feeding rate (amount of feed supplied) is determined in relation to the biomass contained in a fish tank, cage, or pond (16). In the case of tilapia production, as well as in other farmed fish, the feeding is given using standardized feeding tables according to the growth stage (22). Likewise, the companies that manufacture balanced fish feed issue tables of feeding programs, where the suggested handling rate is indicated according to the weight of the organisms and the product. Feeding practices that affect fish welfare also include feeding schedules (23, 24). During production, erroneous management such as underfeeding and overfeeding can occur with negative effects on the welfare of fish, either due to lack of nutrients (25) or deterioration in water quality (25–27).

Epidemiology is the study of disease in populations and of factors that determine its occurrence, the keyword being populations (28). Veterinary epidemiology additionally includes research and assessment of other health-related events, notably productivity (29). All this research involves observing animal populations and making inferences from the observations (29). Epidemiological tools are useful when studying the general status of a certain group, establishing diagnostic criteria to carry out evaluations that allow the prevention, detection, correction, and control of problems, particularly health problems. Epidemiological indicators are calculations used to determine the exposure of a population to a disease or any damage, such as body areas with descaling, hemorrhages, or broken fins (lesion), that is, the probability of the presence of a specific event in a defined time. A cohort study (prospective) is based on the evaluation of the occurrence of an event (in terms of presence/absence) as a result of the follow-up over time of a group, as a consequence of having been exposed or not (comparison groups) to a certain exposure (risk factor) (28, 29). The analysis of the probability of an event occurrence, using epidemiological indicators such as *odds ratio*, to identify risk factors for the presence of bodily injuries and their impact on welfare, has recently been reported in terrestrial animals (30). The incidence (I) represents the number of cases (events) that appear in a population and in each period of time (28). The relative

TABLE 1 | Experimental design: initial values of the number of fish per pond, weight, length, and K.

	Experiments				
	Fingerlings	**Juvenile**	**On-growing 1**	**On-growing 2**	**On-growing 3**
Fish					
n per pond	180	100	100	100	60
Weight (g)[a]	1.26 ± 0.03	9.26 ± 0.19	35.13 ± 0.82	66.89 ± 2.07	144.84 ± 10.14
Length (cm)[a]	3.86 ± 0.31	7.84 ± 0.07	12.00 ± 0.21	14.99 ± 0.22	19.14 ± 0.49
K[1]	2.32 ± 0.53	1.92 ± 0.02	2.04 ± 0.07	1.99 ± 0.04	2.07 ± 0.03
Pond					
Biomass weight (k)[b]	0.22 ± 0.006 2.91%	0.92 ± 0.02 2.07%	3.51 ± 0.08 2.32%	6.68 ± 0.20 3.10%	8.78 ± 0.55 6.32%
Feeding rates treatments, %					
Underfeeding	6.4	4.0	3.2	3.2	2.4
Control*	8.0	5.0	4.0	4.0	3.0
Overfeeding A	9.6	6.0	4.8	4.8	3.6
Overfeeding B	11.2	7.0	5.6	5.6	4.2
Feed					
Characteristics	DM: 95.04 CP: 49.18 GE: 4.51 PS: <0.35	DM: 95.26 PC: 47.66 GE: 4.84 PS: 1.5	DM: 94.21 PC: 44.38 GE: 4.36 PS: 2.4	DM: 94.04 PC: 40.27 GE: 4.14 PS: 3.5	DM: 92.47 PC: 36.51 GE: 3.99 PS: 4.8
Servings a day	6	5	5	5	4
Duration					
Days	24	27	21	29	60

*[a]Values ± SD; [b]Values ± SD, % CV. DM, Dry Matter (%); PC, Protein Crude (%); GE, Gross energy (Kcal/g); PS, Particle Size (mm); balanced specific for the species. *Recommended (22), feeding rate in relation to the pond biomass. Replicates per treatment = 3 ponds.*
Initial biomass per pond, feeding rates treatments (%), feed characteristics and management, and duration (days) of each experiment.

risk (RR) is a measure of the relationship existing between the probability that an event occurs in the exposed group with the same risk factor. The RR is calculated from the cases (events) observed in the group of animals exposed to the risk factor in relation to the cases (events) observed in the group of animals not exposed to the risk factor. It is essential to calculate the corresponding confidence intervals (CIs), which allows for giving greater statistical weight to the calculated RR value. The CI values allow a correct interpretation of the RR result obtained because they approximate to the real value in the population under study since this is inaccessible but it is located inbetween the CI range, with a degree of uncertainty that we can determine (95%) (29, 31). When the RR is equal to or >1.0, then there is a negative effect on the risk factor for the incidence of the event; when it is <1.0, there is no such negative effect on the population (28, 31).

Aquaculture production is generally evaluated considering the population, losing the richness of individual values (8, 13, 17, 32). Therefore, the advantages of analyzing WIs with epidemiological statistical tools could allow measuring the state of a determined group of fish, advancing the knowledge of the animal welfare and the application of WIs in farms or laboratories. Mortality, body condition, damage to the eyes, mouth, opercula, skin, degree of scaling bleeding lesions, and damage such as tears, fraying or bleeding that affects the integrity of the dorsal, lateral, anal, and caudal fins have been part of proposals for the evaluation of welfare indicators in fish (12–14), which have been put into operational practice by applying in *Salmo salar* (10, 33), *Perca fluviatilis* (34), and *Oreochromis niloticus* (35), along with other indicators such as water quality and production rates. Some studies report WIs in the proportion of the damage in an evaluated population (10, 33–35), but in these researches, an analysis is not carried out to determine if the degree of damage registered is considered a situation of population risk, as it would be done in analysis with epidemiological statistical tools. Therefore, the objective of this study was to evaluate the effect of the feeding rate on the welfare indicators of tilapia (*Oreochromis niloticus*) cultivated in recirculating aquaculture systems, using an epidemiological approach.

MATERIALS AND METHODS

Location

The study was carried out at the facilities of the Aquaculture Laboratory of the Veterinary and Zootechnical Department of the Life Sciences Division, Campus Irapuato-Salamanca of the University of Guanajuato, located according to Geo Locator (2021) at 20 ° 44′34.65″N and 101 ° 19′51.78″W, at 1,745 meters above sea level. The study was carried out from April to November 2019.

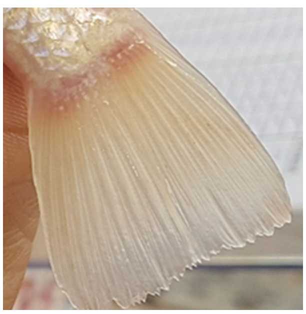
Experimental Systems

Twelve individual and independent experimental conventional aquaculture recirculation systems (RAS) were used ($n = 3$ per treatment), which were located inside a greenhouse and were

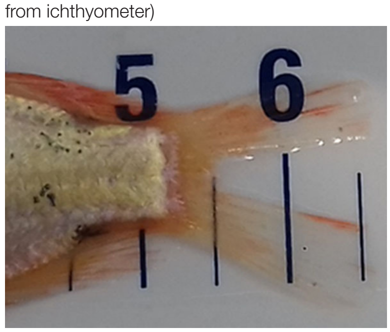
TABLE 2 | Description of the welfare indicator (index description) evaluated.

INCIDENCE	**(nO/nTx)*100**	
Welfare Indicator (Index description)	**Formula application**	**Visual**
Mortality incidence: Number of dead fish	**nO:** no. of individuals from the treatment who died **nTx:** no. of individuals per treatment	Alive | Death 0 | 1
Weight reduction incidence: Number of fish weighing less than the average of the treatments Control	**nO:** no. of individuals in the treatment who presented lower body weight than the average of the control **nTx:** no. of individuals per treatment	0 Higher that / 1 Lower that — Average weight, control treatment **Note:** On farms it is recommended to use control data obtained in successful production cycles
K reduction incidence: Number of fish with lower K than the initial one per pond	**nO:** No. of individuals in the treatment who presented lower K than the initial value **nTx:** no. of individuals per treatment	0 Higher that / 1 Lower that — Initial averange K per pond
Caudal/Anal fin damage incidence: Number of fish with presence of damage to caudal and anal fins	**nO:** No. of treatment individuals who presented damage to the caudal and / or anal fin **nTx:** no. f individuals per treatment	Without damage | Damaged 0 | 1

Caudal fin without damage



Damaged caudal fin (numbers from ichthyometer)



Damaged caudal fin

Anal fin without damage

Hemorrhagic anal fin

Damaged anal fin

built based on the modified design of Timmons and Ebeling (3). At the beginning of each experiment, a total water change, and deep cleaning of the components were carried out. Each system consisted of a 1.5 m^3 fish tank with an integrated filter of four elements with a capacity of 0.2 m^3 each (1 settler-clarifier, 2 physical particle separations with filter material inside, and 1 biological with biospheres). Water lines were 2" hydraulic PVC pipes. The internal movement of water was carried out with a submersible pump (RESUN Model SP3800, Q = 2,000 L/h) placed inside the biological filter. To maintain a constant and suitable temperature for the species, each tank was covered with a dome made of ¼" plastic and PVC, with a small opening. Air was injected at a rate of 40 L/min to each tank and biological filter, using aerating stones connected to a general distribution line to all systems and to a compressor (RESUN GF-750). The RAS were filled to their maximum capacity the same day with water from a

TABLE 3 | Suggested risk limits (%) and color for welfare indicator in relation to the individuals present in the culture pond.

*Welfare indicator %	No risk	Moderate risk	High risk
Fingerlings, from 1 g			
Mortality	<14	14–25	>25
Decreased body weight	<34	34–57	>57
Decreased body condition	<11	11–20	>20
Obvious damage to caudal fin	<6	6–12	>12
Obvious damage to anal fin	<8	8–15	>15
Juveniles, from 10 g			
Mortality	<4	4–5	>5
Decreased body weight	<35	35–60	>60
Decreased body condition	<11	11–21	>21
Obvious damage to caudal fin	<6	6–12	>12
Obvious damage to anal fin	<8	8–14	>14
On-growing 1, from 30 g			
Mortality	<2	2–13	>13
Decreased body weight	<47	47–58	>58
Decreased body condition	<24	24–53	>53
Obvious damage to caudal fin	<11	11–18	>18
Obvious damage to anal fin	<17	17–19	>19
On-growing 2, from 65 g			
Mortality	<2	2–4	>4
Decreased body weight	<50	50–57	>57
Decreased body condition	<34	34–38	>38
Obvious damage to caudal fin	<21	21–30	>30
Obvious damage to anal fin	<15	15–19	>19
On-growing 3, from 130 g			
Mortality	<2	2–7	>7
Decreased body weight	<36	36–66	>66
Decreased body condition	<45	45–53	>53
Obvious damage to caudal fin	<29	29–55	>55
Obvious damage to anal fin	<23	23–72	>72

Welfare Indicator was determined based on the percentage of affected individuals. The ranges of the risk traffic light were determined considering the lowest data (in whole numbers) of % I Tx, according to the stage and the evaluated welfare indicator (Tables 4–8**); if this was too low then the next higher value was used. To consider the lower limit indicator in a situation of moderate risk, the data had to be associated with a RR lower than 0.85 and a present CI in ranges lower than 0.99. As for the upper limit indicator, it was calculated = (% I Tx lower limit * RR constant) / RR, and said RR constant was determined as 0.999.*

single well. The movement and oxygenation of the water began, after 24 hours, adding lyophilized bacteria (AZOO-NitriPro, Nitrosomonas, and Nitrobacter) at a rate of 3 g per 250 L of water, following the protocol described by Espinoza-Moya et al. (36).

Fish and Food

The project was evaluated and approved by the Institutional Committee of Bioethics in Research of the University of Guanajuato (code: CIBIUG-A59-2020). The specimens of *O. niloticus* (males obtained by sexual reversion) were purchased from a commercial farm located in Chupícuaro, Guanajuato. The fish were transported with the farm's water, inside plastic bags with oxygen injection, and placed in a reception tank (quarantine) for their acclimatization (14 days). Five experiments were carried out, in 6 months. Each growth stage of the tilapia (5 stages in total) was considered a separate experiment. The growth stages (experiments) according to the initial weight of the fish were in the following ranges: fingerlings (1.2–1.3 g), juveniles (9.0–9.5 g), on-growing 1 (34–37 g), on-growing 2 (65–70 g) and on-growing 3 (130–150 g). Fish size and density were considered for fish management (37). The experimental design: initial values of the number of fish per pond per experiment (n) and their characteristics (average initial values ± SD of weight, length, and K), initial biomass per pond (average initial weight ± SD and % CV), feeding rates treatments (%), feed characteristics and management (species-specific commercial balance), and duration (days) of each experiment can be seen in **Table 1**.

Data were obtained from a total of 6,480 tilapias. At the beginning and end of each growth stage total fish length was recorded using an ichthyometer (Pentair Aquatic Ecosystems Inc.). Wet live weight (g) was recorded using a digital scale (RHINO, model BAPRE-3). At the end of each experiment, the same measurements were recorded and the same equipment was used to obtain all animal-related data. Due to the high number of specimens used per treatment, metabolism was decreased (lethargy and tranquillization) using cold water to weigh, measure, and check the animals externally. For both initial and end experiment data collection we followed the same pathway. First, we proceeded individually to reduce the individual's metabolism with cold water, making a sudden change in the maintenance temperature to 5°C (where swimming pattern change is visible), this being a recommended method to calm and immobilize fish (38, 39). The data collection and photographs did not exceed 90 seconds for each animal. Immediately after this, tilapias were put in a container with air injection for their recovery, and awakened fish that showed normal respiration and swimming were transported to a community 10,000 L RAS pond (under similar management conditions to the control treatment). It should be noted that the same set of animals did not participate in successive experiments. Four feeding rates were used as treatments: underfeeding (Ufe, 80%), recommended feeding (22) (Control, 100%), and two levels of overfeeding (OfA: 120% and OfB: 140%). Balanced feed samples were taken to determine crude protein (CP) (AOAC) and gross energy (GE) by combustion (IKA Calorimeter System C 2000 Basic). To adjust the amount of feed to be supplied, a sample was taken each week from the animals which were weighed, mortality was also considered for each experimental system.

Production Variables and Water Quality

For each pond, total biomass production (g) was determined as the final harvested weight minus the initial weight. Water quality was measured, at 9:00 am every other day, variables were temperature (°C), dissolved oxygen (mg L^{-1}), electrical conductivity (mS/cm^3), NH_4^+ (mg L^{-1}), and pH, using a multiparameter recorder (YSI Mod. Professional Plus, Cable Quatro). Measurements were carried out directly in the tank in the middle of the water column.

Welfare Indicators (WIs) and Epidemiological Approach

A thorough visual inspection was performed to determine the presence or absence of visible damage to the eyes, mouth, opercula, skin, descaling, bleeding, and dorsal, lateral, anal, and caudal fin damage measured as tears, fraying, or bleeding (16) (without damage: 0, damaged: 1). Mortality (alive: 0, death: 1), weight reduction and body condition index (Fulton's K) were calculated (higher: 0, lower: 1) (**Table 2**).

$$Mortality = no.\ of\ harvested\ fish - no.\ of\ fish\ stocked$$

To determine the number of fish with an increase or decrease in weight or K, at the end of each experiment, per pond per treatment, the average value of control treatment per fish per pond was taken, as follows:

$$Final\ weight\ per\ fish - \bar{x}\ weight\ control\ treatment$$

where the positive values are taken as an increase (0) and the negative values as a decrease (1) in weight per fish (**Table 2**).

$$Fultons\ K = (W/L^3)$$

where, W is the individual wet weight (g), and L is the total length (cm).

To determine the number of fish with an increase or decrease in K at the end of each experiment, per pond per treatment, the average value of initial K per fish per pond was taken (constant of K per pond), as follows:

$$Final\ K\ per\ fish - constant\ of\ K\ per\ pond$$

where the positive values are taken as an increase (0) and the negative values as a decrease (1) in K per fish (**Table 2**).

The following calculations were performed to determine (29) (**Table 2**):

$$Incidence\, Welfare\, Indicator\, in\ the\ treatment\, (I\ Tx) = \left(\frac{nO}{nTx}\right) * 100$$

the incidence of the welfare indicator corresponding to each section according to the treatment, where nO: no. individuals that presented the event and, nTx: no. individuals by treatment; observed treatment individuals presenting the event (0 or 1) accordingly to the corresponding index description.

$$Incidence\ of\ the\ same\ Welfare\ Indicator\ in\ the\ rest\ \ (I) = \left(\frac{\sum\ nO\ other\ \ treatments}{\sum\ nTx\ other\ treatments}\right) * 100$$

$$Relative\ Risk\ (RR) = \left(\frac{\%\ I\ Tx}{\%\ I}\right)$$

The relative risk was taken with 95% confidence intervals (CI). It is important to note that a RR with a value >1.0 indicates that the factor, in this case, the treatment, represents a risk on the WI evaluated, otherwise, a RR <1.0 indicates a NO risk. If the RR value is equal to 1, then the risk is the same between the groups. The CIs allow determining the lower and upper limits where the real RR value is located, therefore, a lower CI with a value >1.0 indicates a risk at a 95% confidence level.

WIs Risk Traffic Light

To facilitate the use and practical application of the results reported in this study, a risk traffic light for WIs was designed (**Table 3**). The ranges of the risk traffic light were determined considering the lowest data of % I Tx, according to the stage and the evaluated welfare indicator (**Tables 4–8**); if this was too low then the next higher value was used. To consider the lower limit indicator in a situation of moderate risk, the data had to be associated with a RR lower than 0.85 and a present CI in ranges lower than 0.99. As for the upper limit indicator, it was calculated = (% I Tx lower limit ∗ RR constant)/RR; the RR constant was determined as 0.999.

Statistical Analysis

To determine the probability of occurrence or not of an event (0 or 1), according to the welfare indicator and index description, being death or, where appropriate, the visible damage observed in the experimental organisms (each dead fish or fish with visible damage is considered an event: nO), the presence/absence data of the welfare indicator were analyzed from an epidemiological approach through a prospective, longitudinal, analytical, experimental study (29). Contingency tables were used to determine the % incidence of the welfare indicators (events) presented in a particular treatment (% I Tx), the % incidence of the welfare indicators presented in the rest of the treatments (% I), the relative risk (RR), and 95% confidence intervals. The independence tests were performed using the Chi-square test (which measures the degree of association or relationship between the treatment and the welfare indicator) (28). The total number of animals per treatment was used to develop the contingency table analysis. A one-way ANOVA was used to analyze the biomass production values, followed by a Duncan test for, bias and kurtosis previously reviewed. To jointly analyze the data of the growth stages, the values were transformed into %, considering the highest value obtained from biomass production as 100%. Statistical program Statgraphics XVI, was used.

RESULTS

Statistically significant associations (Chi-square test, $p < 0.05$) were observed between the treatments and the welfare indicators (WIs) of mortality, weight reduction, K reduction, and presence of evident damage in caudal and anal fins. In the rest of the WIs evaluated, no statistically significant relationships were observed. In all the stages studied, statistically significant associations were observed between the treatments and the five WIs mentioned

TABLE 4 | Contingency table, incidence of mortality, weight reduction, K reduction and caudal and anal fin damage of *O. niloticus* fingerlings according to the feeding rate.

		Records		Epidemiological analysis				
		nO	**(%)**	**% I Tx**	**% I**	**RR**	**(CI 95%)**	***p* value**
Tx	**n Tx**	**Total, *n* = 2,160 (100%)**		**Welfare Indicator**				
		Death fish		**Mortality**				
Ufe	540	112	(5.19)	20.74	25.56	0.81	(0.67–0.97)	0.0240
Control	540	74	(3.43)	13.70	27.90	0.49	(0.39–0.61)	0.0000
OfA	540	130	(6.02)	24.07	24.44	0.98	(0.82–1.17)	ns
OfB	540	210	(9.72)	38.89	19.51	1.99	(1.72–2.30)	0.0000
		Total, *n* = 1,634 (100%)						
		Weight lost cases		**Weight reduction**				
Ufe	428	211	(12.9)	49.30	56.97	0.86	(0.77–0.96)	0.0062
Control	466	160	(9.79)	34.33	63.18	0.54	(0.47–0.62)	0.0000
OfA	410	256	(15.6)	62.44	52.45	1.19	(1.08–1.30)	0.0004
OfB	330	271	(16.6)	82.12	48.05	1.70	(1.58–1.84)	0.0000
		K lost cases		**K reduction**				
Ufe	428	56	(3.43)	13.08	21.56	0.60	(0.46–0.79)	0.0001
Control	466	52	(3.18)	11.16	22.60	0.49	(0.37–0.65)	0.0000
OfA	410	117	(7.16)	28.54	16.26	1.75	(1.43–2.14)	0.0000
OfB	330	91	(5.57)	27.58	17.25	1.59	(1.29–1.97)	0.0000
		Damaged caudal fin cases		**Caudal fin damage**				
Ufe	428	53	(3.24)	12.38	10.20	1.21	(0.89–1.64)	ns
Control	466	28	(1.71)	6.01	12.67	0.47	(0.32–0.69)	0.0001
OfA	410	45	(2.75)	10.98	10.70	1.02	(0.74–1.41)	ns
OfB	330	50	(3.06)	15.15	9.66	1.56	(1.15–2.12)	0.0041
		Damaged anal fin cases		**Anal fin damage**				
Ufe	428	36	(2.20)	8.41	16.00	0.52	(0.37–0.73)	0.0001
Control	466	62	(3.79)	13.30	14.30	0.93	(0.70–1.22)	ns
OfA	410	68	(4.16)	16.59	13.15	1.26	(0.97–1.63)	ns
OfB	330	63	(3.86)	19.09	12.73	1.50	(1.15–1.95)	0.0029

*Tx, treatment; n Tx, no. fish by treatment; nO, no. observed fish who presented the event (cases); %, proportion of individuals who presented the event (cases) in relation to the total n; p-value calculated by means of X^2; I Tx: incidence of the variable corresponding to each section according to the treatment, calculated = (nO / nTx)*100; I: incidence of the same variable in the rest of the individuals, calculated = (sum nO of the other treatments/sum nTx of the other treatments)*100; RR: relative risk, calculated = % I Tx / % I; CI, confidence intervals (95%).*

Treatments: Feeding rate in relation to the pond biomass for underfeeding (Ufe, 80%), control (100%), overfeeding A (OfA, 120%), and overfeeding B (OfB, 140%), respectively.

(**Tables 4–8**). The first section of **Table 4** is described in detail below to facilitate the reading and understanding of the results of this study. Total n = number of fish that were part of the corresponding WI analysis (mortality), which for on-growing 1 was 2,160 fingerlings; n Tx = number of fish per treatment (540 for each); nO = number of observed fish that presented the WI, in this case death, being 112, 74, 130, and 210 dead fish throughout the experimental period, for the Uf, Control, OfA and OfB treatments, respectively; % = number of WIs in relation to Total n (2,160 = 100%), being 5.19, 3.43, 6.02 and 9.72% according to each treatment; *p* value = statistical significance of the Chi-square test analysis; % I Tx = Incidence of the WI in a particular treatment, it is obtained by performing the following operation: (nO / n Tx) ∗ 100 resulting in 20.74, 13.70, 24.07 and 38.89% for the Ufe, Control, OfA and OfB treatments, respectively; % I = Incidence of the WI in the rest of the treatments, therefore, for the treatment of Ufe, the following operation is obtained: (74 + 130 + 210 / 540 + 540 + 540) ∗ 100 = (414 / 1,620) ∗ 100 = 25.56. The RR is the relationship between % I Tx and % I, and RR = 20.74 / 25.56 = 0.81 are obtained for the Ufe treatment, RR = 13.70 / 27.90 = 0.49 for the Control, RR = 24.07 / 24.44 = 0.98 for the treatment OfA, and RR = 38.89 / 19.51 = 1.99 for OfB, respectively. In the following sections of **Table 4**, which correspond to the rest of the WIs evaluated (weight reduction, K reduction, damage to caudal and anal fins), Total n is the number of fish that survived at the end of the experiment, in this case, and accordingly to the calculation made corresponding to the number of surviving fish (1,634 fish), distributed in the treatments (428, 466, 410, and 330, respectively).

In fingerlings, a RR of 1.99 for the WI mortality is observed in the OfB treatment, this means that the probability of observing dead fish under this risk condition is 1.99 times greater than the mortality probability, in the rest of the treatments (**Table 4**). Compared to the Control treatment, where a lower RR was

TABLE 5 | Contingency table, incidence of mortality, weight reduction, K reduction, and caudal and anal fin damage of *O. niloticus* juvenile according to the feeding rate.

		Records		Epidemiological analysis				
		nO	(%)	% I Tx	% I	RR	(CI 95%)	*p* value
Tx	n Tx	Total, $n = 1,200$ (100%)		Welfare Indicator				
		Death fish		Mortality				
Ufe	300	8	(0.67)	2.67	4.11	0.64	(0.30–1.37)	ns
Control	300	8	(0.67)	2.67	4.11	0.64	(0.30–1.37)	ns
OfA	300	11	(0.92)	3.67	3.78	0.97	(0.49–1.89)	ns
OfB	300	18	(1.50)	6.00	3.00	2.00	(1.11–3.57)	0.0179
		Total, $n = 1,155$ (100%)						
		Weight lost cases		Weight reduction				
Ufe	292	251	(21.7)	85.96	48.09	1.78	(1.64–1.94)	0.0000
Control	292	140	(12.1)	47.95	60.95	0.78	(0.69–0.89)	0.0001
OfA	289	100	(8.66)	34.60	65.36	0.52	(0.44–0.62)	0.0000
OfB	282	175	(15.1)	62.06	56.24	1.10	(0.99–1.22)	ns
		K lost cases		K reduction				
Ufe	292	83	(7.19)	28.42	17.15	1.65	(1.31–2.09)	0.0000
Control	292	37	(3.20)	12.67	22.48	0.56	(0.40–0.78)	0.0003
OfA	289	33	(2.86)	11.42	22.86	0.49	(0.35–0.70)	0.0000
OfB	282	78	(6.75)	27.66	17.53	1.57	(1.24–2.00)	0.0002
		Damaged caudal fin cases		Caudal fin damage				
Ufe	292	34	(2.94)	11.64	10.78	1.08	(0.74–1.56)	ns
Control	292	17	(1.47)	5.82	12.75	0.45	(0.27–0.74)	0.0011
OfA	289	36	(3.12)	12.46	10.51	1.18	(0.82–1.70)	ns
OfB	282	40	(3.46)	14.18	9.97	1.42	(1.00–2.01)	0.0490
		Damaged anal fin cases		Anal fin damage				
Ufe	292	24	(2.08)	8.22	15.76	0.52	(0.34–0.78)	0.0013
Control	292	38	(3.29)	13.01	14.14	0.92	(0.65–1.29)	ns
OfA	289	46	(3.98)	15.92	13.16	1.20	(0.88–1.65)	ns
OfB	282	52	(4.50)	18.44	12.37	1.49	(1.10–2.01)	0.0103

Tx, treatment; n Tx, no. fish by treatment; nO, no. observed fish who presented the event (cases); %, proportion of individuals who presented the event (cases) in relation to the total n; p-value calculated by means of X^2*; I Tx, incidence of the variable corresponding to each section according to the treatment, calculated = (nO / nTx)*100; I, incidence of the same variable in the rest of the individuals, calculated = (sum nO of the other treatments/sum nTx of the other treatments)*100; RR: relative risk, calculated = % I Tx / % I; CI, confidence intervals (95%).*

Treatments: Feeding rate in relation to the pond biomass for underfeeding (Ufe, 80%), control (100%), overfeeding A (OfA, 120%), and overfeeding B (OfB, 140%), respectively.

observed, the probability of death is 0.49 times the probability of observing dead fish in the other treatments. This means that the treatment factor OfB represents a mortality risk of practically four times, compared to the mortality risk of the Control treatment. With the WI of weight reduction, a RR of 0.54 was observed in Control, and in the OfB of 1.70, which means that there is 3.49 times more risk that the fish lose weight than with the control treatment. In the WI of K reduction, the OfA treatment obtained a RR of 1.75, while the Control treatment a RR of 0.49, with which in the OfA treatment it is up to 3.5 times more likely that the fish will see their body condition reduced than in the Control treatment. The WI of caudal fin damage showed that in the OfB treatment the RR was 1.56, while in the control treatment the RR was 0.47, with which in OfB the fish have up to 3.32 times the risk of injury to the caudal fins than in the control treatment. The anal fin damage WI presented that the RR of OfB was 1.50 and the Ufe treatment of 0.52, which indicates that in OfB the fish have a risk of 2.88 more lesions in the anal fin than in the Ufe treatment.

In treatment OfB a RR of 2.0 for the WI mortality is observed for the juvenile stage (**Table 5**), meaning that the probability of observing dead fish in the OfB treatment is 2.0 times greater than the probability of mortality in the rest of the treatments. Compared with the Ufe and control treatments, where a lower RR is observed, the probability of death is 0.64 times the probability of observing dead fish in the other treatments. The OfB treatment factor represents a mortality risk of practically 3.12 more, compared to the mortality risk of the Ufe and control treatments. With the WI of weight reduction, the RR of 0.52 was observed in the OfA, and in the Ufe of 1.78, with which there is 3.42 more risk that the fish lose weight than with the OfA. In the WI of K reduction, the Ufe treatment obtained a RR of 1.65, while the OfA treatment a RR of 0.49, with which in the Ufe treatment it is up to 3.36 more likely that the fish will see their

TABLE 6 | Contingency table, incidence of mortality, weight reduction, K reduction, and caudal and anal fin damage of on-growing 1 of *O. niloticus* according to the feeding rate.

Records				Epidemiological analysis				
		nO	(%)	% I Tx	% I	RR	(CI 95%)	*p* value
Tx	n Tx	Total, *n* = 1,200 (100%)		Welfare Indicator				
		Death fish		Mortality				
Ufe	300	1	(0.08)	0.33	4.78	0.07	(0.01–0.50)	0.0004
Control	300	1	(0.08)	0.33	4.78	0.07	(0.01–0.50)	0.0004
OfA	300	2	(0.17)	0.67	4.67	0.14	(0.03–0.58)	0.0014
OfB	300	40	(3.33)	13.33	0.44	30.0	(10.8–83.1)	0.0000
		Total, *n* = 1,156 (100%)						
		Weight lost cases		Weight reduction				
Ufe	299	246	(21.2)	82.27	50.99	1.61	(1.48–1.75)	0.0000
Control	299	140	(12.1)	46.82	63.36	0.73	(0.64–0.84)	0.0000
OfA	298	147	(12.7)	49.33	62.47	0.79	(0.69–0.89)	0.0001
OfB	260	150	(12.9)	57.69	59.49	0.97	(0.86–1.09)	ns
		K lost cases		K reduction				
Ufe	299	72	(6.23)	24.08	58.5	0.41	(0.33–0.50)	0.0000
Control	299	178	(15.4)	59.53	46.2	1.28	(1.14–1.44)	0.0001
OfA	298	177	(15.3)	59.40	46.2	1.28	(1.14–1.44)	0.0001
OfB	260	147	(12.7)	56.54	47.6	1.18	(1.04–1.34)	0.0117
		Damaged caudal fin cases		Caudal fin damage				
Ufe	299	54	(4.67)	18.06	16.5	1.09	(0.82–1.44)	ns
Control	299	32	(2.77)	10.70	19.1	0.55	(0.39–0.79)	0.0008
OfA	298	56	(4.84)	18.79	16.3	1.15	(0.87–1.52)	ns
OfB	260	54	(4.67)	20.77	15.8	1.31	(0.98–1.73)	ns
		Damaged anal fin cases		Anal fin damage				
Ufe	299	54	(4.67)	18.06	19.37	0.93	(0.70–1.23)	ns
Control	299	65	(5.62)	21.74	18.09	1.20	(0.92–1.55)	ns
OfA	298	56	(4.84)	18.79	19.11	0.98	(0.74–1.29)	ns
OfB	260	45	(3.89)	17.31	19.53	0.88	(0.65–1.19)	ns

*Tx, treatment; n Tx, no. fish by treatment; nO, no. observed fish who presented the event (cases); %, proportion of individuals who presented the event (cases) in relation to the total n; p-value calculated by means of X^2; I Tx, incidence of the variable corresponding to each section according to the treatment, calculated = (nO / nTx)*100; I, incidence of the same variable in the rest of the individuals, calculated = (sum nO of the other treatments/sum nTx of the other treatments)*100; RR: relative risk, calculated = % I Tx / % I; CI, confidence intervals (95%).*

Treatments: Feeding rate in relation to the pond biomass for underfeeding (Ufe, 80%), control (100%), overfeeding A (OfA, 120%), and overfeeding B (OfB, 140%), respectively.

body condition reduced than in the OfA treatment. The WI of caudal fin damage showed that in the OfB treatment a RR of 1.42, while in the Control treatment the RR was 0.45, with which in OfB the fish have up to 3.32 times more risk of injury to the caudal fin than in Control. Anal fin damage WI in the OfB treatment presented a RR of 1.49 and in Ufe of 0.52, which indicates that fish in OfB conditions have a 2.88 higher risk of lesions in the anal fin than in Ufe.

For on-growing 1 (**Table 6**), a RR of 30 for WI mortality is observed in the OfB treatment, this means that the probability of observing dead fish for the OfB treatment is 30 times greater than the probability of mortality in the rest of the treatments. Compared with the Ufe and control treatments, where a lower RR is observed, the probability of death is 0.07 times the probability of observing dead fish in the other treatments. This means that the OfB treatment factor represents a mortality risk of practically 428.57 times more, compared to the mortality risk of the Ufe and Control treatments. With the WI of weight reduction, the RR of 0.73 was observed in the Control, and in the Ufe it was 1.61, with which there is 2.20 more risk that the fish lose weight than in the Control. In the WI of K reduction, the control and OfA treatments obtained a RR of 1.28, while the Ufe treatment had a RR of 0.41, with which in the Ufe treatment they are up to 3.12 times more likely that the fish will suffer reduced bodily condition than in the OfA treatment. The WI of caudal fin damage showed that in the control treatment a RR of 0.55, with which in Control the fish have up to 3.32 times less risk of injury to the caudal fin than in the rest of the treatments, which did not present differences. Anal fin damage WI did not present significant RR in any treatment.

For on-growing 2 (**Table 7**), we observed a RR of 8.50 for the WI mortality in the OfB treatment meaning that the probability of observing dead fish for the OfB treatment is 8.50 times greater than the probability of mortality in the rest of the treatments,

TABLE 7 | Contingency table, incidence of mortality, weight reduction, K reduction, and caudal and anal fin damage of on-growing 2 of *O. niloticus* according to the feeding rate.

Records				Epidemiological analysis				
		nO	(%)	% I Tx	% I	RR	(CI 95%)	*p* value
Tx	n Tx	Total, *n* = 1,200 (100%)		Welfare Indicator				
		Death fish		Mortality				
Ufe	300	3	(0.25)	1.00	2.22	0.45	(0.13–1.50)	ns
Control	300	3	(0.25)	1.00	2.22	0.45	(0.13–1.50)	ns
OfA	300	0	(0.00)	0.00	2.56	0.00	n/a	0.0052
OfB	300	17	(1.42)	5.67	0.67	8.50	(3.38–21.8)	0.0000
		Total, *n* = 1,177 (100%)						
		Weight lost cases		Weight reduction				
Ufe	297	175	(14.8)	58.94	60.00	0.98	(0.88–1.09)	ns
Control	297	149	(12.6)	50.17	62.95	0.79	(0.70–0.90)	0.0001
OfA	300	198	(16.8)	66.00	57.58	1.14	(1.03–1.26)	0.0103
OfB	283	181	(15.3)	63.96	58.39	1.09	(0.98–1.21)	ns
		K lost cases		K reduction				
Ufe	297	135	(11.4)	45.45	38.0	1.19	(1.02–1.38)	0.0246
Control	297	102	(8.67)	34.34	41.8	0.82	(0.68–0.97)	0.0229
OfA	300	117	(9.94)	39.00	40.2	0.96	(0.82–1.14)	ns
OfB	283	116	(9.86)	40.99	36.6	1.03	(0.88–1.21)	ns
		Damaged caudal fin cases		Caudal fin damage				
Ufe	297	113	(9.60)	38.05	27.0	1.40	(1.17–1.68)	0.0003
Control	297	91	(7.73)	30.64	29.5	1.03	(0.84–1.26)	ns
OfA	300	62	(5.27)	20.67	32.9	0.62	(0.49–0.79)	0.0001
OfB	283	85	(7.22)	30.04	29.7	1.00	(0.82–1.23)	ns
		Damaged anal fin cases		Anal fin damage				
Ufe	297	46	(3.91)	15.49	20.8	0.74	(0.55–1.00)	0.0457
Control	297	82	(6.97)	27.61	16.7	1.65	(1.30–2.09)	0.0000
OfA	300	49	(4.16)	16.33	20.5	0.79	(0.59–1.06)	ns
OfB	283	52	(4.42)	18.37	19.8	0.92	(0.70–1.22)	ns

*Tx, treatment; n Tx, no. fish by treatment; nO, no. observed fish who presented the event (cases); %, proportion of individuals who presented the event (cases) in relation to the total n; p-value calculated by means of X^2; I Tx, incidence of the variable corresponding to each section according to the treatment, calculated = (nO / nTx)*100; I, incidence of the same variable in the rest of the individuals, calculated = (sum nO of the other treatments/sum nTx of the other treatments)*100; RR: relative risk, calculated = % I Tx / % I; CI, confidence intervals (95%).*

Treatments: Feeding rate in relation to the pond biomass for underfeeding (Ufe, 80%), control (100%), overfeeding A (OfA, 120%), and overfeeding B (OfB, 140%), respectively.

where no significant RR was reported. With the WI of weight reduction, the RR of 0.79 was observed in the Control, and in the OfA of 1.14, with which there is 1.44 more risk that the fish lose weight than in the control. In the WI of K reduction, the Ufe treatment obtained a RR of 1.19, while the control treatment a RR of 0.82, with which in the Ufe treatment they are up to 1.45 more likely that the fish will see their body condition reduced than in the OfA treatment. The WI of caudal fin damage showed that in the Ufe treatment a RR of 1.40, while in the OfA treatment the RR was 0.62, with which in OfB the fish have up to 2.25 times the risk of injury to the caudal fins than in control. Anal fin damage WI showed that the control RR was 1.65 and that of Ufe was 0.74, which indicates that in Control the fish have a risk of 2.22 more of presenting lesions in the anal fin than in Ufe.

For the on-growing 3 (**Table 8**), a RR of 9.27 for WI mortality is observed in the OfB treatment meaning that the probability of observing dead fish for the OfB treatment is 9.27 times greater than the probability of mortality in the rest of the treatments. Compared with the control treatment, where a lower RR is observed, the probability of death is 0.21 times that of observing dead fish in the other treatments. This means that the treatment factor OfB represents a mortality risk of practically 44.1 more, compared to the mortality risk of the control treatment. With the WI of weight reduction, a RR of 0.49 was observed in the Ufe, and the OfA and OfB both observed a 1.46 RR, with which there is 2.97 more risk that the fish lose weight than in the Ufe. In the WI of K reduction, the OfA treatment obtained a RR of 1.48, while the Ufe treatment a RR of 0.76, which means the OfA is up to 1.94 more likely that the fish will see their body condition reduced than in the Ufe treatment. The WI of caudal fin damage showed that in the OfA treatment a RR of 1.39, while in the OfB treatment the RR was 0.48, with which in OfA the fish have up to 2.89 times the risk of tail fin injury than in OfB. Anal fin damage WI showed that the RR of OfB was 0.29, while in the others the

TABLE 8 | Contingency table, incidence of mortality, weight reduction, K reduction, and caudal and anal fin damage of on-growing 3 of *O. niloticus* according to the feeding rate.

Records				Epidemiological analysis				
		nO	(%)	% I Tx	% I	RR	(CI 95%)	*p* value
Tx	n Tx	Total, *n* = 720 (100%)		Welfare Indicator				
		Death fish		Mortality				
Ufe	180	7	(0.97)	3.89	7.04	0.55	(0.25–1.21)	ns
Control	180	3	(0.42)	1.67	7.78	0.21	(0.06–0.68)	0.0034
OfA	180	1	(0.14)	0.56	8.15	0.06	(0.009–0.49)	0.0003
OfB	180	34	(4.72)	18.89	2.04	9.27	(4.79–17.91)	0.0000
		Total, *n* = 675 (100%)						
		Weight lost cases		Weight reduction				
Ufe	173	62	(9.19)	35.84	72.91	0.49	(0.39–0.60)	0.0000
Control	177	95	(14.0)	53.67	66.87	0.80	(0.69–0.93)	0.0017
OfA	179	148	(21.9)	82.68	56.45	1.46	(1.32–1.62)	0.0000
OfB	146	123	(18.2)	84.25	57.66	1.46	(1.32–1.61)	0.0000
		K lost cases		K reduction				
Ufe	173	77	(11.4)	44.51	58.37	0.76	(0.63–0.91)	0.0016
Control	177	87	(12.8)	49.15	56.83	0.86	(0.73–1.02)	ns
OfA	179	129	(19.1)	72.07	48.59	1.48	(1.30–1.68)	0.0000
OfB	146	77	(11.4)	52.74	55.39	0.95	(0.80–1.13)	ns
		Damaged caudal fin cases		Caudal fin damage				
Ufe	173	51	(7.56)	29.48	30.28	0.97	(0.74–1.27)	ns
Control	177	60	(8.89)	33.90	28.71	1.18	(0.92–1.51)	ns
OfA	179	68	(10.0)	37.99	27.22	1.39	(1.10–1.76)	0.0071
OfB	146	24	(3.56)	16.44	33.84	0.48*	(0.33–0.71)	0.0000*
		Damaged anal fin cases		Anal fin damage				
Ufe	173	40	(5.93)	23.12	18.72	1.23	(0.89–1.71)	ns
Control	177	41	(6.07)	23.16	18.67	1.24	(0.89–1.71)	ns
OfA	179	43	(6.37)	24.02	18.35	1.30	(0.95–1.80)	ns
OfB	146	10	(1.48)	6.85	23.44	0.29*	(0.15–0.54)	0.0000*

*Tx, treatment; n Tx, no. fish by treatment; nO, no. observed fish who presented the event (cases); %, proportion of individuals who presented the event (cases) in relation to the total n; p-value calculated by means of X^2; I Tx, incidence of the variable corresponding to each section according to the treatment, calculated = (nO / nTx)*100; I, incidence of the same variable in the rest of the individuals, calculated = (sum nO of the other treatments/sum nTx of the other treatments)*100; RR: relative risk, calculated = % I Tx / % I; CI, confidence intervals (95%). *False positive, as an effect of the high mortality that occurred in said treatment.*

Treatments: Feeding rate in relation to the pond biomass for underfeeding (Ufe, 80%), control (100%), overfeeding A (OfA, 120%), and overfeeding B (OfB, 140%), respectively.

RR was the same. It should be noted that in these last two WIs where OfB came out with a lower RR than the other treatments, it was due to the poor quality of the water, which affected the fish behavior.

The registered water quality values during the study are in **Table 9**. The biomass production data per pond concerning the treatments are in **Figure 1**. In the fingerling stage, we observed highly significant differences between the treatments ($p < 0.01$). The Control treatment showed the highest value (95.47%), followed by Ufe (79.90%), and the lowest value in OfB (40.33%). In the juvenile phase, there were significant differences between feeding rates ($p < 0.05$). OfA being the maximum value recorded (94.03%) after the Control treatment (83.63%), the lowest biomass production was observed in OfB. In the group of fish with on-growing 1, significant statistical differences were found between the treatments ($p < 0.05$), with OfA where the highest production occurred and the lowest in the overfeeding treatments (OfA and OfB). In on-growing 2, highly significant differences were observed between treatments ($p < 0.01$). In the control, Ufe and OfA, the highest values were observed (88.50, 82.33, and 80.33%, respectively), and the lowest value was in the OfB treatment (45.60%). Regarding on-growing 3 phase, the ANOVA analysis did not show significant statistical differences between the treatments, however, in **Figure 1** it can be observed that the highest productions were recorded in the Ufe and control treatments, and the lowest in OfA and OfB. In the lower part of **Figure 1**, the application of the proposed epidemiological traffic light and the qualification of welfare by treatment according to the evaluated indicators can be observed. To determine the degree of welfare, the color of the traffic light was categorized as follows:

Green = 2 points
Yellow = 1 point
Red = 0 points

TABLE 9 | Water quality values during the study.

Treatments	Underfeeding	Control	Overfeeding A	Overfeeding B
Temperature (°C)				
Fingerlings	26.4 ± 1.8	26.2 ± 2.1	26.4 ± 1.8	28.7 ± 2.1
Juveniles	25.3 ± 1.5	25.0 ± 1.8	25.2 ± 1.4	25.6 ± 1.6
On-growing 1	24.9 ± 1.2	24.5 ± 1.7	24.6 ± 1.3	25.1 ± 1.6
On-growing 2	26.7 ± 1.5	25.3 ± 1.8	25.4 ± 1.4	25.8 ± 2.2
On-growing 3	23.6 ± 1.6	23.2 ± 1.6	23.4 ± 1.4	23.1 ± 2.1
Disolved Oxigen (mg L^{-1})				
Fingerlings	5.5 ± 0.5	4.9 ± 0.6	4.7 ± 0.7	3.5 ± 0.6
Juveniles	5.4 ± 0.6	4.9 ± 0.7	4.7 ± 0.8	3.3 ± 1.1
On-growing 1	5.8 ± 0.8	5.2 ± 0.8	5.1 ± 0.7	3.7 ± 0.9
On-growing 2	5.5 ± 0.6	4.9 ± 0.6	4.7 ± 0.8	3.5 ± 0.9
On-growing 3	5.7 ± 0.8	5.4 ± 0.9	4.6 ± 1.0	3.7 ± 1.0
Electrical Conductivity (mS/cm^3)				
Fingerlings	699.3 ± 31.2	725.6 ± 39.5	863.4 ± 44.7	896.7 ± 50.8
Juveniles	792.4 ± 39.2	790.5 ± 46.0	815.9 ± 45.6	813.3 ± 48.2
On-growing 1	777.0 ± 37.9	760.9 ± 107.9	791.2 ± 44.7	808.2 ± 52.2
On-growing 2	919.7 ± 137.2	952.6 ± 153.9	978.0 ± 182.4	1070.9 ± 198.1
On-growing 3	947.9 ± 221.0	943.1 ± 209.8	921.7 ± 198.4	951.7 ± 235.8
NH_4^+ (mg L^{-1})				
Fingerlings	0.1 ± 0.1	0.3 ± 0.2	0.3 ± 0.2	0.7 ± 0.1
Juveniles	0.3 ± 0.2	0.4 ± 0.4	0.8 ± 0.6	0.9 ± 0.3
On-growing 1	0.5 ± 0.1	0.7 ± 0.2	0.8 ± 0.7	0.9 ± 0.1
On-growing 2	0.5 ± 0.1	0.6 ± 0.4	0.8 ± 0.4	0.9 ± 0.3
On-growing 3	0.2 ± 0.1	0.2 ± 0.2	0.3 ± 0.2	0.3 ± 0.2
pH				
Fingerlings	7.2 ± 0.1	7.3 ± 0.1	7.1 ± 0.2	7.5 ± 0.2
Juveniles	7.5 ± 0.2	7.3 ± 0.2	7.3 ± 0.1	7.3 ± 0.2
On-growing 1	7.3 ± 0.1	7.2 ± 0.1	7.2 ± 0.1	7.2 ± 0.1
On-growing 2	7.3 ± 0.1	7.2 ± 0.1	7.3 ± 0.1	7.3 ± 0.1
On-growing 3	7.4 ± 0.1	7.3 ± 0.1	7.3 ± 0.1	7.3 ± 0.1

Means ± SD.

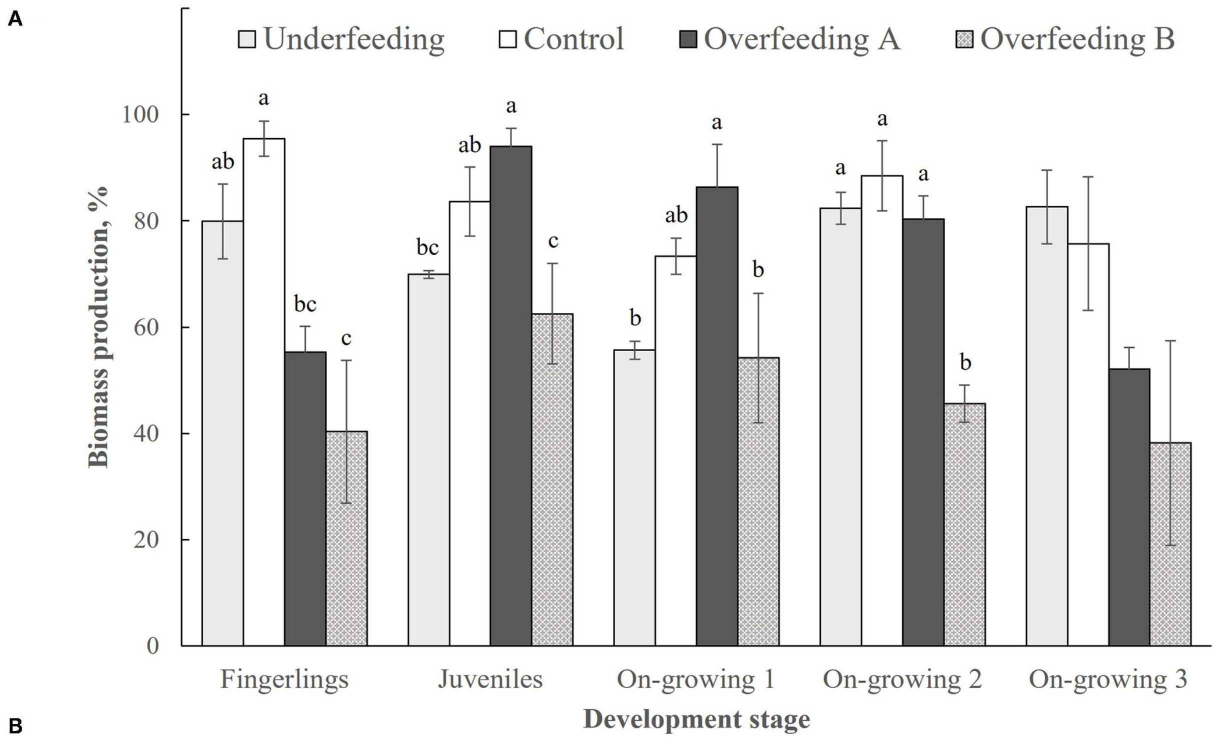
Subsequently, the value of the evaluated indicators was added to obtain a comprehensive qualification (fingerlings to on growing 3) of the welfare state according to the following:

10 points = Excellent (EX)
8 and 9 points = Very Good (VG)
6 and 7 points = Well (WE)
4 and 5 points = Regular (RE)
≤ 3 points = Poor (PO)

DISCUSSION

To the authors' knowledge, this is the first report on the analysis of welfare indicators (WIs) using an epidemiological population approach tools at various stages of the tilapia life cycle. To determine if the production conditions that arise because of feed management, in particular the feeding rate used, can be a risk factor on the welfare status of a fish population in a defined time. Fish welfare is an issue that concerns fish farmers in daily practice, whose objective is to be able to produce organisms properly and follow the correct guidelines for growing this species (8, 14), carrying out responsible and sustainable management. Currently, the consumer is willing to pay an additional price for fish products from farms that are identified with quality and welfare standards (40), therefore, it is important to identify according to the species, which WI's can be used as a standard on laboratories and farms, that is, as laboratory welfare indicators or operational welfare indicators (10).

In the fingerling stage, the water quality indicators were observed within the optimal ranges for tilapia cultivation, except NH_4^+ in the OfB that already presented levels higher than those recommended for the species (41, 42). In the juvenile stages, on-growing 1 and on-growing 2, NH_4^+ was recorded above the optimal values lower than 0.70 mg L^{-1} (41, 42) in the OfA and OfB treatments and the control treatment in the on-growing 1. In on-growing 3, all the water indicators were optimal. High feeding rates rapidly affect water quality. As a fish health indicator, this is important, as ponds exceeding the limits for ammonium, nitrates, and nitrites compromise the development and survival of the organisms (1, 43–45). In addition, a higher concentration of



B

Welfare Indicator (Index)	Incidence Results / Risk Limits, (%)																													
	Fingerlings				<	>	Juveniles				<	>	On-growing 1				<	>	On-growing 2				<	>	On-growing 3				<	>
Mortality	21	14	24	39	14	28	3	3	4	6	4	6	0,3	0,3	1	13	2	14	1	1	0,5	6	2	4	4	2	1	19	2	8
Weight reduction	49	34	62	82	34	64	86	48	35	62	35	67	82	47	49	58	47	64	59	50	66	64	50	64	36	54	83	84	36	73
K reduction	13	11	29	28	11	23	28	13	11	28	11	23	24	60	59	57	24	59	45	34	39	41	34	42	45	49	72	53	45	59
Caudal fin damage	12	6	11	15	6	13	12	6	12	14	6	13	18	11	19	21	11	19	38	31	21	30	21	33	29	34	38	16*	29	61
Anal fin damage	8	13	17	19	8	16	8	13	16	18	8	16	18	22	19	17	17	21	15	28	16	18	15	21	23	23	24	7*	23	80

Grades values	2	1	0

C

Welfare Indicator	Welfare status grading																			
Mortality	1	2	1	0	2	2	2	1	2	2	2	1	2	2	2	0	1	2	2	0
Weight reduction	1	2	1	0	0	1	2	1	0	2	1	1	0	2	0	0	2	1	0	0
K reduction	1	2	0	0	0	1	2	0	2	0	1	0	0	2	0	0	2	1	0	1
Caudal fin damage	1	2	1	0	1	2	1	0	1	2	1	0	0	1	2	1	2	1	1	2
Anal fin damage	2	1	1	0	2	1	1	0	1	0	1	2	2	0	1	1	2	2	1	2
Total grade (∑)	**6**	**9**	**4**	**0**	**5**	**7**	**8**	**2**	**6**	**6**	**6**	**4**	**4**	**7**	**5**	**2**	**9**	**7**	**4**	**5**
Qualification	**WE**	**VG**	**RE**	**PO**	**RE**	**WE**	**VG**	**PO**	**WE**	**WE**	**WE**	**RE**	**RE**	**WE**	**RE**	**PO**	**VG**	**WE**	**RE**	**RE**

General welfare status									
10	**EXCELLENT (EX)**	**8 and 9**	**VERY GOOD (VG)**	**6 and 7**	**WELL (WE)**	**4 and 5**	**REGULAR (RE)**	**≤ 3**	**POOR (PO)**

FIGURE 1 | (A) Biomass production per pond per treatment (means ± SE) in the 5 experiments carried out according to the treatments: underfeeding (80%), control (100%), overfeeding A (120%), and overfeeding B (140%). **(B)** Each cell in color is the value resulting from the % I Tx of the epidemiological analysis (integer) according to the welfare indicator of each treatment (from **Tables 4–8**). White cells are risk limits (%) for welfare indicators in relation to the individuals present in the culture pond of each treatment (from **Table 3**; < and >). **(C)** The application of the proposed epidemiological traffic light and the qualification of the welfare state by treatment according to the evaluated indicators. Color traffic lights are categorized as Green (2 points), Yellow (1 point), and Red (0 points). The value of the evaluated indicators added (summative) to obtain a comprehensive/overall qualification (fingerlings to on growing 3) of the welfare state according: Excellent (EX: 10 points), Very Good (VG: 8 and 9 points), Well (WE: 6 and 7 points), Regular (RE: 4 and 5 points) and Poor (PO: ≤ 3 points). *False positive, as an effect of the high mortality occurred in said treatment. Means ± SE.

nitrogenous products in the system can favor higher productivity in the pond, which is usually accompanied by a decrease in dissolved oxygen in the water during the early morning hours (46). According to the results, in fingerlings a greater growth was observed using the control feeding rate (8% with 49% CP), in addition to this, that treatment, it was where the best values of

WIs were observed. It is important to mention that the higher mortality and lower production occurred in the overfeeding treatments, showing that the fingerlings are very sensitive to water conditions (41). Feed management directly impacts the quality of the culture water (47) as can be seen in the results presented here, the concentration of NH_4^+ and dissolved solids in pond water increases as more food to the fish. Contrarily, in the following two stages (juveniles and on-growing 1), the highest biomass obtained was registered when the fish had an overfeeding of 20% greater than that indicated for the species. It should be noted that it is recommended that tilapia diets decrease the concentration of crude protein as the size of the fish increases (48, 49) because young animals need nutrients steadily, the feeding rate is also higher, as well as the frequency of feeding the diet. As the fish grow, the feeding rates and frequencies decrease, since their metabolism is more efficient to take advantage of the nutrients in the feed (20, 50, 51). Although the commercial diets used in the present study, for the juvenile and on-growing 1, are similar in the contribution of protein and energy, the recommended rate (5 and 4%, respectively) (22), could not be covering the nutritional requirements of the species, for which, and as a result of this study, a rate between 5 and 6% (with 47–48% of CP) and 4 and 4.8% is suggested from the point of view of obtaining biomass (with 44–45% CP), respectively. These values are like those studied by El-Dakar et al. (52) in fingerlings and juveniles of hybrids of *O. niloticus* and *O. mossambicus*. In addition, it was in these two growth stages where greatest the values in % of fish with lesions were recorded, in a moderate risk range of welfare indicators evaluated.

In on-growing 2, the best growths were observed in the control treatment, as were the WIs, except in the incidence of lesions in the anal fin, which suggests a change in behavior concerning age. Tilapia is a cichlid, so aggressiveness is part of its normal behavior, and one of the targets of social injuries is the anal fins (53). Social lesions also occur in other types of fish, such as Siluridae, where the objective is to attack the flanks (54). The purpose of these injuries is to warn other members of their species about the hierarchy and occupation of space, not to hurt (53), however, these injuries can be aggravated if the environment of culture is not suitable (55). Regarding on-growing 3, the highest biomass obtained was recorded in the Ufe treatment, indicating a better use of the feed nutrients (20, 50, 51). The survival of tilapia depends on the type of facilities available, the temperature, the feeding, and the age of the culture (42). In RAS, the survival of the fish is greater, since the conditions are more stable than in open-air cultivation (earthen ponds), as there is a lower proportion of incidences due to the action of predators and diseases (1, 56). In this type of system, 0 to 20% mortality has been observed (17, 32), results like those observed in the OfB treatments in the present study.

The decrease in fish weight is one of the main concerns among aquaculturists; it is very difficult to observe growth with the naked eye but, feasible to follow the development of the pond over time, during cultivation, in the unfolds and pond changes. According to Rey et al. (57), the weight and the comparison of this value must be done by pond because each cage or pond behaves differently. They have their environment. The most advisable action is periodacally to monitor the population and consider the average weight of the pond, instead of making comparisons against databases, especially if they are from regions with different conditions. Therefore, using indicators such as the incidents proposed in this study will allow monitoring of the physiological condition and welfare of the organisms during the production cycle. To identify possible higher-risk situations and make timely and consistent decisions with each stage of growth. Body condition (K) is a variable factor and fluctuates throughout the life cycle of the fish. It is difficult to define exact values that are indicative of reduced welfare, but <0.9 is usually indicative of emaciation (21). Body condition in fish has been used as a productive indicator, making it possible to measure fish growth over time. It has also been mentioned that it is an indicator of welfare in salmon (10), and in perch has been compared between manual and automatic feeding methods (34). What is absent in the previous studies is that there is no average initial body condition. So, it is not known how much it increased or decreased over the experiment time, a fact that is obtained in the present study and that was used as a comparative in the final body condition of the individuals. Thus, body condition can be used as a measurable indicator of welfare, particularly if it is based on the premise that fish entering a culture stage are expected to increase or at least maintain their body condition.

In the present study, was determined that the relationship between the incidence of tilapia caudal fin damage arises because of the used feeding rate. When feed is restricted, frequent and significant damage to fins caused by aggression from dominant individuals is observed, in addition to a decrease in body condition (33). It has been reported that feeding management directly affects the integrity of the fins of *Perca fluviatilis* L. (34) and *Salmo salar* (33), depending on the stage of the culture. However, these studies evaluated the relationship between the feeding management factor and the damage caused to the fins but, did not determine whether feeding management is a risk factor related to the proportion of animals that present evident damage to the fins. The fish fins are part of their anatomy since these limbs allow them to move in their environment (58). The fins are prone to damage their integrity can be affected by various elements, both biotic and abiotic, which can be detrimental to welfare. These factors include population density (59, 60), presence of disease (61), abrasions from pond and cage surfaces (62), aggressions among members of the population (63, 64), feeding management (65–67) and poor water quality, which is related to directly to various circumstances such as low dissolved oxygen concentration, and high concentration of NH4+ and dissolved solids (55, 68). One of the most important fins for fish is the caudal fin since they are propelled by this member and allow them to measure the force with which they move (58).

According to a study in salmon (33), where two experimental groups were used: control and with food restriction, it is reported that 7.5 and 12.5% of the population presented damage, for the control and food restriction, respectively. It should be mentioned that, at first glance, it can infer that there is an effect of the treatment on the number of animals that presented damage. Analyzing the reported data using the Chi-square test, there is no statistically significant relationship between each treatment and the number of observed events. In this case, fish with dorsal fin damage (p = 0.4561). It is important to emphasize that the authors mentioned that, in the food restriction treatment, fins with a greater degree of damage were observed than those that were presented in the control treatment. Although in the present study, damage to the dorsal fin was not recorded in the tilapias. According to the risk traffic light proposed here (**Table 8**), fish weighing 66 g (on-growing 2 in tilapia), similar to those in the study by Cañon-Jones et al. (33) (initial weight 61.7 ± 6.4 g), is considered a risky situation when from 15 and 21% of the population presented damage to the anal and caudal fins, respectively, values higher than those observed in salmon (7.5 and 12%), which is probably why the Chi-square test did not show a statistically significant relationship, which is consistent with the present study.

WIs have been determined in various species (10, 15), and are usually specific to the physiological or life stage (15) and the type of culture system used (10). The results obtained in the present study indicate that the 5 experiments allow us to see, as a whole, the effect of the feeding rate in the different growth stages of *O. niloticus*. By integrating the risk limits (%) in a final assessment (Excellent, Very Good, Well, Regular and Poor; **Figure 1**), a congruence is observed in the condition or general welfare status of the fish analyzed through the monitoring of survival, weight, Fulton index and damage to fins (anal and caudal). Although, in all the stages these same indicators were found where statistically significant relationships were observed, the level of risk changes due to the interaction between the growth stage and the feeding treatment. Mortality, for example, reaches a high risk (cells in red **Figure 1**), in the overfeeding treatment B for the phases of fingerlings, and on-growing 2 and 3, and only alert or moderate risk (cells yellow), for the juvenile and on-growing 1 phase. This indicates that, despite being the same species, the fish in the early growth phases behave differently in their development and physiology of the amount, quality, and characteristics of the food provided (20, 25, 47, 50).

Gutierrez-Rabadan et al. (15), established WI's in the lumpfish (*Cyclopterus lumpus*), using two experimental groups: animals that were in the hatchery and those in cages, with 60 and 35 animals, respectively. Of the fish in the first group ($n = 60$), 31 presented caudal fin damage, 32 with pelvic fin damage, and in the second group ($n = 35$), 6 fish with tail fin damage and 6 with fin injuries. Proportionally, it is reported that 52 and 53% of the population present damage to the caudal and pelvic fin respectively. In the hatchery, and 6 and 6% to the caudal and anal fin respectively, when fish are kept in cages. When analyzing these same data using the Chi-square test, we observe that there is a statistically significant relationship between the treatment (hatchery) and the number of observed events (fish with damage to the fins) ($p = 0.0009$ for caudal fin; $p = 0.0005$ for pelvic fin). Fin damage can result from aggression but also stress (60). Also, can cause detrimental effects on growth and survival by increasing susceptibility to opportunistic infections. Although in the tilapias of the present study, damage to the pelvic fins was not recorded. According to the risk traffic light proposed here (**Table 8**), in fish weighing 5 to 152 g (growth stages 2 to 5 in tilapias in this study), like those from Gutierrez-Rabadan et al. (15) (weight range 5–152 g), is considered a risky situation when 12% of the population show damage to the anal and caudal fins, respectively, a lower value than those observed in lumpfish (52 and 53%), which is probably why the Chi-square test showed a statistically significant relationship. When calculating the RR (5.16 for tail fin with CI of 1.87–14.24; 3.11 for pelvic fin with CI of 1.44–6.69), it is observed that culture management directly impacts fish welfare indicators, that is, it is a risk factor to the population.

The integration of the indicators allows qualifying of the general condition of wellbeing obtained in each treatment. Thanks to this analysis tool are possible to observe that the most appropriate feeding protocol depends on the phase of the life cycle of the tilapia. In the present study, the control treatment followed the recommendation proposed for the species (22). Using this regimen, the best growth was obtained in fingerlings and on-growing 2 phases which coincide with the best general welfare state rating. (Very well and well, respectively). However, for the juvenile and on-growing 1 phase, the best biomass production, and general welfare rating were obtained with the overfeeding treatment A (120%), indicating a higher nutritional requirement, particularly protein, in these phases (49). In practice, this form of data visualization and analysis could be useful for making and correcting decisions in crop management and planning an adequate feeding regimen, thanks to the evaluation of the general welfare status of the animals with operational indicators.

In a high-risk productive activity such as aquaculture, increasing efficiency and reducing risks are permanent goals. Usage of operational welfare indicators offers the use of easily obtained biological information. Allowing the identification of "red flags" promptly to avoid breaks in the production cycles. The epidemiological analysis showed its potential application and the methodology to obtain specific alarm values related to the characteristics of each farm (growth stage, feeding amount, the season of the year, etc.). Generating animal welfare programs proposes the opportunity to systematically test the inputs for production (genetic lines, type of feed, tolerance to environmental factors, etc.) and increase efficiency with a focus on constant improvement.

CONCLUSIONS AND ANIMAL WELFARE IMPLICATIONS

To have information that allows people responsible for tilapia production, either on the farm or in experiments to make prompt decisions and evaluations is a must. Mortality incidence, weight reduction, K reduction, and damage to caudal and anal fins could be used as laboratory welfare indicators or operational welfare indicators in the cultivation of *O. niloticus* applying an epidemiological approach. The feeding rate used directly affects production and the welfare indicators, and in a different way depending on the growth phase. As a result of this study, the epidemiological approach seems to be a valuable tool for production. The proposed risk traffic light method could have great potential, with the suggested limits for WI's concerning the individuals present in the culture pond, allowing progressive evaluation and decision-making to correct risky situations that arise.

DATA AVAILABILITY STATEMENT

The datasets presented in this article are not readily available because The dataset is part of ongoing research and is still being used to complete it. Requests to access the datasets should be directed to ar.martinez@ugto.mx.

ETHICS STATEMENT

The animal study was reviewed and approved by Institutional Committee of Bioethics in Research of the University of Guanajuato (code: CIBIUG-A59-2020).

AUTHOR CONTRIBUTIONS

LF-G: investigation, data obtaining, data analysis, and writing—original draft preparation. JC-C: investigation and data collection. CP-J: data analysis, writing, and partial economical support. PA-R: data analysis and writing—review and editing. JM-L: data analysis. PA-A: design, construction of experimental systems, and writing—review and editing. RM-Y: conceptualization, methodology, formal analysis, investigation, writing—original draft preparation, and project administration. All authors contributed to the article and approved the submitted version.

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Use of male-to-female sex reversal as a welfare scoring system in the protandrous farmed gilthead sea bream (*Sparus aurata*)

Paul G. Holhorea[1], Alicia Felip[2], Josep À. Calduch-Giner[1], Juan Manuel Afonso[3] and Jaume Pérez-Sánchez[1]*

[1]Nutrigenomics and Fish Growth Endocrinology Group, Institute of Aquaculture Torre de la Sal, CSIC, Castellón, Spain, [2]Group of Fish Reproductive Physiology, Institute of Aquaculture Torre de la Sal, CSIC, Castellón, Spain, [3]Aquaculture Research Group, Institute of Sustainable Aquaculture and Marine Ecosystems (IU-ECOAQUA), University of Las Palmas de Gran Canaria, Las Palmas, Spain

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EDITED BY
Pablo Almazan Rueda,
Consejo Nacional de Ciencia y Tecnología (CONACYT), Mexico

REVIEWED BY
Jose Fernando Lopez-Olmeda,
University of Murcia, Spain
Li Shuisheng,
Sun Yat-sen University, China

*CORRESPONDENCE
Jaume Pérez-Sánchez
✉ jaime.perez.sanchez@csic.es

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Gilthead sea bream is a highly cultured marine fish throughout the Mediterranean area, but new and strict criteria of welfare are needed to assure that the intensification of production has no negative effects on animal farming. Most welfare indicators are specific to a given phase of the production cycle, but others such as the timing of puberty and/or sex reversal are of retrospective value. This is of particular relevance in the protandrous gilthead sea bream, in which the sex ratio is highly regulated at the nutritional level. Social and environmental factors (e.g., contaminant loads) also alter the sex ratio, but the contribution of the genetic component remains unclear. To assess this complex issue, five gilthead sea bream families representative of slow/intermediate/fast growth were grown out with control or a plant-based diet in a common garden system from early life to the completion of their sexual maturity in 3-year-old fish. The plant-based diet highly enhanced the male-to-female sex reversal. This occurred in parallel with the progressive impairment of growth performance, which was indicative of changes in nutrient requirements as the result of the different energy demands for growth and reproduction through development. The effect of a different nutritional and genetic background on the reproductive performance was also assessed by measurements of circulating levels of sex steroids during the two consecutive spawning seasons, varying plasma levels of 17β-estradiol (E_2) and 11-ketotestosterone (11-KT) with age, gender, diet, and genetic background. Principal component analysis (PCA) of 3-year-old fish displayed a gradual increase of the E_2/11-KT ratio from males to females with the improvement of nutritional/genetic background. Altogether, these results support the use of a reproductive tract scoring system for leading farmed fish toward their optimum welfare condition, contributing to improving the productivity of the current gilthead sea bream livestock.

KEYWORDS

protandrous fish, sex reversal, sex steroids, welfare scoring, plant-based diets, nutrition and genetics interactions

Introduction

Fish farming has evolved as one of the most sustainable production sectors because of its high feed conversion efficiency and its lower carbon footprint when compared with other animal production systems (1). Nonetheless, aquaculture production is becoming more intensified to meet the increased global demand for fish protein aquaculture (2). It is, thereby, important to encompass the development of aquaculture with novel and stricter criteria of welfare for the simultaneous improvement of aquaculture productivity and welfare of farmed fish (3, 4). Certainly, important research efforts are now conducted within the AquaIMPACT H2020 project for integrating information from fish breeding and nutrition to promote the production of healthier and more robust fish with higher phenotypic plasticity to cope with a challenging environment. This includes the use of gut microbiota as a reliable criterion to evaluate the success of selective breeding for improving the performance and competitiveness of European aquaculture (5, 6). At the same time, novel fish feed formulations, and epigenetic and behavioral approaches are widely applied to assure a more ethical and sustainable aquaculture production with the increase of water temperature and hypoxia as major environmental problems in coastal marine ecosystems (7–9). Thus far, fish welfare assessment is still in the infancy state due to the limited understanding of the diverse fish species' welfare-relevant biology (10). However, several benchmarking systems on key performance indicators (KPIs) based on growth performance, survival rates, and external tissue damage (skin/fin erosion) have been currently validated in salmon, but also in Mediterranean fish species, to ensure that farmed fish are not far from their optimum welfare (11–14). Otherwise, behavioral indicators are becoming especially useful for alerting farmers that something is potentially wrong and warrants investigation before significant welfare issues can occur (15, 16). In any case, the best monitoring solution, especially those based on telemetry techniques and bio-loggers for tracking swimming activity and/or heart or breathing rates, highly depends on the asked question, species biology, and culture system (17).

A common feature of welfare indicators is their accuracy for a given time and culture condition, reflecting immediacy rather than a historical background. However, the success of reproductive performance, measured by means of fecundity, puberty onset, and sex reversal, can also have a high value from a retrospective point of view, as it is the end point of a complex cascade of developmental events that encompass a wide range of biotic and abiotic factors (18–22). In particular, the sex ratio in gonochoristic fish tends to be balanced in optimal culture conditions (23), although it can be affected by chemicals (24) and other environmental factors such as rearing density, temperature, pH, oxygen, and diet composition (25–29). Similarly, sex reversal in hermaphrodite species, such as in the protandrous gilthead sea bream, is socially controlled and endocrine-regulated by the circulating levels of estradiol (E_2) and 11-ketotestosterone (11-KT) (30), and intriguingly the exposure to synthetic estrogens (e.g., 17α-ethynylestradiol) prevents the male-to-female sex reversal (31). Stress may also influence the onset of sex change through the mediation of cortisol, although the exact mechanisms in which it may act as a mediator in sex change remain to be fully established (32). Otherwise, puberty onset is determined by genetic factors and controlled by the nutritional status and/or the body's growth (33). Thus, similar to what occurs in humans, better welfare conditions for fish entail an increase in their growth before reaching their first sexual maturation (34, 35). However, early puberty, in particular in males, occurs in several species kept under aquaculture conditions and is often associated with a final growth retardation or health risks (36, 37). Moreover, the age of puberty can be controlled in farmed fish by selective breeding and feeding level (38–40), and a recent gilthead sea bream study stated that plant-based diets have the potential to alter the sex steroid profile during the pre-spawning and spawning period, promoting the enhanced male-to-female sex reversal when the presence of powerful functional females is compromised by the diet (41).

Taking into account all the above findings, we had herein a double objective: (i) to assess how the male-to-female sex reversal is affected by nutrition and genetics in the protandrous gilthead sea bream and (ii) to provide new insights into the use of male-to-female sex reversal and population sex ratio as a reliable best practice framework for animal welfare certification of a highly cultured farmed fish in all the Mediterranean basin. The rationale for this procedure is that the complex balance of environmental variables that regulate animal welfare conditions can also affect sex change in sequential hermaphrodites. To pursue this issue, sex reversal was monitored in fish families with different nutritional backgrounds and different heritable growth within the PROGENSA® selection program (42), which co-selected among other traits with changes in gut microbiota composition and metabolic plasticity (5, 43), as well as swimming performance and aerobic scope (44, 45).

Materials and methods

Diets

Two extruded diets were formulated and produced by BioMar (BioMar Process Innovation Technical Center, Brande, Denmark), at a range of pellet sizes corresponding to the respective fish size as fish grew (i.e., 1.9, 3, 4.5, and 6.5 mm). Both diets were isonitrogenous, isolipidic, and isoenergetic and met all known nutritional requirements of gilthead sea bream. Fish meal (FM) was included at 23% in the control diet (D1) and at 3% in the experimental diet (D2). The addition of fish oil (FO) was 14.1% for D1, and 3.9% for D2 with the replacement of rapeseed oil, decreasing EPA+DHA content from 3.8 to 1.02%. Lysine, methionine, choline, lecithin, and monocalcium phosphate were added to D2 to reach D1 levels (Supplementary Table S1).

Experimental setup and sample collection

Broodstock crossings of eight (two females and six males) and five (three females and two males) fish from the gilthead sea bream PROGENSA® selection program rendered sixteen families with differences in heritable growth, as described elsewhere (42). Briefly, juvenile fish of these families, previously genotyped by DNA fin analysis, were individually tagged (dorsal muscle) with passive integrated transponders (PITs) (ID-100A 1.25 Nano Transponder, Trovan, Madrid, Spain) and maintained in a common garden system fed D1 or D2 diets in replicate 3,000-L tanks under the natural photoperiod and temperature conditions (latitude 40° 5′ N; 0° 10′ E) at the Institute of Aquaculture Torre de la Sal (IATS), over the course of a 12-month feeding trial (September 2017 to September 2018). At this end, five families were selected by their growth trajectories during this period as a representative of fast growth (e5e2, e6e2; 158 and 49 individuals, respectively), intermediate growth (c2c7, e4e1; 91 and 174 individuals, respectively), and slow growth (c4c3; 60 individuals), and were distributed at similar family in the common garden system. Growth performance and reproductive status were assessed in these families until the completion of sexual maturation in 3-year-old fish (December 2020).

Over the course of the entire trial, the concentration of water oxygen was always higher than 80% saturation. Fish were fed by automatic feeders 1–2 times per day and 3–7 days per week according to fish size and season, with the ratio adjusted weekly to a level close to satiation. The final rearing density was 19–20 kg/m^3. Fish body weight and body length were measured individually using an FR-200 FishReader W (Trovan, Madrid, Spain) at different monthly intervals during the first (Age +1), second (Age +2), and third year (Age +3) of the production cycle. At the time of a maximum number of spermiating fish (December), overnight-fasted fish were anesthetized with 100 mg/L MS-222 (Sigma, Saint Louis, MO, USA) for blood extraction and sexing by stripping. It is a non-lethal and accurate sexing method at this time and developmental stage as almost all males are fluent by stripping, in coincidence with the annual peak of E_2 in females and 11-KT in males that resulted in a minimum presence (<5%) of intersex fish (41). Blood was collected (100 fish/diet for Age +2 fish, 150 fish/diet for Age +3 fish) from caudal vessels using heparinized syringes and centrifuged at 3,000 × g for 20 min at 4°C, and plasma aliquots were stored at −20°C until sex steroid analyses.

All procedures were approved by the Ethics and Animal Welfare Committee of IATS and CSIC. They were carried out in the IATS's registered aquaculture infrastructure facility (code ES120330001055) in accordance with the principles published in the European Animal Directive (2010/63/EU) and Spanish Laws (Royal Decree RD53/2013) for the protection of animals used in scientific experiments.

Sex steroids

Quantification of plasma sex steroids was performed by enzyme immunoassays (EIAs) as described by Rodríguez et al. (46) for 11-KT and by Molés et al. (47) for E_2. Briefly, steroids were extracted from 100 µl plasma in 1 ml methanol and supernatants were dried and reconstituted in EIA buffer (0.1 M potassium phosphate, pH 7.4 containing 0.01% sodium azide, 0.4 M NaCl, 0.001 M EDTA, and 0.1% BSA). Steroid standards were purchased from Sigma-Aldrich. Mouse anti-rabbit immunoglobulin monoclonal antibody (Ab), rabbit steroid Abs (T-Ab, 11-KT-Ab, and E_2-Ab), and enzymatic tracers [steroid acetylcholinesterase (AChE) conjugates: T-AchE, 11-KT-AChE, and E_2-AChE] were obtained from Vitro S.A. (Sevilla, Spain). Samples and standard curves of 11-KT (0.0001–1.0 ng/ml) and E_2 (0.005–9.0 ng/ml) were run in duplicate. Optical density was read at 405 nm using a microplate reader (Bio-Rad 3550). The inter-assay coefficients of variation at 50% of binding were 5.02% ($n = 10$) with a 0.88 slope for 11-KT and 5.97% ($n = 10$) with a 0.68 slope for E_2.

Statistical analysis

Statistical analysis was performed using SigmaPlot version 14.0 (Systat Software, San Jose, CA, USA) with all *P*-values set to 0.05 for significance determination. Body weight and sex ratio differences between both dietary groups were assessed by means of the Student's *t*-test. Male and female body weight differences within each age, diet, and family (or grouped families) were determined by means of the Student's *t*-test. One-way ANOVA, followed by a Holm-Sidak *post-hoc* test, was conducted in order to assess significant differences in male-to-female sex steroids (11-KT and E_2) concentration between families of the same diet and age. Sex steroid differences between diets within each family were assessed by means of a Student's *t*-test. For evidencing gradation in sex steroids and body weight between diets, families, and males/females of each family, a principal component analysis (PCA) was performed using EZinfo version 3.0 (Umetrics, Umea, Sweden). Differences in E_2/11-KT quotient between families and diets were assessed

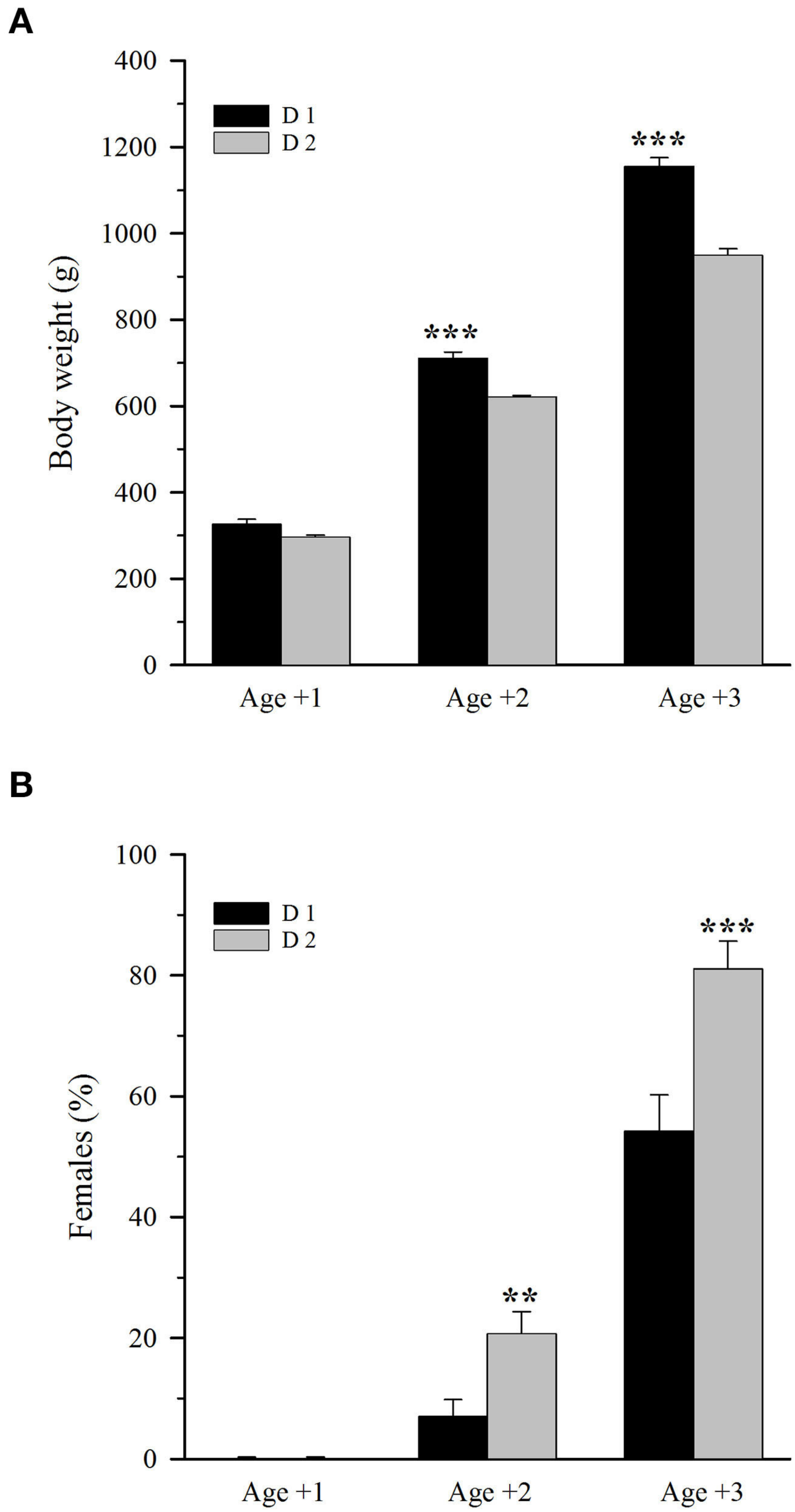

FIGURE 1
(A) Body weight and **(B)** female percentage in 1-, 2-, and 3-year-old gilthead sea bream fed an FM/FO diet (D1) or a plant-based diet (D2). Values are the mean ± SEM of three tanks per diet (n = 261 (D1) –271 (D2) fish/diet at Age +1, 202 (D1) –207 (D2) fish/diet at Age +2, 138 (D1) –146 (D2) fish/diet at Age +3). Asterisks indicate significant differences between the experimental diets within each age (Student's t-test, $^{**}P < 0.01$, $^{***}P < 0.001$).

by means of a one-way ANOVA, followed by a Holm-Sidak *post-hoc* test.

Results

Growth and sex ratio progression

Dietary treatment had a clear effect on fish size regardless of their genetic background. Thus, considering all fish families as a whole under each dietary treatment, fish fed D2 consistently showed a lower body weight than that fed D1. Differences in body weight were not statistically significant at Age +1 (Figure 1A). However, at Age +2 and Age +3, the body weight difference between both dietary groups was 12.5 and 18%, respectively, with the growth performance negatively affected ($P < 0.001$) by the plant-based diet. Regarding sex, all fish were males at Age +1 regardless of diet (Figure 1B). However, the plant-based diet largely enhanced the male-to-female sex reversal, resulting in a significantly ($P < 0.01$) higher female percentage at both Age +2 (20.9 vs. 7.2%) and Age +3 (81.2 vs. 54.4 %).

Sexual dimorphism: Body weight and sex steroids

Data on body weight at Age +2 showed no significant differences between males and females fed D1, neither when considering all fish families as a whole nor analyzing each family separately (Figure 2A). However, in fish fed D2, a significant body weight sexual dimorphism toward larger females was evidenced comparing male and female populations (Figure 2B). It must be noted that this feature was significant only for the fast-growing family e6e2 (Figure 2B).

At Age +3, gilthead sea bream females had approximately 15% more body weight than their male counterparts regardless of diet (Figure 3). In fish fed D1, this clear sexual dimorphism was mostly observed in families c2c7, e5e2, and e6e2 (Figure 3A), whereas for fish fed D2, it was supported by a significantly higher body weight of females of families e4e1, e5e2, and e6e2 (Figure 3B).

E_2 plasma levels in Age +2 males were quite similar, with the only significant difference between families e5e2 and e4e1 when fed the D1 diet (Figure 4A). For Age +2 and Age +3, plasma levels of E_2 increased around 130% in males fed D1, whilst those of males fed D2 decreased to around 50% (Figure 4B). Age +3 male fish fed D1 showed higher E2 plasma levels than fish fed D2, with no differences among families for the same dietary group. Male plasma levels of 11-KT at Age +2 showed a gradually decreasing trend from slow- to fast-growing families, with e5e2 and e6e2 families significantly different from the slow and intermediate families within both diets (Figure 4C). Comparison between diets showed that fish fed D2 had significantly higher levels of 11-KT in the case of slow- and intermediate-growing families, and the same trend, although non-significant, was maintained in fast-growing families (Figure 4C). Male 11-KT levels generally increased from Age +2 to Age +3, keeping the gradual decrease of 11-KT from slow- to fast-growing families (Figure 4D). Four of five families of Age +3 males displayed higher 11-KT levels when fed D2.

Results of female sex steroids at Age +2 were not conclusive due to the low number of gilthead sea bream that underwent sex change from male to female (Figures 5A, C). Nonetheless, at Age +3, female E_2 plasma levels showed a clear diet effect, with fish fed D1 having significantly higher levels within all families (Figure 5B). A genetic effect was reduced to fish fed D2, with higher circulating levels of E_2 in slow-growing fish than in fast- and intermediate-growing families (Figure 5B). 11-KT plasma levels of all 3-year-old female families were below 0.05 ng/ml, and no differences were observed between families and dietary groups (Figure 5D).

Sex steroids ratio

The PCA of plasma sex steroid levels at Age +3 showed that >86% of the total variance was explained by the two first components (Figure 6A). Each fish was categorized according to its diet, sex, and family group. For better representation, $e_x e_y$ families were joined as a unique fast-growth family group, while $c_x c_y$ families were joined as a unique slow-growth family group. Movement along the X-axis (60.32% of total variance) accounted for plasma sex steroid levels, with the highest values of E_2 on the left and the maximum values of 11-KT on the right. This sex steroid distribution clearly discriminated females (black and orange boxes) on the left and males (green and blue boxes) on the right. The Y-axis (25.85% of total variance) accounted for body weight changes, separating the fast-growth families at the top from the slow-growth families at the bottom. In other words, the resulting plasma E_2/11-KT ratio was affected by both diet and genetics, increasing this hormonal quotient with the improvement of both the nutritional and genetic background (Figure 6B). In males, a genetic effect was not seen, but the diet effect persisted with a decreased E_2/11-KT ratio in fish fed the plant-based diet. This occurred in parallel with a genetically regulated male-to-female sex reversal, displaying fast-growing families fed D1 a significantly lower percentage of phenotypic females (54%) than slow-growing families (65%) fed the same diet (Figure 6C). This percentage of phenotyped females reached a plateau (79–81%) in fish fed D2 regardless of their genetic background, which is indicative of a genetic and nutrition interaction according to which the plasma E_2/11-KT ratio becomes more fine-regulated in females than in males, and in fish fed D1 diet rather than in fish fed D2 diet. Moreover, it is noteworthy that the highest plasma E_2/11-KT

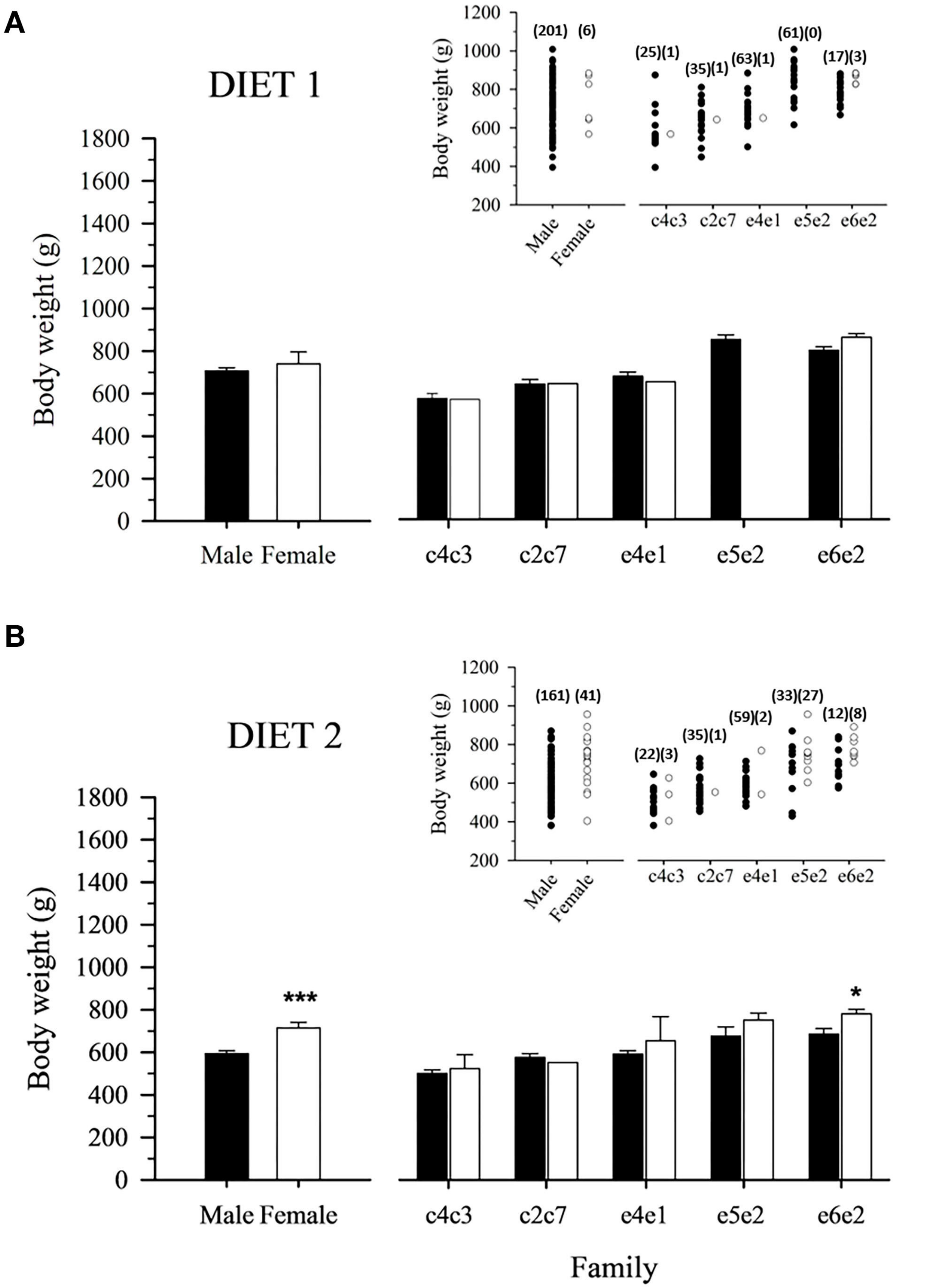

FIGURE 2
Mean male and female body weight of 2-year-old gilthead sea bream-fed control **(A)** or experimental diet **(B)** as a whole population and separated by family. Values are the mean ± SEM of three tanks per diet ($n = 207$ fish for diet 1, 202 for diet 2). Asterisks indicate significant differences (Student's t-test, $P < 0.05$) between males and females body weight. Inserts indicate individual body weight in each population. The number of males and females for the populations and families is indicated in parenthesis.

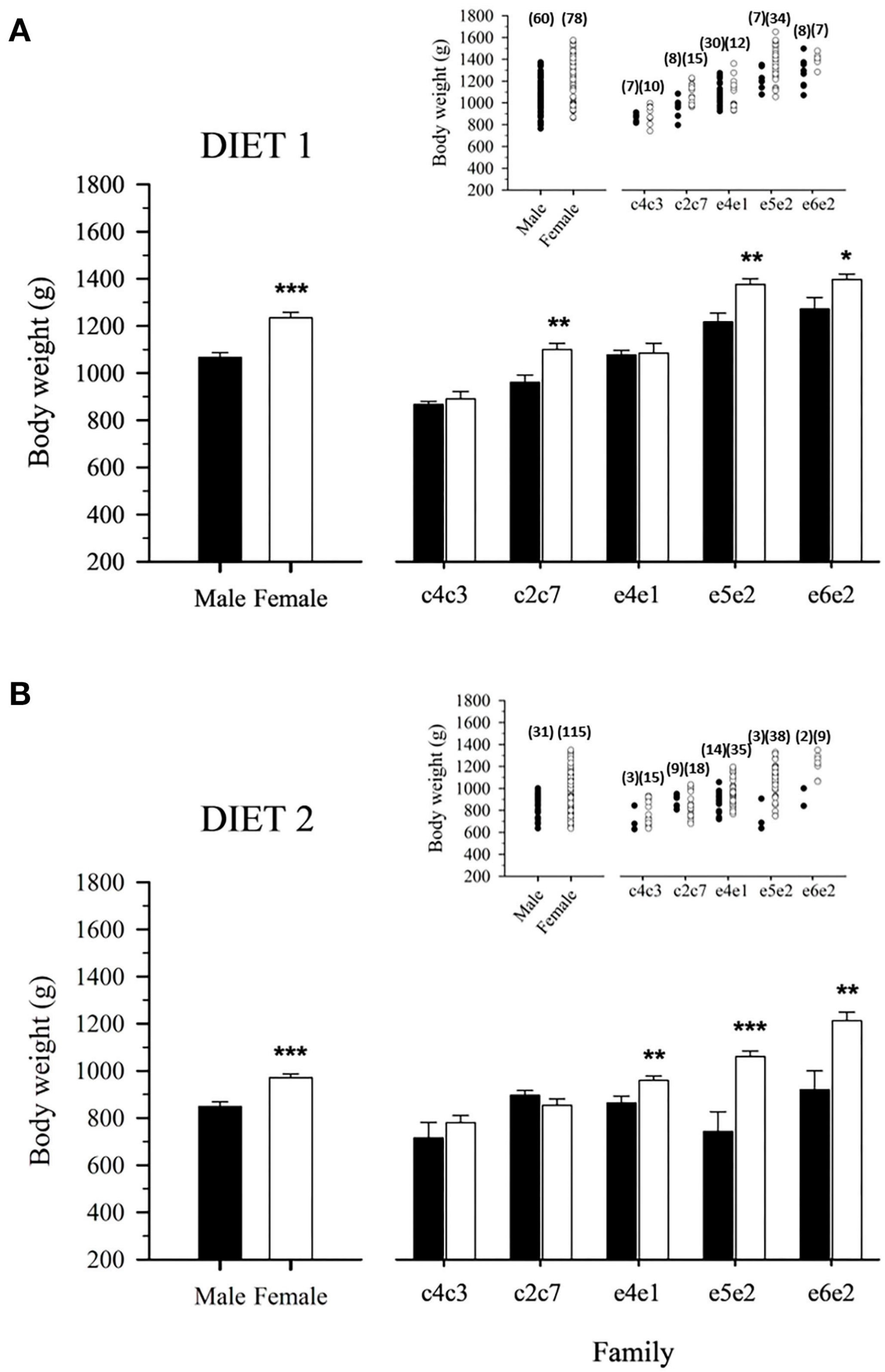

FIGURE 3
Mean male and female body weight of 3-year-old gilthead sea bream-fed control **(A)** or experimental diet **(B)** as a whole population and separated by family. Values are the mean ± SEM of three tanks per diet ($n = 138$ fish for diet 1, 146 for diet 2). Asterisks indicate significant differences (Student's t-test, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$) between males and females body weight. Inserts indicate individual body weight in each population. The number of males and females for the populations and families is indicated in parenthesis.

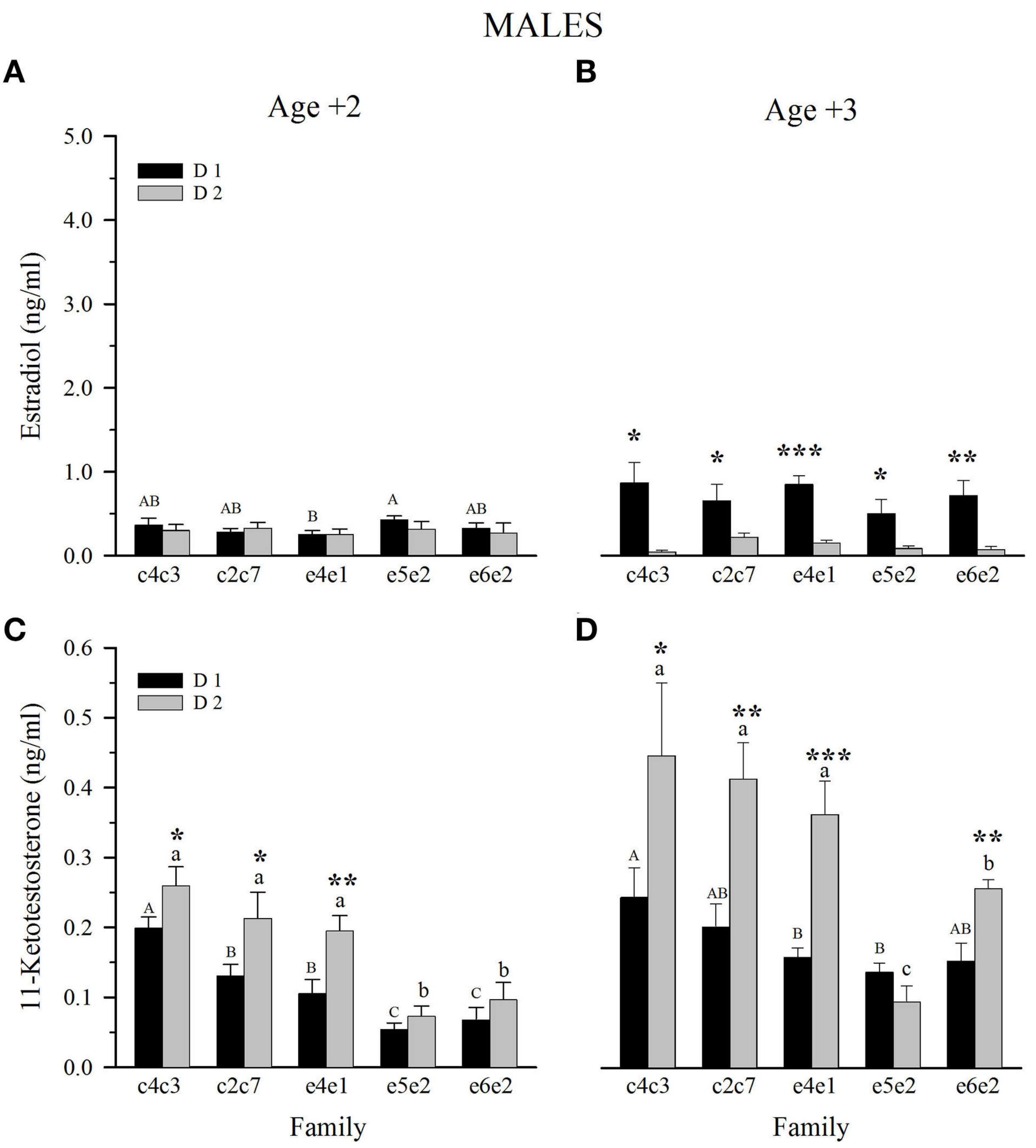

FIGURE 4
Plasma levels at spawning season of estradiol **(A, C)** and 11-ketotestosterone **(B, D)** in 2 and 3-year-old males fed control (D1) or experimental diet (D2). Values are the mean ± SEM. Different capital letters indicate significant differences between families fed D1. Different lowercase letters indicate significant differences between families fed D2. Asterisks indicate significant differences between dietary groups within each family (Student's *t*-test, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

ratio (powerful sex female steroid profile) was concurrent with a lower abundance of functional/powerful females when fish from fast- (e_xe_y) or slow-growth (c_xc_y) fish families were grouped and analyzed together as two different experimental groups in our common garden rearing system.

Discussion

The present study underlines the effect of different nutrition and genetic backgrounds in the plasma sex steroids profile and male-to-female sex reversal in gilthead sea bream with

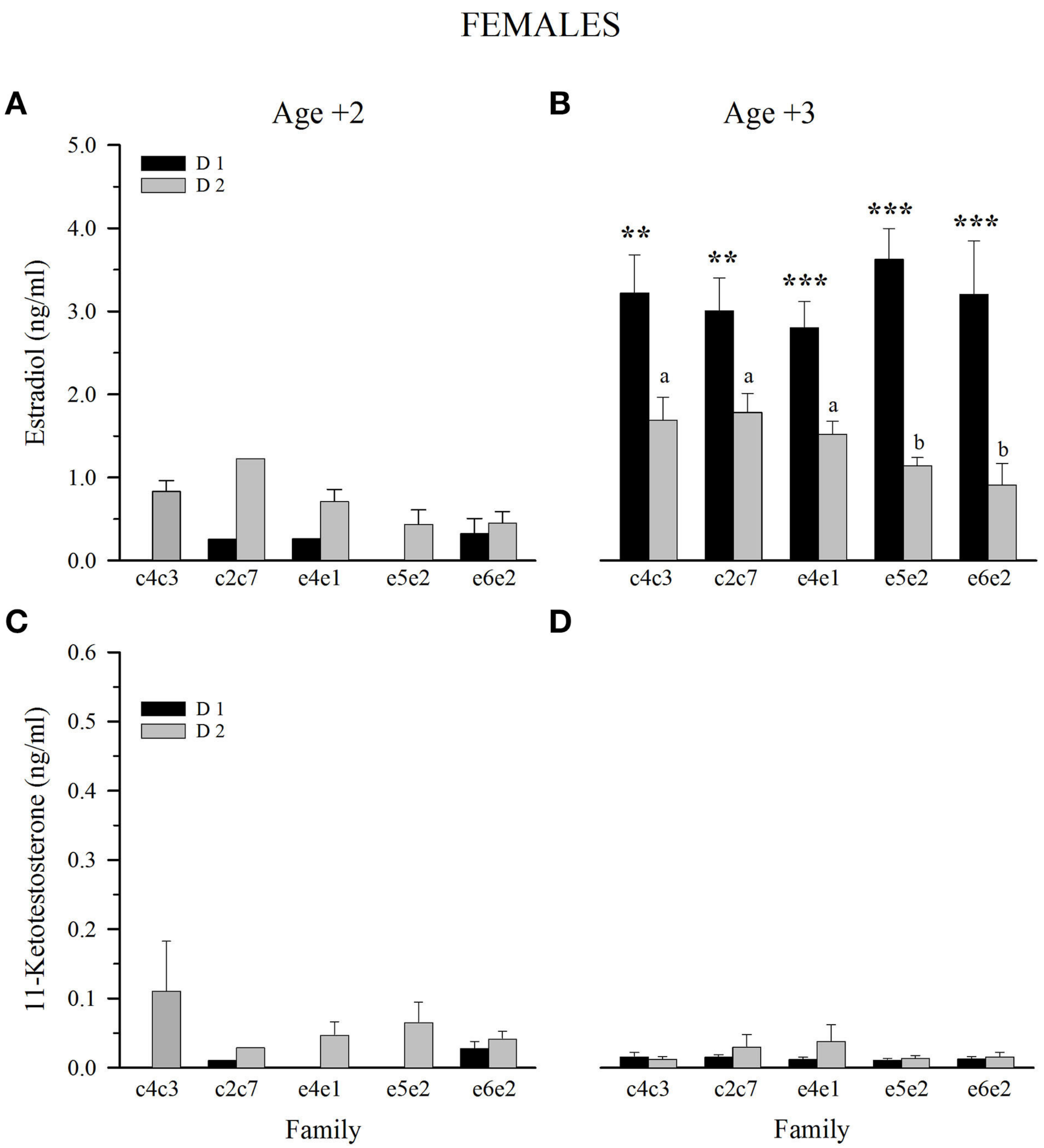

FIGURE 5
Plasma levels at spawning season of estradiol **(A, C)** and 11-ketotestosterone **(B, D)** in 2 and 3-year-old females fed control (D1) or experimental diet (D2). Values are the mean ± SEM. Different letters indicate significant differences between families fed D2. Asterisks indicate significant differences between dietary groups within each family (Student's *t*-test, $^{**}P < 0.01$, $^{***}P < 0.001$).

differences in heritable growth and a sexual growth dimorphism, which was exacerbated in fast-growing fish by feeding a plant-based diet. Indeed, when fish attained 2 years of age, a sexual growth dimorphism was only observed in fish fed D2. However, body weight differences intensified as fish grew, and were equally significant and visible with both diets, especially in fast-growing families. Besides, previous studies have highlighted that families with a fast growth phenotype within the PROGENSA® selection program displayed a plastic gut microbiota to cope better with changes in diet composition, also contributing to a better disease progression of parasitic enteritis in fish challenged with the myxozoa *Enteromyxum*

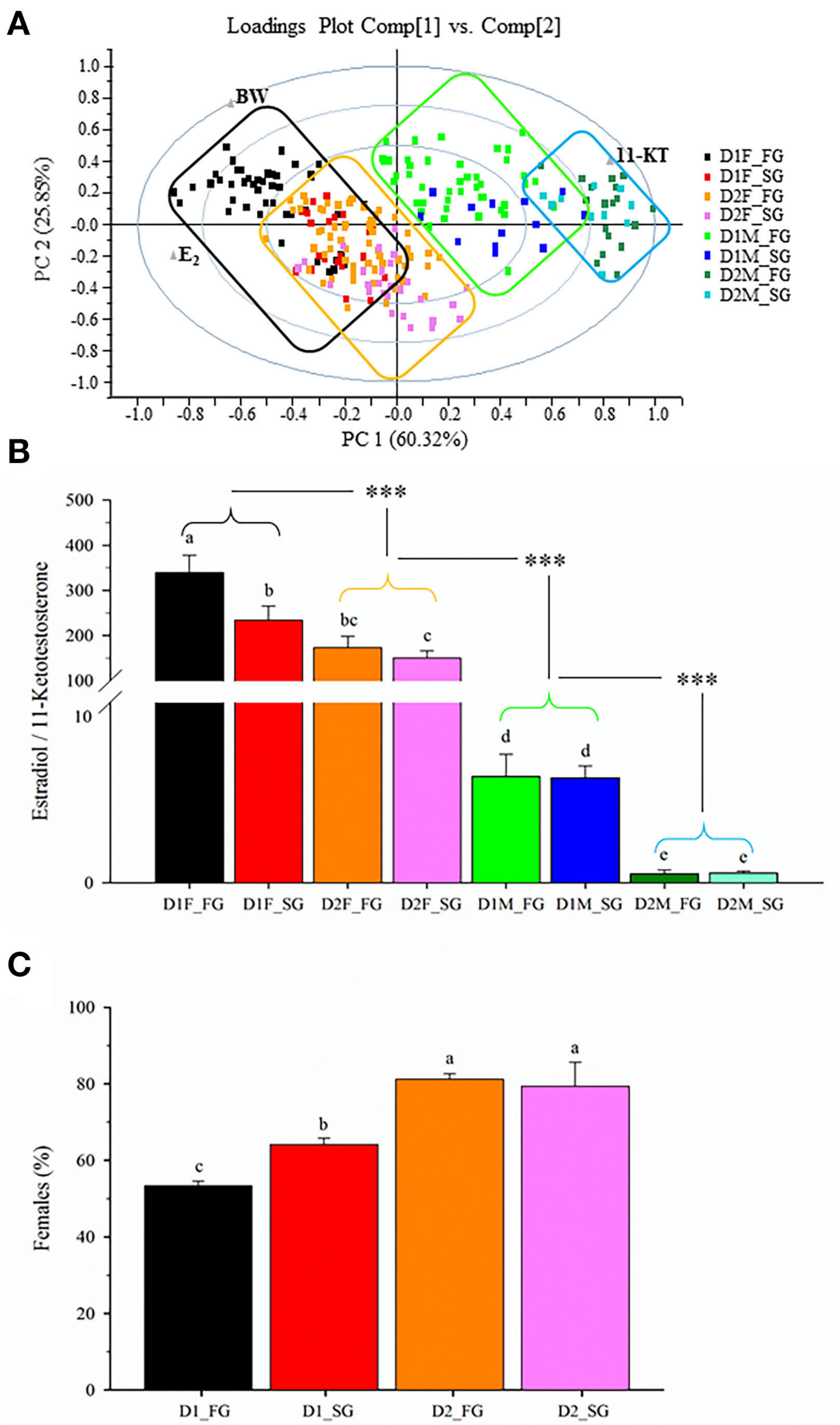

FIGURE 6
(A) Principal component analysis of body weight and plasma sex steroids levels during the second spawning period (3-year-old fish). **(B)** Sex steroids quotient of females (F) and males (M) of each dietary group (D1–D2), and fast (FG) or slow (SG) growth family groups. Values are the mean ± SEM of the E_2/11-KT quotient. Different letters indicate significant differences between each group. Asterisks indicate significant differences between males and female groups with different dietary regimes (Holm-Sidak *post-hoc* test, $^{***}P < 0.001$). **(C)** Female percentage of fast and slow growth family groups.

leei (5, 43). Available studies also evidenced that such selective breeding for growth and skeletal deformities has an impact on humoral immune markers (48), carcass and morphometric traits (49, 50), energy partitioning between growth and swimming activity (44), and even more in reproductive success as recently evidenced in fish with a normal phenotype but with a genetic background of skeletal deformities (51). It appears, thereby, that selection for growth and deformity traits co-selects in the PROGENSA® selection program for a number of relevant traits, including the sex ratio, which is becoming nutritionally and genetically regulated through the life cycle. The ultimate physiological mechanism remains elusive, but this study aimed to provide new insights into the sex ratio in a protandrous fish for its use as an operational welfare scoring system in a challenging environment.

Sex reversal in fish is defined as a mismatch between the phenotypic and the genetic sex (52). Thus, in gilthead sea bream, in particular, the general thinking is that individuals of this fish species act as functional males by the end of the first-second year of life, and sex reversal generally takes place 1 year after the first male sexual maturation (53). The percentage of male-to-female sex reversal can vary from 15 to 80% during the second year of life (53), but the possibility of a later sex reversal was reported by Brusléa-Sicard and Fourcault (54) and further corroborated by Chaoui et al. (55). Herein, the male-to-female sex reversal was accomplished by a relatively high percentage of individuals (50–85%) regardless of diet, which confirms the notion that a sex change in gilthead sea bream is cued by social and environmental factors when a critical age or size is attained (30, 56, 57). Indeed, the removal of functional females from the population drives the feminization of the remaining males (53, 58), probably *via* the production and release of specific pheromones that can activate or block some sex-specific networks. The aquatic environment may also contain a wide range of endocrine-disrupting chemicals that reduce reproductive performance and can even inhibit the sex reversal in gilthead sea bream (31, 59, 60). However, xenobiotic-induced sex reversal did not appear to be our case, because fish grew from early life stages in an eco-friendly environment where a wide-screening of undesirable compounds in fish edible matter revealed bio-contaminant loads to be much lower than the maximum established residue level (61).

The net balance between gonadal estrogen and androgen production directs sexual differentiation and gonadal development in fish (62). Indeed, E_2 and 11-KT are typically considered the predominant steroids in the regulation of sex change in most fish species (63). Thus, we found herein that both E_2 and 11-KT increased over time, with males and females displaying the highest plasma levels of 11-KT or E_2 at Age +3, respectively. A nutritionally mediated effect was also reported, although it is difficult to deconvolute the extent to which this observation is due to a specific nutrient or to a different loading of plant phytoestrogens with both estrogenic and antiestrogenic effects on vertebrates (64). In tilapia farming, in particular, herbal extracts could be used as safe alternative agents to control precocious tilapia maturity and prolific breeding in production (65). In the present study, the inclusion level of soy protein concentrate, a rich source of phytoestrogens, varied between 16% in D1 and 25% in D2, although it is within the tolerance range for gilthead sea bream (65). In any case, plasma levels of fish fed D2 were lower for E_2 and higher for 11-KT, displaying these fish a masculinized sex steroid profile that would promote the male-to-female sex reversal in the absence of high powerful functional females that ensure reproduction success, as stated before by Simó-Mirabet et al. (41). Masculinization of gonochoristic fish populations also occurs as a result of elevated temperatures and other environmental stressors. This would be mediated, at least in part, by the increase of circulating cortisol, which is now recognized as a universal mediator of sex reversal in fish due to its implication in delaying ovarian meiosis and increasing 11-KT (52). For instance, in the protogynous three-spot wrasse, cortisol treatment had a masculinizing effect (66). This feature was also reported by us in the protandrous gilthead sea bream-fed plant-based diets, regardless of the well-known hypocholesterolemic effect of plant ingredients in most farmed fish (67). In fact, since cholesterol is the precursor of cortisol, its reduced dietary supply or intestinal absorption could initially lead to a female-biased sex ratio. Nonetheless, there are more factors at play in this process, and Nile tilapia fry fed with saponin-supplemented diets (hypocholesterolemic diets) displayed a significant male-biased population (68). In other words, the sex ratio can be influenced by a number of nutritional factors, including changes in the dietary fatty acid composition as a result of a high replacement of marine feedstuffs by vegetable oils (69). However, all this is the result of a complex trade-off, which is also indicative of the amazing diversity and evolution of sex determination in vertebrates, and fish in particular (70). Similarly, global warming due to climate change would affect the offspring quality of a wide range of animals (71–73). This is especially important for aquatic ectotherms, where temperature values above the optimal for each species and fish strain can shift the sex ratio toward either the male or female phenotype in gonochoristic fish (74–76) and perhaps hermaphroditic fish.

Moreover, in the present study, discriminant analysis of sex steroids and body weight displayed a clear separation of phenotypic males and females, with also a differentiation of slow- and fast-growth families. Thus, families of fast heritable growth displayed a more mature/advanced sex steroid phenotype, resulting in a higher E_2/11-KT ratio (feminization phenotype) that would trigger the inhibition of male-to-female sex reversal of the remaining males. This hormonal quotient, rather than the circulating amount of a given sex steroid, would determine what sex-specific network is activated or suppressed, leading or not to the sex reversal in the individual. Indeed, the rise of the E_2/11-KT ratio was associated with a female bias in painted turtles (77), and we found herein that this plasma sex steroid ratio was progressively increased from males to females with both the nutritional and genetic improvement. However, the increased plasma E_2/11-KT ratio in female fish fed D1 was negatively associated with the rate of male-to-female sex reversal in genetically improved fish, whereas this genetically mediated response was mostly masked in fish fed D2. This finding highlighted a nutrition and genetic interaction on the progression of sexual maturation, as reported for gut microbiota composition and function. Indeed, fast-growing fish families in the PROGENSA® program are more resilient to changes in gut microbiota composition, but at the same time, metatranscriptomic analyses confirm and extend the notion that the core microbiota of genetically improved fish is able to modulate their metabolic activity to cope better with changes in diet composition (5, 43). Otherwise, there is no evidence in gilthead sea bream that the modulating effects of gut microbiota by feed additives are a specific feature of each additive and genetic background (unpublished results).

As indicated earlier, reproductive physiology and spawning are sensitive processes to changes in environmental conditions and physiological stress, and how and to what extent external and internal factors have an impact on broodstock welfare is of relevance to assure reproduction success, but also the offspring plasticity and quality (78, 79). Concretely, in gilthead sea bream, several attempts at nutritional programming are made through changes in the parental nutrition (80–83). Such an approach is not unique to fish, as parental nutrition and hormonal status of humans and terrestrial animals directly impact all stages of gamete maturation, fetal development, and long-term offspring health (84, 85). Therefore, one of the challenges of modern aquaculture is to assure the welfare of breeders, which could also inform the welfare condition from a retrospective point of view. Certainly, we can conclude that fast-growing fish families fed a control diet became powerful females, whilst fish of slow-growth families and/or fish fed plant-based diets experienced a pseudo-feminization effect (i.e., fish with a weaker female signal, which enhances the ratio of sex reversal). Therefore, it appears that the progression of sex reversal is directly regulated by both nutritional and genetic background among many other environmental and social factors. Thus, the study of sex reversal as a biological endpoint is becoming a reliable tool of relevance for the animal welfare certification of a highly cultured protandrous fish such as gilthead sea bream. Several items support this assumption. First, fish growing with plant-based diets from early life stages shared an enhanced onset of puberty and sex reversal in concurrence with some growth impairment of sexually mature fish. Second, genetically improved fish for growth are more resilient to the progression of male-to-female sex reversal with the use of alternative fish feed formulations, but further research should be directed toward the effect of specific nutrients on reproductive performance and maturation as a means to enhance the offspring quality. Finally, a decreased plasma E_2/11-KT ratio is becoming indicative of a negative welfare status in the long term, supporting this finding the use of such reproductive tract scoring systems for leading a protandrous farmed fish toward their optimum welfare condition.

Conclusion

Results of this long-term dietary and genetics trial disclose that sex steroids profile and male-to-female sex reversal are nutritionally and genetically regulated in the protandrous gilthead sea bream. Moreover, the sex ratio is proposed as a reliable welfare indicator alerting of disturbances in reproductive performance, and perhaps overall growth and offspring quality. Such a scoring system is becoming, thereby, an exploitable finding for the certification of animal welfare in a given gilthead sea bream production system.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The animal study was reviewed and approved by Ethics and Animal Welfare Committee of the Institute of Aquaculture Torre de la Sal and CSIC Ethics Comittee.

Author contributions

JP-S: conceptualization. PH, AF, JC-G, and JP-S: formal analysis. AF, JC-G, and JP-S: funding acquisition. PH, AF, and JP-S: methodology. AF, JA, and JP-S: resources. PH, JC-G, and JP-S: draft writing. PH, AF, JC-G, JA, and JP-S: draft review and edition. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2022.1083255/full#supplementary-material

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EDITED BY
Rosario Martínez-Yáñez,
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Jorge Francisco Monroy López,
National Autonomous University of Mexico, Mexico
Pedro Luis Castro,
University of Las Palmas de Gran Canaria, Spain

*CORRESPONDENCE
Ana Silvia Pedrazzani
✉ anasilviap@ufpr.br

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From egg to slaughter: monitoring the welfare of Nile tilapia, *Oreochromis niloticus*, throughout their entire life cycle in aquaculture

Ana Silvia Pedrazzani[1]*, Nathieli Cozer[1,2,3], Murilo Henrique Quintiliano[4], Camila Prestes dos Santos Tavares[1,3,5], Vilmar Biernaski[3] and Antonio Ostrensky[1,3]

[1]Wai Ora—Aquaculture and Environmental Technology Ltd., Curitiba, State of Paraná, Brazil, [2]Graduate Program in Animal Science, Federal University of Paraná, Curitiba, State of Paraná, Brazil, [3]Integrated Group of Aquaculture and Environmental Studies (GIA), Department of Animal Science, Agricultural Sciences Sector, Federal University of Paraná, Curitiba, State of Paraná, Brazil, [4]FAI Farms, Londrina, State of Paraná, Brazil, [5]Graduate Program in Zoology, Federal University of Paraná, Curitiba, State of Paraná, Brazil

The primary aim of this study was to comprehensively evaluate the welfare of Nile tilapia *(Oreochromis niloticus)* throughout their entire life cycle within aquaculture, spanning from reproduction to slaughter. The methodology was structured to identify welfare indicators closely aligned with the principles of animal freedoms defined by the Farm Animal Council, encompassing environmental, health, nutritional, behavioral, and psychological freedom. Notably, psychological freedom was inherently considered within the behavioral and physical analyses of the animals. To accomplish this, an integrative systematic literature review was conducted to define precise indicators and their corresponding reference values for each stage of tilapia cultivation. These reference values were subsequently categorized using a scoring system that assessed the deviation of each indicator from established ideal (score 1), tolerable (score 2), and critical (score 3) ranges for the welfare of the target species. Subsequently, a laboratory experiment was executed to validate the pre-selected health indicators, specifically tailored for the early life stages of tilapia. This test facilitated an assessment of the applicability of these indicators under operational conditions. Building on the insights gained from this experimentation, partial welfare indices (PWIs) were computed for each assessed freedom, culminating in the derivation of a general welfare index (GWI). Mathematical equations were employed to calculate these indices, offering a quantitative and standardized measure of welfare. This approach equips tilapia farmers and processors with the tools necessary for the continuous monitoring and enhancement of their production systems and stimulate the adoption of more sustainable and ethical practices within the tilapia farming.

KEYWORDS

grow-out, larviculture, fish welfare, welfare indices, protocols, sustainable aquaculture

1. Introduction

The international scientific community's recent recognition of fish as sentient beings (1–4) has encouraged various countries to implement norms and regulations and enact laws to protect these animals when commercially farmed in captivity (5–8). Simultaneously, multiple actors in the food sector—such as importers, retail chains, restaurants, and their respective representative entities—began to request sustainability and animal welfare certificates as a prerequisite for purchasing processed or unprocessed aquaculture products (9, 10). This transformation has occurred in harmony with the aquaculture sector's recognition of the relevance of launching marketing and advertising campaigns focussed on animal welfare and integrating them into the industry's main collective corporate social responsibility commitments (11, 12). As a result, albeit at an early stage, the issue of "fish welfare" is gradually being incorporated into economic actors' social attitudes and practices (13).

However, many challenges must be overcome for welfare to become an inseparable element of farmed fish production. The international recommendations and guidelines currently focus on animal transport and slaughter stages and establish only the minimum animal protection standards (7, 8, 14, 15). In this way, the strict aspects of each species and the critical points in the welfare of these animals end up being neglected. The lack of scientifically based information on tested, standardized, and validated instruments for each farmed fish species and restrictions on their applicability in field situations are often cited as some of the reasons to explain these gaps (13, 16).

Amongst the ∼350 species of fish farmed in aquaculture worldwide (17), tilapia, in particular, have shown significant volume growth. According to recent FAO data (18), Nile tilapia, *Oreochromis niloticus*, is currently amongst the three most farmed fish species globally, with China, Indonesia, Egypt, Bangladesh, and Brazil emerging as the largest producers (19). Aquaculture sectors have been under pressure to produce more with fewer resources—increasing production using less feed, water, and space to meet the growing global demand for fish proteins (20). However, this pressure tends to affect the environment and the welfare of farmed fish (21). In this context, the assessment of the health and welfare of tilapia becomes an increasingly relevant challenge in a global scenario. That is why these issues emerge as a central focus in the search for the development of management alternatives aimed at improving the quality of life of the animals and the quality of the final product made available by the industries to their consumers (9, 22).

The first protocol for assessing tilapia welfare, developed by Pedrazzani et al. (23) was limited to the grow-out phase of Nile tilapia. In the present study, our goal is to review the indicators proposed in that protocol, in addition to identifying and validating specific health, environmental, nutritional, and behavioral indicators for all other phases of the development cycle of *O. niloticus* in captivity, including the stages of reproduction, nursery, and transport. This approach will, for the first time, enable a more comprehensive, accurate, and personalized analysis of the evaluation and promotion of tilapia welfare throughout its entire production cycle rather than just the final grow-out stage. Moreover, in the present study, we also applied a method to establish quantitative and standardized welfare indices for each tilapia cultivation phase.

2. Materials and methods

2.1. Organization of the welfare protocol for tilapia into categories

The operational welfare indicators for tilapia were organized according to four of the five freedoms for animals established by the Farm Animal Council (24): environmental, health, nutritional, and behavioral. The fifth freedom, psychological, is intrinsically evaluated through the behavioral and physical analysis of the animals. The methodology employed followed the same principles already used in the creation of the protocols previously developed by our group for *O. niloticus* (23) and grass carp, *Ctenopharyngodon idella* (25), during the grow-out phase of both and also for white-leg shrimp, *Penaeus vannamei* throughout its entire production cycle (26).

2.2. Systematic review for the definition of indicators and their respective reference values

An integrative systematic review (27) was conducted using the Google Scholar platform as the research base. The aspects related to the environment, health, nutrition, and behavior associated with the species, as well as the specific welfare indicators and their respective reference values for each cultivation stage (Figure 1), were studied and defined. For the grow-out phase of tilapia, emphasis was given to cultivations carried out in earth ponds, the primary fish farming system used globally (18). The research included books, technical and scientific articles, case studies, manuals, and technical reports developed by international institutions, as well as theses and dissertations. Materials containing the following terms were selected: "*Oreochromis niloticus*" AND welfare indicator AND production stage, in the titles, abstracts, or keywords. The welfare indicators defined for each stage of the cultivation process are listed in Table 1. The search period extended from 1985 to 2023.

The reference values for each indicator were classified through a system involving three possible scores (1, 2, or 3). A score of 1 indicates the limits of variation of a particular indicator within the ranges considered ideal for the target species. A score of 2 pertains to variations the animals tolerate, which can cause deleterious effects, provided they are non-lethal. A score of 3 indicates significant levels of variation in a specific indicator that significantly compromises the health and even the survival of the animals, which is deemed unacceptable from an animal welfare perspective. The maximum tolerated mortality rates, the primary indicator of the degree of welfare of the fish, were set at levels much lower than those found in nature, taking into account each stage of the production cycle. The reference values for each indicator and score were established based on the literature available for the species.

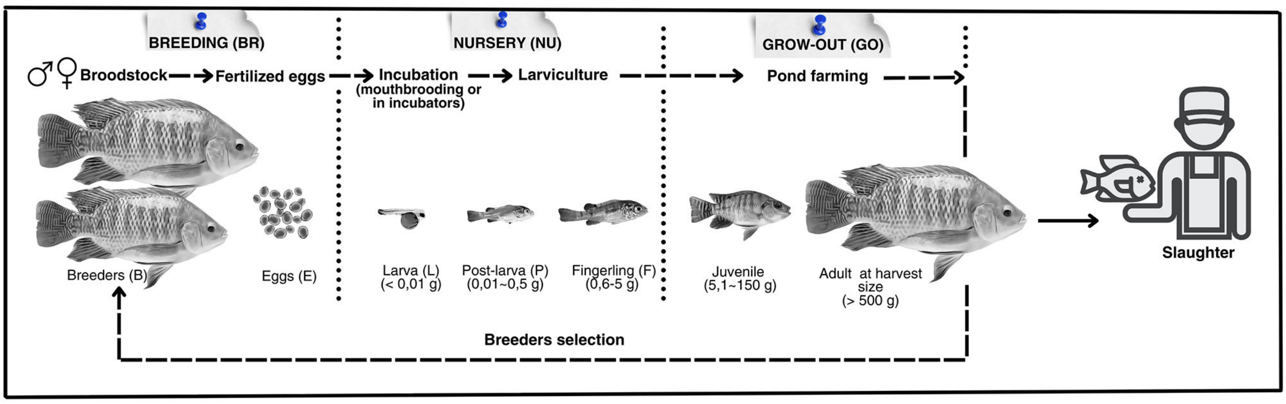

FIGURE 1
Cultivation stages (breeding, nursery, and grow-out) and development stages (breeders, eggs, larvae, post-larvae, fry, juveniles, and adults) of Nile tilapia, *Oreochromis niloticus*.

2.3. Preliminary assessment of the indicators for the early life stages of tilapia

An experimental trial was conducted to establish health protocols for the early life stages of tilapia and to test the pre-selected indicators, allowing for the assessment of their applicability under operational conditions and the evaluation techniques for each indicator. The experiment was conducted at the Laboratory of Research with Aquatic Organisms (LAPOA) of the Integrated Group of Aquaculture and Environmental Studies (GIA) at the Federal University of Paraná, Curitiba, Brazil. All husbandry and experimental procedures were approved by the Animal Use Ethics Committee of the Agricultural Sciences Campus (CEUA) of the Federal University of Paraná (protocol number 021/2023).

A total of 480 newly hatched tilapia larvae were used, subdivided into 12 aquariums of 30 liters in volume, each linked to a chemical–biological filtration system, under controlled conditions of temperature (27.01 ± 1.0°C), pH (7.83 ± 0.2), and dissolved oxygen (5.40 ± 0.4 mg/L). Over 15 days, a tilapia larva from each aquarium was randomly selected using a catch net and carefully and individually transferred to cell culture dishes, duly labeled, containing 10 mL of water on a daily basis. This procedure ensured that the samplings were representative, giving greater precision to the results. The collected fish were anesthetized with clove oil at a concentration of 500 mg/L and kept until they reached stage V anesthesia (∼5 min). At that point, the fish have a medullary collapse and permanent unconsciousness (28). The cell culture dishes containing the collected animals were then transferred to the imaging laboratory, where the animals underwent physical evaluation procedures and photographic recording to determine their health status and evaluate the welfare indicators. The following organs were assessed under a stereo microscope (Figure 2): eyes, mouth and jaws, skin, fins, gill covers, spinal column, and yolk sac. Next, an individual and bilateral photographic record of each individual was made. The indicators that proved unfeasible to measure under operational conditions and on a commercial scale were excluded from the final version of the protocol. After this final verification, the protocols were reorganized in the format and content presented in Tables 1–10.

2.4. Application of welfare indices

Calculation of tilapia welfare indices utilized the same mathematical equations proposed by Pedrazzani et al. (25) for evaluating the welfare degree of *C. idella*. The weights assigned to each indicator in the respective indices were established based on the number of valid bibliographic references found for *O. niloticus* in each production phase, using Google Scholar as the research platform. The variable "Y" was calculated as the integer part of the natural logarithm (ln) of the number of articles identified through specific keywords, as shown in Equation 1. Consequently, partial welfare indices (PWIs) were proposed for each category, along with the general welfare index (GWI), calculated from the PWIs. The PWIs were computed using Equation 2, which considers the weights assigned to each indicator and their respective scores.

$$Y = INT\,(\ln(n)) \tag{1}$$

$$PWI_x = \left(\frac{\sum Y}{\sum (S \times Y)} \times (1.4925 - 0.4925)\right) \tag{2}$$

where:

PWI: Partial welfare index standardized to vary continuously between 0 (critical risk of harm to farmed fish welfare) and 1 (maximum welfare or, otherwise, minimum risk of injury to animal welfare), regardless of the number of indicators used in each freedom.

X: Freedom (En, environmental; Be, behavioral; Nu, nutritional, or He, health).

Y: Weight assigned to the specific indicator.

S: Score assigned to the indicators in the analyzed fish farm.

10.3389/fvets.2023.1268396

TABLE 1 Welfare indicators organized according to animal freedoms and production stages of Nile tilapia (BR, breeding; NU, nursery; GO, grow-out).

Freedom	Indicator	Breeding (BR)	Nursery (NU)	Grow-out (GO)
Environmental	Alkalinity	✓	✓	✓
	Aquatic predators and other interspecific inhabitants	✓	✓	✓
	Dissolved oxygen	✓	✓	✓
	Hapas net cleaning	✓		
	Nitrite	✓	✓	✓
	Non-ionized ammonia	✓	✓	✓
	pH	✓	✓	✓
	Photoperiod	✓	✓	
	Sex ratio (male: female)	✓		
	Temperature	✓	✓	✓
	Terrestrial predators	✓		✓
	Transparency	✓		✓
Health	Breeding control	✓		
	Conditioning/ breeding interval (days)	✓		
	Eggs–macroscopical aspects	✓		
	Emaciation state	✓	✓	
	Eyes	✓	✓	✓
	Fins		✓	✓
	Gills	✓		✓
	Hatching rate		✓	
	Invasive procedures	✓		✓
	Jaws/lips/head	✓	✓	✓
	Mortality (%)	✓	✓	✓
	Operculum	✓	✓	✓
	Sexual maturation	✓		
	Skin	✓	✓	✓
	Spine	✓	✓	✓
	Tail		✓	
	Yolk sac		✓	
Nutritional	Amount of feed	✓	✓	✓
	Feed conversion ratio (FCR)			✓
	Feed crude protein	✓	✓	✓
	Feeding frequency	✓	✓	✓
	Food distribution	✓	✓	✓
Behavioral	Anesthesia	✓		✓
	Feed intake	✓	✓	✓
	Swimming behavior	✓	✓	✓
	Stunning during slaughter–reflexes			✓

(Continued)

TABLE 1 (Continued)

The general welfare index (GWI) was calculated as the arithmetic mean of the PWIs, multiplied by an elimination factor (kl, Equation 3). The kl is defined based on the observed mortality rate. Thus, mortality becomes mathematically the most critical indicator for measuring the welfare degree of farmed fish in captivity. By definition, if the mortality rate exceeds 30%, the value of the elimination factor will be equal to zero ($kl = 0$), automatically indicating a "critical" classification for the GWI of the evaluated fish. If the mortality rate is below 30%, the elimination factor will be equal to 1 (kl=1), and the welfare of the fish will be determined based on the respective indicators analyzed, their scores, and weights.

$$WGI = \frac{((PWI_{En} + PWI_{Be} + PWI_{Nu} + PWI_{He}) \times kl)}{4} \quad (3)$$

where:

WGI: General welfare index, que varia de 0 (critical risk of harm to farmed fish welfare) and 1 (maximum welfare or, otherwise, minimum risk of injury to animal welfare).

kl: Knockout level (risk of total impairment of the degree of welfare).

The partial confidence levels (CLx) proposed for each PWI are determined based on the number of indicators effectively analyzed in the field. The more indicators evaluated compared to the proposed indicators, the higher the confidence level of the results. The general confidence level (GCL) is calculated as the arithmetic mean of the CLx (Equation 5). Finally, the PWIx, GWI, CLX, and GCL are classified and interpreted according to the obtained values.

$$CL_x = \left(\frac{\sum W_{An}}{\sum W_{\max}} \right) \quad (4)$$

CL_X: PWIx confidence level.

$\sum W_{An}$: Sum of the weights of the indicators analyzed for the freedom x.

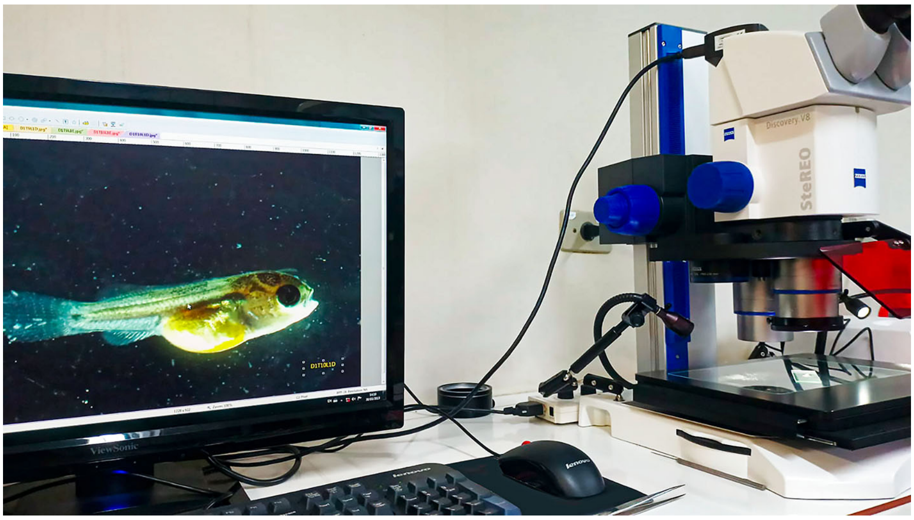

FIGURE 2
Image analysis system used to evaluate and record any health alterations in larvae and post-larvae of *Oreochromis niloticus*.

$\sum W_{max}$: Sum of the weights of all the defined indicators for the freedom x.

$$GCL = \frac{(IR_{En} + IR_{Be} + IR_{Nu} + IR_{He})}{4} \tag{5}$$

3. Results

The general protocol is divided into four categories/freedoms analyzed (environmental, health, nutritional, and behavioral), each with indicators applicable to their respective cultivation phases (Table 1).

3.1. Environmental welfare indicators for Nile tilapia

In the scope of environmental freedom, a set of 12 indicators has been established for different cultivation phases (Table 2). The physicochemical indicators of water have been standardized across all stages. This allows for the prevention of shocks during the transfer of animals between phases whilst maintaining strict adherence to the adopted environmental indicator reference values. Predators and aquatic and terrestrial cohabitants have also been included as environmental welfare indicators, with scores assigned based on their control or presence/absence. A score of 1 should be considered when there is no evidence of other terrestrial or aquatic species in the fishpond. In the case of terrestrial predators, a score of 2 should be applied when the fish farmer adopts control measures, such as filters or screens, but indirect contact still occurs (e.g., a visual connection between tilapia and their predators). For aquatic interspecific predators or cohabitants, a score of 2 should be applied to polyculture systems. The evaluator should assign a score of 3 when there is no control over predators or the presence of cohabitants from other species. The photoperiod should be considered a relevant indicator during the reproduction and larviculture phases. In the reproduction phase, additional indicators such as hapa cleanliness and the proportion of males and females used in tanks and fishponds were considered (Table 2).

3.2. Health welfare indicators for Nile tilapia

3.2.1. Breeding (BR) and grow-out (GO) phases

Health indicators were established considering the morphological abnormalities in tilapia during their ontogenetic development, observing aspects corresponding to different life and production phases (Tables 3, 4). For breeders (BRs) and animals in the grow-out phase (GO), welfare should be assessed based on physical features such as eye appearance, jaw and lip condition, gill covers, fins, skin, gills, spine, as well as mortality rates and the conduct of invasive procedures with the fish (e.g., vaccination, microchipping, mouth cutting, and removal of dorsal spines). These procedures should be scored as 2 or 3, depending on whether anesthesia is used during execution. For breeders, other important factors for assessing welfare include the stage of sexual maturation, the interval between conditioning periods for mating, and the use of techniques to prevent inbreeding during stock formation and reproductive management. For eggs, only the macroscopic aspect was identified as a practical health indicator, with eggs that are translucent and uniform in appearance considered healthy.

3.2.2. Nursery phase

The health indicators during the nursery phase were subdivided according to the ontogenetic developmental stage of the fish: larvae (L), post-larvae (PL), and fingerlings (F). The laboratory experiment proved essential for evaluating the feasibility of applying the pre-selected indicators in each phase (Table 4). The health status of the eyes, jaw/lips, and head as a whole, as well as the skin, spinal column, and fish mortality, could be easily observed

TABLE 2 Environmental welfare reference values for different tilapia production phases (BR, breeding; NU, nursery; GO, grow-out).

Production stages			Indicators	Scores	Reference values	References
BR	NU	GO				
✓	✓	✓	Temperature (°C)	1	24.0–31.0	(29–35)
				2	21.0–23.9 or 31.1–34.9	
				3	$\leq$ 20.9 or $\geq$ 35.0	
✓	✓	✓	pH	1	6.0–8.5	(29, 30, 34, 36)
				2	5.5–5.9 or 8.6–9.5	
				3	$\leq$ 5.4 or $\geq$ 9.6	
✓	✓	✓	Oxygen saturation (%)	1	$\geq$ 6	(29, 37, 38)
				2	40–59	
				3	$\leq$ 39	
✓	✓	✓	Non-ionized ammonia (mg/L of NH_3)	1	0.00–0.05	(30, 36, 39–42)
				2	0.06–0.09	
				3	$\geq$ 0.10	
✓	✓	✓	Nitrite (mg/L of NO_2^-)	1	0.00–0.30	(43–47)
				2	0.31–0.49	
				3	$\geq$ 0.50	
✓	✓	✓	Alkalinity (mg/L of $CaCO_3$)	1	30–100	(48–51)
				2	$\geq$ 101	
				3	$\leq$ 29	
✓	✓	✗	Photoperiod (Light: Dark)	1	Natural or 12L: 12D 16L:8D	(32, 39, 52–55)
				2	17L:7D−18L:6D	
				3	19L:5D or lighter; 11L:13D or darker	
✓	✗	✓	Transparency (cm)	1	30–4	(29, 48, 56)
				2	21–29 or 46–60	
				3	$\leq$ 20 or $\geq$ 61	
✓	✗	✓	Terrestrial predators	1	Absence	(30)
				2	Controlled presence	
				3	Uncontrolled presence	
✓	✓	✓	Aquatic predators and other interspecific inhabitants	1	Absence	(30)
				2	Controlled presence	
				3	Uncontrolled presence	
✓	✗	✗	Hapas net cleaning	1	7–15	(34, 57–59)
				2	$\leq$ 6	
				3	$\geq$ 16	
✓	✗	✗	Sex ratio (male: female)	1	1:2–1:3	(30, 34, 39)
				2	1:1 or 1:4	
				3	Any other configuration	

Indicators included (✓) or not included (✗) in each production phase.

with the aid of a stereoscopic loupe (10x magnification) until the animals reached ~2 cm in standard length. After that, it is possible to evaluate the external organs of the fish using only a handheld loupe with a 3x magnification capability.

TABLE 3 Health welfare reference values for tilapia breeding (BR, breeding; NU, nursery; GO, grow-out) phases.

Production phase			Indicators	Scores	Description or reference values	References
BR		GO				
✓	✗	✓	Eyes	1	Normal and healthy appearance	(39, 60, 61)
				2	Unilateral hemorrhage, exophthalmos, or traumatic injury	
				3	Bilateral bleeding, exophthalmos, or traumatic injury; chronic condition, impaired vision	
✓	✗	✓	Jaw/lips	1	Normal and healthy appearance	(60, 62, 63)
				2	Mild injury or deformity (without affecting eating)	
				3	Bleeding, redness, severe injury or deformity (affecting eating)	
✓	✗	✓	Operculum	1	Normal and healthy appearance	(63–65)
				2	Absence of tissue (<25%)	
				3	Bleeding, redness, absence of tissue (≥ 25%)	
✓	✗	✓	Skin	1	Normal, healthy appearance, scar tissue	(39, 66, 67)
				2	Punctual loss of scales, ulcers, or superficial lesions <1 cm^2	
				3	Generalized bristling or loss of scales, ulcers, or superficial lesions >1 cm^2, redness, necrosis, darkening or lightening, bleeding, swelling, presence of parasites	
✓	✗	✓	Fins	1	Normal and healthy appearance	(67–69)
				2	Scar tissue, mild necrosis, or splitting	
				3	Severe necrosis, splitting or bleeding, redness, exposure to rays, adhered foreign body, ectoparasite	
✓	✗	✓	Gills	1	Normal and healthy appearance	(40, 60, 61, 66, 68, 70, 71)
				2	Light injury, mild necrosis, splitting or thickening	
				3	Bleeding, redness, pallor, severe necrosis, splitting or thickening, excess of mucus, spots, swelling, deformation, adhered foreign body, ectoparasite	
✓	✗	✓	Spine	1	Normal and healthy appearance	(61, 63, 65)
				2	Light deformity (kyphosis, lordosis or scoliosis, normal body weight)	
				3	Severe deformity (kyphosis, lordosis or scoliosis, emaciation)	
✓	✗	✗	Conditioning/breeding interval (days)	1	≥10	(34, 39, 72, 73)
				2	5–9	
				3	≤ 4	
✓	✗	✓	Invasive procedures	1	No invasive procedure	(23, 34, 74)
				2	Microchipping with anesthesia, mouth egg collection	
				3	Microchipping without anesthesia; mouth clipping; up-rooting of dorsal spines	
✓	✗	✗	Sexual maturation	1	Mature animals. Male: Reddish colouration under the jaw; release milt when slight pressure is applied to the abdomen. Female: Ready to spawn grayish colouration under the jaw, pink to red and protruding genital papilla, opened genital pore, distended abdomen	(29)
				2	Male: do not release milt when the abdomen is pressed. Female: Pink to yellow, slightly opened genital pore, slightly distended abdomen	
				3	Male: do not release milt when the abdomen is pressed. Female: Spawned: Red genital papilla, compressed abdomen aspect or; Immature: White to clear and flat genital papilla, regular abdomen aspect	

(Continued)

TABLE 3 (Continued)

Production phase			Indicators	Scores	Description or reference values	References
BR		GO				
✓	✗	✗	Breeding control	1	Microchipping and family physical restrain	(74, 75)
				2	Family physicals restrain without individual identification	
				3	No breeding control	
✓	✗	✓	Mortality (%)	1	≤ 10	(23, 25)
				2	11–24	
				3	≥ 25	
✗	✓	✗	Macroscopical aspect	1	≥ 90 spherical and translucent, with yellowish colouration; the remaining with an opaque aspect	(76–79)
				2	70–89 spherical and translucent, with yellowish colouration; the remaining with an opaque aspect	
				3	≤ 69% spherical and translucent eggs and or detection of some reddish or clustered eggs; presenting white or yellow spots	

Indicators included (✓) or not included (✗) in each production phase.

The indicators exclusively adopted for the larvae were hatching rate, caudal fin formation, and yolk sac. On the other hand, gill covers and fins could only be observed in the post-larvae and fingerlings, as these structures were not fully developed in the larvae. Similarly, fish emaciation was observed only after yolk consumption i.e., in the post-larval stage. Therefore, the "emaciation" indicator was included in the protocols for assessing the welfare level of post-larvae and fingerlings. Due to the difficulty of handling fish during the early life stages and the fragility of organs during physical examination, it was impractical to evaluate the gill condition during the nursery phase. Simply manipulating the larvae during physical examination in the early days of life can cause damage to the skin, eyes, and internal organs, thereby biasing the assessment results. Thus, the convenience and feasibility of applying the protocol for tilapia larvae should be evaluated on a case-by-case basis.

3.3. Nutritional welfare indicators for Nile tilapia

Four relevant nutritional indicators applicable to all cultivation phases have been defined: crude protein content in the feed provided to the fish, feed quantity with the biomass of the batch, feeding frequency, and feeding distribution range in the respective cultivation system. The reference values for these indicators were determined based on the weight of tilapia (Table 5). Considering the physiological and energy demands and the management practices adopted during the reproduction phase, two sub-stages were established for calculating the amount of feed provided to the broodstock: "maintenance" and "breeding". In the grow-out phase, the feed conversion rate (FCR) was incorporated in addition to the mentioned indicators.

3.4. Behavioral welfare indicators for Nile tilapia

3.4.1. Breeding (BR) and grow-out (GO)

Behavioral welfare indicators have been established under the management practices commonly adopted during the breeding (BR) and grow-out (GO) phases (Table 6). In both phases, the selected indicators include the effectiveness of anesthesia during invasive procedures and feeding behavior. Regarding feeding, monitoring the time required for fish to capture and entirely consume the provided food is essential. Additionally, during the grow-out phase, the swimming behavior of tilapia during harvesting was considered, along with the total time until the loss of consciousness during slaughter. This latter indicator encompasses the period from the start of the procedure until the point where the animal demonstrates a complete absence of clinical reflexes.

3.4.2. Nursery (NU) phase

Swimming behavior was selected as the sole practical and viable indicator for the visual analysis of larvae and post-larvae, establishing swimming characteristics across different phases (Table 7). The feeding behavior was also included as a welfare indicator for the post-larval stage.

3.5. Weights for calculating the partial (PWIs) and general welfare index (GWI)

The weights assigned to determine the importance of each adopted welfare indicator were established based on the number of publications identified in the Google Scholar platform. These values were obtained through a combination of general search

TABLE 4 Health welfare reference values for tilapia nursery (NU) phase, more specifically during larvae (L), post-larvae (P), and fingerlings (F) stages.

Stages			Indicators	Score	Reference values	References
L	P	F				
✓	✗	✗	Hatching rate (% of eggs)	1	≥ 9	(77)
				2	75–89	
				3	≤ 74	
✓	✓	✓	Eyes	1	Normal and healthy appearance	(40, 80–82)
				2	Unilateral: malformation or absence; exophthalmos, redness, darkening, corneal opacity, impaired vision	
				3	Bilateral: malformation or absence; exophthalmos, redness, darkening, corneal opacity, impaired vision	
✓	✓	✓	Jaws/lips/head	1	Normal and healthy appearance	(63, 83, 84)
				2	Malformation without possible feeding restriction	
				3	Malformation with possible feeding restriction, injury, ulcers, necrosis	
✓	✗	✗	Skin	1	Fully pigmented (melanophores throughout the dorsal, ventral, and mediolateral region of the body)	(60, 63, 82, 83, 85)
				2	Partially pigmented (melanophores for some regions of the body)	
				3	Completely translucent or grayish-pale body; redness, paleness, darkening, ectoparasites, white or black spots, bleeding, swelling, ectoparasites, or increase in mucus secretion	
✗	✓	✓	Skin	1	Normal and healthy appearance	(39, 66, 67)
				2	Scar tissue, ulcers, or superficial lesions	
				3	Severe ulcers or lesions, redness, necrosis, white or black spots, cysts, darkening or lightening, bleeding, swelling, ectoparasites, or increase in mucus secretion	
✓	✗	✗	Tail	1	Normal and healthy appearance	(82, 83)
				2	Malformation without movement restriction	
				3	Malformation with movement restriction, darkening, redness	
✓	✓	✓	Spine	1	Functional and healthy appearance	(83)
				2	Malformation without movement restriction	
				3	Malformation with movement restriction	
✓	✗	✗	Yolk sac	1	Functional and healthy appearance	(81, 86)
				2	Malformation without size reduction	
				3	Malformation with size reduction (atrophy), hemorrhage, red spots, sub-epithelial oedema	
✗	✓	✓	Operculum	1	Normal and healthy appearance	(63, 84)
				2	Malformation or lesion causing the absence of tissue (<25% of gills covering)	
				3	Bleeding, swelling, redness, absence of tissue (≥ 25% of gills covering)	
✗	✓	✓	Fins	1	Functional and healthy appearance	(71, 82–85)
				2	Malformation (partial absence), ray deviations	
				3	Necrosis, redness, darkness, white spots, total absence	

(Continued)

TABLE 4 (Continued)

Stages			Indicators	Score	Reference values	References
L	P	F				
✗	✓	✓	Emaciation state	1	No signs of emaciation	(83, 87)
				2	Discrete emaciation	
				3	Advanced emaciation	
✓	✓	✓	Mortality of the batch (%)	1	≤ 10	(81, 88, 89)
				2	11–15	
				3	≥ 16	

Indicators included (✓) or not included (✗) in each production phase.

terms ("Oreochromis niloticus" AND "aquaculture" AND the respective life phase) and specific search terms presented in Tables 8–10.

Figure 3 illustrates the application of the welfare protocol for *O. niloticus* during the grow-out phase. The data were simulated using a Microsoft Excel spreadsheet and are derived from a hypothetical but commonly observed scenario in commercial tilapia farming. Based on the example, partial welfare indices related to the environment (PWI_{En}), health (PWI_{He}), nutrition (PWI_{Nu}), and behavior (PWI_{Be}) of the fish are calculated. In the analyzed, the first three indices indicate a moderate level of welfare for the cultivated fish. However, the behavioral index suggests a low level of welfare due to improper management practices during harvesting and slaughter. The simulated scenario calculated the general welfare index (GWI) at 0.59, considered moderate. The confidence level in this result was the highest, as all indicators were analyzed. Examples of these index calculations during the breeding and nursery phases are included in Supplementary Figures 1, 2.

4. Discussion

The current paradigm regarding the welfare of farmed fish suggests that the evaluation parameters used should be specific to each species, considering the animals' developmental stage and the system in which they are raised. These parameters should also encompass indicators that address the fish's physical, nutritional, environmental, and behavioral aspects (13, 23, 25, 26, 87, 119, 122). However, the understanding and investigation of the psychological dimensions, although constituting one of the animal freedoms, represent a field of scientific knowledge still in its early and nascent stages, especially when compared to the progress achieved regarding animals involved in terrestrial agriculture. Notably, the impacts of domestication on the welfare of farmed fish are more complex to analyse than those faced in welfare studies of land animals that serve as human food sources (123). This is because fish have significantly different genetic, physiological, and behavioral characteristics compared to land animals, as well as experiencing a completely different sensory universe (124, 125). Thus, developing empathy for fish and understanding their needs entails a series of challenges to be evaluated in the field, which makes it impractical to include them in operational welfare protocols for tilapia.

The welfare of any organism is a dynamic state (126, 127), and the systems used to monitor it should be flexible enough to adapt to changes in its welfare state (17). There is also an understanding of the need for protocols to consider the interaction between different indicators (119, 122, 128), providing relevant information about the overall quality of life of the animals (129, 130). In this context, despite the recent trend of an increasing number of physiological or molecular parameters being tested and recommended and despite the effectiveness of these indicators in laboratory conditions (131–135), the proposed welfare indicators should apply to the practical requirements and routines of commercial fish breeding, larviculture, and grow-out operations (13, 17, 136).

Thus, good operational welfare indicators for farmed fish can be defined as those that address biologically relevant aspects, are easy to use, preferably non-invasive and low-cost (130, 137); reliable, comparable, suitable for aquaculture practices, and appropriate for specific systems or routines (16). They should identify welfare problems and risks to animal welfare and serve as a basis for technical decision-making by producers (138), enabling timely corrections. In contrast, it is highly unlikely that expensive, complex, unreliable, or time-consuming tools or techniques will be adopted and incorporated by the aquaculture industry (139).

4.1. Changes in the welfare protocol developed for the grow-out phase of tilapia

When we originally proposed the method and indices that our group has already applied to assess the degree of welfare in grass carp and white-leg shrimp (25, 26), we emphasized that the indicators, their reference values, scores, and weights would need to be periodically reviewed and updated as scientific knowledge advances on the subject. In this article, we put this concept into practice by applying our metrics to assess the welfare of tilapia not only during the grow-out phase but throughout all life stages, whilst also revising and advancing the knowledge generated previously. The indicators now applied to tilapia in the grow-out phase had already been tested and validated by our group under field

TABLE 5 Nutritional welfare reference values for tilapia breeding (BR), nursery (NU), and grow-out (GO) phases and different weights*.

Indicators	Phases							References
	Scores	BR	NU		GO			
			(≤ 0.5 g)	(0.6–5.0 g)	(5.1–30 g)	(31–150 g)	(151–1,000 g)	
Feed crude protein (%)	1	30–45	40–50	32–40	35–40	28–36	28–	(32, 39, 90–99)
	2	25–29	28–39	28–31	28–34	20–27	20–27	
	3	≤ 24–≥ 46	≤ 27–≥ 51	≤ 27–≥ 41	≤ 27–≥ 41	≤ 19–≥ 37	≤ 19–≥ 37	
Amount of feed (% biomass)**	1	⊗	15–30	4–15	4–8	3–6	≥ 2	(30, 45, 88, 99–104)
	2	⊗	10–14	3–14	3	2	1	
	3	⊗	≤ 10–≥ 31	≤ 2	≤ 2	≤ 1	< 1	
Amount of feed during maintenance (% biomass)*	1	≥ 2	⊗	⊗	⊗	⊗	⊗	(94, 105–107)
	2	1						
	3	< 1						
Amount of feed during mating (% biomass)*	1	3–5						(94, 105–107)
	2	2	⊗	⊗	⊗	⊗	⊗	
	3	≤ 1						
Feeding frequency (times/day)	1	⊗	5–8	≥ 3	≥ 3	≥ 2	≥ 2	(108–111)
	2	⊗	2–4	2	2	1	1	
	3	⊗	≤ 1	≤ 1	< 1	< 1	< 1	
Feeding frequency during maintenance (times/day)	1	≥ 2						(108–110)
	2	1	⊗	⊗	⊗	⊗	⊗	
	3	< 1						
Feeding frequency during mating (times/day)	1	> 1						(112, 113)
	2	1	⊗	⊗	⊗	⊗	⊗	
	3	< 1						
Food distribution (% of water surface area reach)	1				≥ 75 of surface area			(23, 111)
	2				50–74 of surface area			
	3				≤ 49 of surface area			

(Continued)

TABLE 5 (Continued)

Indicators	Phases							References
	Scores	BR	NU		GO			
			(≤ 0.5 g)	(0.6–5.0 g)	(5.1–30 g)	(31–150 g)	(151–1,000 g)	
FCR	1	✗	✗	✗	≤ 1.0	≤ 1.3	≤ 1.6	(110, 114, 115)
	2				1.1–1.6	1.4–1.7	1.7–2.0	
	3				≥ 1.7	≥ 1.8	≥ 2.1	

Not included (✗) in each production phase.
*Fish weight adapted from Borges (116). **Always, when rounding a number from one decimal place to none, if the first number after the decimal point is 5 or greater, add 1 to the number before the decimal point; if it is <5, keep the number before the decimal point unchanged.

TABLE 6 Behavioral welfare reference values for tilapia breeding (BR) and grow-out (GO) phases.

Stages		Management	Indicators	Scores	Reference values	Reference
BR	GO					
✓	✗	Invasive procedures (chipping, tagging, clipping)	Anesthesia–surgical stage (lack of balance and swimming; reduction of the opercular rate	1	Induction in 1–3 min; recovery in ≤ 5 min	(117, 118)
				2	Induction and or recovery in > 5 min	
				3	No induction or no recovery; death	
✓	✓	Feeding	Feed intake (minutes)	1	180–300	(23)
				2	120–179 or 301–419	
				3	≤ 119 or ≥ 420	
✗	✓	Invasive procedure (Vaccination)	Anesthesia–surgical stage (lack of balance and swimming; reduction of the opercular rate	1	Induction in 1–3 min; recovery in ≤ 5 min	(117, 118)
				2	Induction and or recovery in > 5 min	
				3	No induction or no recovery; death	
✗	✓	Harvest (partial or total)	Swimming behavior	1	Most fish with regular swimming and or few body parts on the surface	(23, 119)
				2	Most of the fish show restless swimming behavior, swimming in different directions and or jumping	
				3	Most fish with decreasing activity; fish trapped against the net or swimming sideways; exposure of the body to air; exhaustion	
✗	✓	Stunning during slaughter	Reflexes*	1	Instantaneous loss of EQ, TGR, VER, OR	(119–121)
				2	Instantaneous loss of EQ and TGR, progressive loss of VER and OR in ≤ 30s	
				3	Progressive loss of E.Q., T.G.R., VER and OR in ≥ 31s	

*EQ, equilibrium; TGR, tail grab reflex; OR, opercular beating rate; VER, vestibule-ocular reflex.

conditions (23). In this article, in addition to revising and updating the indicators and reference values for this cultivation phase, we simplified the evaluation structure by reducing the number of scores for each indicator from four to three. This approach improves field evaluation, making it more objective and dynamic than protocols with higher scores (87, 140). This reduction in scores regarding changes affecting eyes, gills, and fins, for example, which often indicate pain and significant diseases, reduces subjectivity in interpreting moderate lesions, thus enhancing evaluation accuracy. However, the blood glucose indicator was removed from the current protocol due to the invasiveness of the method. Additionally, it should be considered that the blood sampling procedure itself can alter the parameters, causing acute stress to the animals.

Regarding environmental indicators such as temperature, pH, and dissolved oxygen, it is necessary to consider that these parameters naturally vary throughout the day in cultivation ponds. However, the animals can adapt (141) as long as the changes occur within tolerable limits for the species. To avoid conflicts between different values considered acceptable in the literature, we adjusted the new scores to reflect these possible changes in water quality in tilapia cultivation ponds.

The stocking density indicator was removed from the current protocol. Although it is evident that stocking density directly influences the degree of welfare and fish health (142–144), establishing fixed values for this parameter is highly subjective. Defining the ideal density regarding welfare depends significantly on the characteristics of the fish, environmental and nutritional resources provided to them, management practices, fish size, genetic characteristics, and other factors (145).

We also found several operational and conceptual difficulties regarding the shading indicator proposed in the previous protocol. Recent studies present conflicting data that do not accurately reflect the reality of pond cultures, mainly due to inadequate consideration of light intensity and its impact on water quality (148, 149). Initially, we proposed a uniform percentage of shading over the surface of the net pen or pond, typically achieved by using protective and shading screens. However, ponds often have localized shade caused by trees or topographic features in their surrounding areas. This led to misinterpretations during the application of the protocol since localized shading is detrimental to the welfare of tilapia (150). Therefore, we chose to exclude this indicator from the current protocol.

In the original protocol for tilapia grow-out, we established crude protein content (CP), feed conversion ratio (FCR), condition factor (K), and feeding behavior as nutritional indicators. However, after reviewing the data from applying the protocol in various countries (Pedrazzani, unpublished data), we found significant genetic variability amongst cultivated *O. niloticus* strains, which led to morphological variations in the fish. Sometimes the strains were naturally broader than long, and *vice versa*, which affected the value of the condition factor (K) without any relation to fish welfare. Therefore, adjusting the formula for each population or strain of the same species proved impractical for standardization.

The nutritional freedom indicator has now been adjusted to calculate welfare indices by category. Thus, to facilitate tracking the feeding history on farms, we standardized four indicators for all cultivation phases: feed quantity, protein content, feeding frequency, and feed distribution within the pond. Due to the difficulty of capturing and weighing fish in early cultivation, FCR monitoring was suggested only for the grow-out phase.

The behavioral indicators during feeding, harvesting, and slaughter were kept the same as in the original protocol, but their scores were readjusted, aiming to reduce subjectivity during the evaluation, as previously discussed regarding animal suffering associated with health freedom.

TABLE 7 Behavioral welfare reference values for tilapia during the nursery (NU) phase.

Indicators	Score	Reference values	References
Larvae swimming behavior	1	Most of the sampled animals presented active swimming against the current	(70, 71, 146, 147)
	2	Most of the sampled animals presented reduced swimming activity against the current	
	3	> 10% of the sample gathered and remained immobile in the center of the container; swimming rapidly, loose equilibrium; present sideways swimming; rubbing against hard surfaces; gasping at the surface	
Post-larvae and fingerlings' swimming behavior	1	Most of the sampled animals presented active swimming against the current and swimming vertically or horizontally in the water column and with short periods at the tank bottom	(40, 70, 71, 146)
	2	Most of the sampled animals presented reduced swimming activity in the water column, or < 10% of the sample present at the tank bottom	
	3	> 10% of the sampled animals presented spiral swimming, efforts to swallow air or float on the water surface; rubbing against hard surfaces; gasping at the surface or being immobile at the tank bottom	
Post-larvae and fingerlings' feed intake (min)	1	180–300	
	2	120–179 or 301–419	(23)
	3	≤ 119 or ≥ 420	

An essential conceptual aspect concerns fish slaughter, which we associate with the grow-out phase. Although the industrial-scale slaughter of tilapia is already a reality (151, 152), globally, this practice remains an exception rather than the rule. Transporting water and live tilapia to the processing plants is costly and requires complex logistics and appropriate slaughter facilities. In most cases, fish are sold alive in markets or slaughtered on-site at the farms where they are cultivated (29, 39, 57, 152–154). Under these circumstances, it is common for animals to be slaughtered without prior stunning, often asphyxiated in the air or ice (155–157). Thus, we believe that a more practical way to improve fish welfare is to consider slaughter as part of the grow-out process. However, considering the expansion of international tilapia trade, the evolution of industrial aquaculture, and the increasing interest of consumers in the quality of the product and the welfare of farmed fish, it is plausible to project that slaughter will soon become a genuinely autonomous stage in the tilapia production cycle.

TABLE 8 Number of documents and the respective weights of the indicators, established from the general search terms ("*Oreochromis niloticus*" AND "aquaculture" AND "breeding"-larva -nursery AND the specific search terms used in Google Scholar in July 2023).

Freedom	Indicator	Specific search terms	Number of documents (*n*)	Weight [ln(*n*)]
Environmental	Alkalinity	"alkalinity"	1.510	7
	Aquatic predators and other interspecific inhabitants	"aquatic predators" OR "interspecific inhabitants"	43	4
	Dissolved oxygen	"dissolved oxygen"	6.780	9
	Hapas net cleaning	"hapa" AND "clean"	76	4
	Nitrite	"nitrite"	2.560	8
	Non-ionized ammonia	"ammonia"	5.250	9
	pH	"pH"	13.500	10
	Photoperiod	"photoperiod"	3.460	8
	Sex ratio (male: female)	"sex ratio"	2.550	8
	Temperature	"temperature"	15.500	10
	Terrestrial predators	"terrestrial" AND "predator"	627	6
	Transparency	"transparency"	976	7
Health	Breeding control	"breeding control"	10	2
	Conditioning/breeding interval (days)	"conditioning" OR "breeding interval"	729	7
	Eggs–macroscopical aspects	"eggs" AND "macroscopic"	240	5
	Eyes	"eyes"	674	7
	Fins	"fins"	2.480	8
	Gills	"gills"	4.000	8
	Invasive procedures	"chipping" OR "tagging" OR "clipping."	847	7
	Jaws/lips	"jaw" OR "lips"	857	7
	Mortality (%)	"mortality"	8.020	9
	Operculum	"operculum"	573	6
	Sexual maturation	"maturation"	5.510	9
	Skin	"skin"	5.090	9
	Spine	"spine"	657	6
Nutritional	Amount of feed	"amount of feed"	910	7
	Feed Crude Protein	"crude protein"	4.700	8
	Feeding frequency	"feeding frequency"	834	7
	Food distribution	"food distribution"	91	5
Behavioral	Anesthesia–surgical stage	"anesthesia" OR "anesthesia"	486	6
	Feed intake	"feed intake"	3.290	8
	Swimming behavior	"swimming behavior" OR "swimming behavior"	432	6

4.2. First protocol of tilapia welfare for the reproduction and nursery stages

The sanitary indices employed during the grow-out phase were maintained with those developed for adult fish and further expanded. The additions were intended to integrate essential management practices commonly used in commercial tilapia reproduction and larviculture facilities. Indicators such as the stage of sexual maturation, the time interval adopted for the recovery/conditioning of breeders between reproductive cycles, and the assessment of the adoption or not of methods or mechanisms of control to avoid inbreeding amongst breeder batches were included. In addition to these, other less intuitive indicators were proposed, such as, for instance, the maximum

TABLE 9 Number of documents and the respective weights of the indicators, established from the general search terms ("*Oreochromis niloticus*" AND "aquaculture" AND nursery" OR "larviculture" – breeding AND the specific search terms used in Google Scholar in July 2023 + specific search terms).

Freedom	Indicator	Specific search terms	Number of documents (*n*)	Weight [ln(*n*)]
Environmental	Alkalinity	"alkalinity"	932	7
	Aquatic predators and other interspecific inhabitants	"aquatic predators" OR "interspecific inhabitants"	9	2
	Dissolved oxygen	"dissolved oxygen"	2.810	8
	Nitrite	"nitrite"	1.550	7
	Non-ionized ammonia	"ammonia"	2.620	8
	pH	"ph"	4.850	8
	Photoperiod	"photoperiod"	979	7
	Temperature	"temperature"	4.660	8
Health	Emaciation state	"emaciation"	35	4
	Eyes	"eyes"	273	6
	Fins	"fins"	569	6
	Hatching rate	"hatching"	1.010	7
	Jaws/lips/head	"jaw" OR "lips" OR "head"	1.350	7
	Mortality (%)	"mortality"	2.780	8
	Operculum	"operculum"	134	5
	Skin	"skin"	1.230	7
	Spine	"spine"	134	5
	Tail	"tail"	523	6
	Yolk sac	"yolk sac"	364	6
Nutritional	Amount of feed	"amount of feed"	550	6
	Feed crude protein	"crude protein"	2.090	8
	Feeding frequency	"feeding frequency"	62	4
	Food distribution	"food distribution"	37	4
Behavioral	Feed intake	"feed intake"	13,50	7
	Swimming behavior	"swimming behavior"	918	7

intervals adopted for cleaning the hapas where the breeders are kept during reproduction—an essential factor to prevent the obstruction of the screens since this hinders the renewal of water and compromises the health of the breeders and can cause not only a reduction of zootechnical indices but also a decrease in immunity and the emergence of diseases (30, 57, 158). The proportion between males and females used during reproduction was another indicator included in the protocol since it interferes with population dynamics and the degree of aggressiveness of the males during the mating phase (30).

For the behavioral assessment of the breeders, we emphasize the recommendation for using anesthesia during any invasive procedures. We kept the "feeding behavior" indicator, although it is relevant to note that female tilapias reduce their food intake during the mating and spawning periods by incubating the eggs in the mouth, whilst males can increase their feed intake in the same period (159). This protocol included the photoperiod due to its central role in the natural induction of sexual maturation and in defining reproductive rates (160, 161).

Concerning the welfare of eggs, larvae, and post-larvae, considering that this is still a controversial topic, that it is in its initial stages of unveiling scientific knowledge, and that there is a tremendous natural vulnerability of larvae and post-larvae to handling, which requires even greater caution and rigor; we advocate for the implementation of the protocol proposed in this phase of tilapia cultivation. However, at the same time, we suggest that the suitability and necessity of its application be determined on a case-by-case basis, considering the overall conditions of the batch and the local structural and operational capacity to assess the proposed indicators.

The welfare of eggs and larvae involves the parents' nutritional, social, and environmental experiences during their development (162). The environment likely influences the epigenetic pattern of gametes, embryos, or adult organisms (163). During gametogenesis, the DNA is reprogrammed, and

TABLE 10 Number of documents and the respective weights of the indicators established from the general search terms ("*Oreochromis niloticus*" AND "aquaculture" AND "farming" AND "pond" AND the specific search terms used in Google Scholar in July 2023).

Freedom	Indicator	Specific search terms	Number of documents (*n*)	Weight [ln(*n*)]
Environmental	Alkalinity	"alkalinity"	2.850	8
	Aquatic predators and other interspecific inhabitants	"aquatic predators" OR "interspecific inhabitants"	44	4
	Dissolved oxygen	"dissolved oxygen"	8.500	9
	Nitrite	"nitrite"	4.310	8
	Non-ionized ammonia	"ammonia"	7.380	9
	pH	"pH"	12.500	9
	Temperature	"temperature"	12.900	9
	Terrestrial predators	"terrestrial" AND "predator"	533	6
	Transparency	"transparency"	202	5
Health	Eyes	"eyes"	196	5
	Fins	"fins"	255	6
	Gills	"gills"	370	6
	Invasive procedures	"chipping" OR "tagging"	505	6
	Jaws/lips	"jaw" OR "lips"	467	6
	Mortality (%)	"mortality"	7.740	9
	Operculum	"operculum"	449	6
	Skin	"skin"	3.830	8
	Spine	"spine"	355	6
Nutritional	Amount of feed	"amount of feed"	1.710	7
	Feed conversion ratio (FCR.)	"F.C.R."	4.550	8
	Feed crude protein	"crude protein"	107	5
	Feeding frequency	"feeding frequency"	1.260	7
	Food distribution	"food distribution"	199	5
Behavioral	Feed intake	"feed intake"	87	4
	Harvest (partial or total)—swimming behavior	"swimming behavior" OR "swimming behavior"	330	6
	Invasive procedure (vaccination)—Anesthesia—surgical stage	"vaccination"	1.060	7
	Stunning during slaughter—reflexes	"stunning"	129	5

this information will be transmitted to the offspring, resulting in transgenerational effects that directly impact the quantity, viability, social status, neurogenesis, and adaptation of future generations (162). Sneddon et al. (1) highlight that fish larvae have various brain structures that process emotions and learning, although they are not identical to the human brain. Lopez et al. (164) demonstrated that zebrafish larvae at 5 days post-fertilization (5 daf) respond to harmful and potentially painful stimulation caused by environmental acidification, exhibiting similar behaviors to adult fish and reducing their activities. This response was alleviated by analgesic drugs such as lidocaine and morphine. Furthermore, different larval rearing protocols can have a significant impact on larval size and mass, survival rates, and the sex ratio of larvae (165). Therefore, it is necessary to consider that welfare should be understood as continuous and intergenerational, as there is a direct link between offspring adaptation and the resources provided by parents (162, 166). On the other hand, the quality of the eggs and larvae will also significantly impact the welfare and health of tilapias throughout their lives (167).

Another argument to be considered is that, despite the legislation and regulatory frameworks of the vast majority of countries still not protecting fish larvae, we must consider the scientific arguments linked to the presence of sentience in larvae and fry (1, 162, 164). In this sense, we advocate the application of the proposed protocol based on the precautionary principle, which establishes that when evidence of sentience is inconclusive, we should "give the benefit of the doubt" to the animal or "err on the side of caution" (168). Thus, we understand that the theme "welfare in the early stages of fish life" has relevance to be applied in current aquaculture but, mainly, that it will have a significant

General Welfare Index (GWI) for the Nile tilapia *(Oreochromis niloticus)*
Grow-out Stage (GO)

Environmental (En)	Score (S)	Weight (Y)	S X W		
				ΣY_{An}	69
Alkalinity	1	8	8	ΣY_{Max}	69
Aquatic predators and other interspecific inhabitants	1	4	4	$\Sigma(SxY)$	102
Dissolved oxygen	3	9	27	*nS*	9
Nitrite	1	8	8	*tS*	9
Non-ionized ammonia	1	9	9	PWI_{En}	0,51
pH	1	9	9	CL_{En}	1,00
Temperature	2	9	19		
Terrestrial predators	2	6	13		
Transparency	1	5	5		

Health (He)	Score (S)	Weight (Y)	S x Y		
				ΣY_{he}	58
Eyes	1	5	5	ΣY_{Max}	58
Fins	2	6	11	$\Sigma(SxY)$	76
Gills	1	6	6	*nS*	9
Invasive procedures	1	6	6	*tS*	9
Jaws/lips	1	6	6	PWI_{He}	0,65
Mortality (%)	1	9	9	CL_{He}	1,00
Operculum	3	6	18		
Skin	1	8	8		
Spine	1	6	6		

Nutritional (Ne)	Score (S)	Weight (Y)	S x Y		
				ΣY_{Nu}	33
Amount of feed	1	7	7	ΣY_{Max}	33
Feed convertion ratio (FCR)	2	8	17	$\Sigma(SxY)$	41
Feed Crude Protein	1	5	5	*nS*	5
Feeding frequency	1	7	7	*tS*	5
Food distribution	1	5	5	PWI_{Ne}	0,70
				CL_{Ne}	1,00

Behavioral (Be)	Score (S)	Weight (Y)	S x Y		
				ΣY_{He}	22
Feed intake	1	4	4	ΣY_{Max}	22
Harvest (partial or total) - Swimming behaviour	2	6	12	$\Sigma(SxY)$	33
Invasive procedure (Vaccination) - Anaesthesia surgical stage	1	7	7	*nS*	4
Stunning during slaughter - Reflexes	2	5	10	*tS*	4
				PWI_{Be}	0,51
				CL_{Be}	1,00

Mortality (%)	10	
kl	1	
General Walfare Index (*GWI*)	0,59	(min= 0,
General Confidence Level (*GCL*)	1,00	máx =1)

Legend		
Colours	Fill the number	Cell filling
	Automatic filling	
	High	Interpretation
	Medium	
	Low	
	Critical	
Score (S)	0	Data not obtained
	1-3	Data obtained
Terms	PWI_x	Partial Welfare Indice for *x* liberty
	CLI_x	Confidence level for *x* liberty
	kl	Knockout value
	GWI	General Walfare Index (GWI)
	GCL	General Confidence Level

FIGURE 3
Example of calculating partial welfare indices for tilapia during the grow-out phase in land-based ponds and the overall welfare index using a calculation model developed in the Microsoft Excel application. In this hypothetical case, the model indicates a moderate level of welfare and a high level of confidence concerning this result.

impact on the aquaculture that will be practiced in the coming years, possibly under a scenario of regulatory restrictions and rigorous governance practices (169). Therefore, be it for biological, ethical, moral, or commercial reasons, and even recognizing the fragility of the current stage of knowledge on the subject and the need for subsequent discussions on the effectiveness of the application of welfare protocols for the larval and post-larval stages of *O. niloticus*, we understand it to be recommendable and, at the same time, almost "inevitable" that the early stages of life be included in animal welfare assessment protocols in fish farming.

We tested and validated their operational feasibility in the laboratory to assess the indicators proposed here for the early stages of tilapia life. The experiments carried out made it possible to identify the most suitable indicators and, at the same time, exclude those that did not meet the established prerequisites.

Some health indicators were not incorporated into the larval protocols, as structures such as fins and gills are still in development during the ontogenetic processes that occur during the nursery phase, making their visual assessment difficult. For post-larvae and fry, it is relevant to consider the "degree of emaciation" as an indirect indicator of feeding effectiveness. The "yolk sac" indicator was included in the assessment of larvae, as organisms at this stage of life still have endogenous energy reserves and, therefore, do not show apparent signs of emaciation (76). In all life stages, we kept the mortality rate as an indicator of welfare, as most fish deaths in captivity are likely preceded or accompanied by suffering. Thus, long-term mortality rates may serve as indicators of the degree of retrospective welfare and signal possible future impacts on the success rates of the enterprise (170).

In the analysis of tilapia larvae and post-larvae, we identified a significant number of articles focussed solely on swimming behavior and the changes commonly related to water contamination or the occurrence of diseases. Therefore, we included only this behavioral indicator for the larval stage once they have endogenous feeding. We also excluded some indicators from this stage, as larvae are produced in laboratories, rendering indicators such as the presence of "terrestrial predators" or "water transparency" irrelevant, for instance.

4.3. Partial and general welfare index (PWIs) and general welfare index (GWI)

Animal welfare should not be linked to cultural differences or subjective criteria but to the species' biology (171). Therefore, quantitative animal welfare assessment is essential for promoting humane and responsible management practices in the animal production industry (172, 173). Moreover, using quantitative and standardized approaches in welfare measurement allows producers to tangibly demonstrate their commitment to animal welfare, which helps build consumer trust and generate new market opportunities (174, 175).

The metrics proposed in this article aim, pioneeringly, to provide a holistic and quantifiable assessment of the welfare of Nile tilapia throughout all stages of its captive life cycle. These metrics were established based on indicators that were simultaneously simple, understandable, and already part of the routine production of the species on a commercial scale. To achieve this, we used indicators representing the nutritional, behavioral, health, and critical environmental conditions to which tilapia are exposed throughout their production process.

Furthermore, this study's partial (PWI) and overall (GWI) welfare indices offer an objective animal welfare assessment. They are based on data and scientifically supported metrics rather than opinions or subjective factors, providing excellent reliability and accuracy. The proposed indices can provide producers with a valuable tool for retrospective and prospective analyses within the same production cycle, enabling informed strategic decisions for the welfare of farmed fish and the profitability and efficiency of their businesses.

There is already recognition within the scientific community that a single score simplifies data interpretation and constitutes a valuable tool for researchers, producers, certifying bodies, and regulatory agencies (87, 122). This characteristic allows the proposed indices to establish a solid foundation for developing animal welfare regulations and guidelines, enabling authorities to define clear and measurable standards to ensure ethical and humane treatment in tilapia farming operations, one of the most critical species in global aquaculture (18).

In this study, PWIs and GWIs follow the same conceptual and mathematical logic applied and extensively discussed concerning grass carp (25) and white-leg shrimp (26). However, the indicators and their respective reference values, scores, and weights are specific to *O. niloticus*, covering aspects of breeding, larviculture, fingerling rearing, and the grow-out phase—in this case, in earthen ponds. Like in previous studies, the weights assigned to each indicator were identified using Google Scholar. These weights ranged from 2 to 10 and were determined based on the number of scientific documents related to each indicator. It should be noted, however, that despite being practical, this method has limitations. For example, the number of publications on a specific topic may not reflect its relevance in a practical context; it may underestimate the importance of less-researched welfare indicators; it can be influenced by the availability of funding for research in specific areas, which may not reflect the importance of those areas for animal welfare; there may also be variations due to the language used in search terms and differences in the consulted databases. Despite these limitations, the approach is robust, standardisable, and encompasses several advantages, including the comprehensiveness and objectivity of evaluating welfare in tilapia farming, recognizing differences that each indicator presents in fish welfare, and the ability to update and refine as new research is published. Subsequent analysis can explore alternative methods for assigning weights to welfare indicators and further examine how the relative importance of these indicators may vary across different life stages.

5. Conclusion

In this study, we have proposed a comprehensive and quantitative approach to assess the welfare of Nile tilapia throughout their life cycle (eggs, larvae, post-larvae, juveniles, and adults) and all cultivation phases (breeding, nursery, and grow-out) in captivity. This approach has generated a valuable and standardized tool for aquaculturists to monitor and improve their production systems, with the potential to enhance the welfare of *O. niloticus* in aquaculture significantly.

The proposed methods will allow for a comprehensive, precise, and tailored analysis of welfare throughout the entire life cycle and all stages of tilapia farming. The developed quantitative indices will enable a standardized comparison of animal welfare amongst different enterprises, locations, and periods, serving as relevant tools to evaluate the effectiveness of other management practices and identify areas that need improvement. This approach can potentially enhance farming practices and promote the welfare of tilapia whilst providing a valuable tool for advancing more sustainable and ethical aquaculture practices.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The animal study was approved by Animal Use Ethics Committee of the Agricultural Sciences Campus (CEUA) of the University of Paraná under protocol 021/2023. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

AP: Conceptualization, Data curation, Formal analysis, Investigation, Validation, Writing—original draft, Writing—review and editing. NC: Investigation, Methodology, Resources, Validation, Writing—original draft. MQ: Project administration, Supervision, Writing—review and editing. CT: Investigation, Validation. VB: Validation. AO: Conceptualization, Methodology, Investigation, Formal analysis, Writing—original draft, Writing—review and editing, Supervision, Project administration.

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Conflict of interest

AP was employed by Wai Ora Aquaculture and Environmental Technology Ltd. MQ was employed by FAI Farms from Brazil.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2023.1268396/full#supplementary-material

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EDITED BY
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University of Santo Tomas, Chile
Carlos Rosas,
National Autonomous University of Mexico,
Mexico

*CORRESPONDENCE
Timothy Robert Wiese
✉ trw1@stir.ac.uk
Sonia Rey Planellas
✉ Sonia.reyplanellas@stir.ac.uk
Francoise Wemelsfelder
✉ Francoise.Wemelsfelder@sruc.ac.uk

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Qualitative Behavioural Assessment as a welfare indicator for farmed Atlantic salmon (*Salmo salar)* in response to a stressful challenge

Timothy Robert Wiese[1]*, Sonia Rey Planellas[1]*, Monica Betancor[1], Marie Haskell[2], Susan Jarvis[3], Andrew Davie[4], Francoise Wemelsfelder[2]* and James F. Turnbull[1]

[1]Institute of Aquaculture, University of Stirling, Stirling, United Kingdom, [2]Scotland's Rural College SRUC, Edinburgh, United Kingdom, [3]Global Academy of Agriculture and Food Systems, University of Edinburgh, Midlothian, United Kingdom, [4]Aquascot Ltd., Alness, United Kingdom

Animal welfare assessments have struggled to investigate the emotional states of animals while focusing solely on available empirical evidence. Qualitative Behavioural Assessment (QBA) may provide insights into an animal's subjective experiences without compromising scientific rigor. Rather than assessing explicit, physical behaviours (i.e., what animals are doing, such as swimming or feeding), QBA describes and quantifies the overall expressive manner in which animals execute those behaviours (i.e., how relaxed or agitated they appear). While QBA has been successfully applied to scientific welfare assessments in a variety of species, its application within aquaculture remains largely unexplored. This study aimed to assess QBA's effectiveness in capturing changes in the emotional behaviour of Atlantic salmon following exposure to a stressful challenge. Nine tanks of juvenile Atlantic salmon were video-recorded every morning for 15 min over a 7-day period, in the middle of which a stressful challenge (intrusive sampling) was conducted on the salmon. The resultant 1-min, 63 video clips were then semi-randomised to avoid predictability and treatment bias for QBA scorers. Twelve salmon-industry professionals generated a list of 16 qualitative descriptors (e.g., relaxed, agitated, stressed) after viewing unrelated video-recordings depicting varying expressive characteristics of salmon in different contexts. A different group of 5 observers, with varied experience of salmon farming, subsequently scored the 16 descriptors for each clip using a Visual Analogue Scale (VAS). Principal Components Analysis (correlation matrix, no rotation) was used to identify perceived patterns of expressive characteristics across the video-clips, which revealed 4 dimensions explaining 74.5% of the variation between clips. PC1, ranging from 'relaxed/content/positive active' to 'unsettled/stressed/spooked/skittish' explained the highest percentage of variation (37%). QBA scores for video-clips on PC1, PC2, and PC4 achieved good inter- and intra-observer reliability. Linear Mixed Effects Models, controlled for observer variation in PC1 scores, showed a significant difference between PC1 scores before and after sampling ($p = 0.03$), with salmon being perceived as more stressed afterwards. PC1 scores also correlated positively with darting behaviours ($r = 0.42$, $p < 0.001$). These results are the first to report QBA's sensitivity to changes in expressive characteristics of salmon following a putatively stressful challenge, demonstrating QBA's potential as a welfare indicator within aquaculture.

KEYWORDS

emotional state, aquaculture, positive welfare, behavioural analysis, qualitative behaviour assessment

1. Introduction

Animal welfare science has faced the challenge of addressing all aspects of welfare without compromising objectivity and the need for empirical evidence. Physical health has long been recognised as an essential component of animal welfare (1–3). However, a widely held perspective now is that animal welfare is ultimately a state that is perceived by the animal itself, and we should therefore also include concerns for the animal's mental well-being (4–6). There is thus a growing demand that welfare assessments, including those for fish, adopt a more holistic approach that places additional focus on monitoring the animal's positive experiences (2, 6–11). Welfare appraisals that adopt this integrated approach, however, inevitably enter the murky waters that are an animal's subjective experiences (12, 13). Despite decades of research trying to resolve this issue, the only progress thus far has been reaching a consensus that there is no single "measure" that can adequately cover what welfare entails (13–16). This dilemma has resulted in the mental well-being of fish often being overlooked in welfare assessments (17).

In 2018, Atlantic salmon accounted for 4.5% of global aquaculture production by tonnage (18). In 2021, production of Scottish Atlantic salmon reached an all-time high of 205,393 tonnes, with more than 50 million smolts transferred to sea in the same year (19). Total tonnage of Scottish farmed salmon, relative to the number of employees on-site, has increased 11-fold within seawater and 6-fold within freshwater between 1985 and 2016 (20). This increase in the numbers of fish relative to farm staff, unavoidably reduces the time available for monitoring the salmon. There is also mounting scientific evidence supporting the sentience of fish (21–24). A UK National survey, involving 1963 members of the public, found that 77% agreed or strongly agreed that fish can feel pain, and 80% agreed that this should therefore be of concern (25). Considering the scale of this industry, there is a clear ethical and economic incentive to develop welfare indicators that are not only practical, but attempt to include aspects of mental well-being (both positive and negative) in their assessment.

To achieve such an assessment, a framework was proposed in which welfare assessments are viewed in the context of a simple question: "Is the animal healthy, and does it have what it wants?" (13). Answering the second, difficult part of this question (i.e., delving into an animal's subjective experiences) may require accepting two arguments. Firstly, that consciousness still presents an impasse for scientific study (3). Secondly, given that animals cannot express their desires/needs in human language, behavioural analysis may provide some of the best insights into what they "want" (12). Behaviours exhibited by an animal are, in essence, the final product of all its own decision-making processes (1, 26). They are the "final common path," as described by Sherrington (27), or, in Charles Darwin's words, the "ultimate phenotype" and "expression of the emotions" (27). Behavioural analysis provides a number of additional advantages over physiological/morphological measures in welfare assessments. Such analyses are frequently non-intrusive (the animal is unaware it is being assessed), and are often quick to observe (1, 28, 29). Behaviour is also gaining recognition as a general, pre-clinical 'early warning system' for issues that may be emerging within the stock (1, 28, 30–32).

Observant farmers are capable of detecting changes in the demeanour and behaviour of their animals (33). Such knowledge, typically gained through years of experience, enables farmers to detect subtle shifts in how animals express themselves when issues arise, even though the exact nature of the problem may remain unclear (34). Qualitative Behavioural Assessment (QBA) is a behavioural assessment tool that benefits from this approach, with its reproducibility and validity demonstrated in previous research (35). QBA is an integrative assessment of the "whole-animal," where observations are made on the animal's dynamic body language (including their appearance, behaviour, and interaction with others and the surrounding environment) as an indicator of its welfare state (36–39). Different aspects of this body language are summarised through a number of 'descriptors' (or terms) such as: relaxed, inquisitive, agitated, or stressed (17, 36, 40). Such terms focus not so much on what an animal does (e.g., feeding), as on how it does this; the expressive characteristics shown in the way it moves (40). Descriptions of such characteristics typically have an emotional connotation, indicating how the animal is experiencing the situation it is in, and QBA is therefore hypothesized to be able to contribute valuable information on an animal's emotional state to studies of animal welfare. Interest in the study of fish emotion has grown in recent years. A variety of reviews conclude that fishes are neuro-physiologically and behaviourally similar enough to mammals to warrant assuming a comparable emotional range in fishes, both in terms of negative and positive emotions (6, 41, 42). Indeed, fish are found to be so intelligent that they are frequently used as models for the study of cognition in mammals (43). There is thus a need for developing and testing methods able to address emotional behaviour in fish, and QBA has been recognised as one such potentially promising method (11).

Previous studies for various livestock species have validated the use of QBA against other welfare indicators, and demonstrated high degrees of inter-observer reliability between observers (33, 44). Additionally, QBA allows for simple, time-efficient, and non-intrusive assessments of an animal's well-being (35, 39). QBA is also the only measure currently included in the EU Welfare Quality® welfare assessment protocols to assess positive emotional states in cattle, pigs, and poultry (45, 46). To date, however, the only QBA study to be applied to fish examined solely the inter/intra-observer reliability and QBA's association with ethograms of salmon behaviour, without the inclusion of any treatments (17). No studies have yet examined fish exposed to stressors, or compared QBA scores in this context to other welfare indicators. Comparing QBA scores against other welfare indicators for salmon may help to further explore what potential role QBA may have as a welfare assessment tool. Darting represents a behavioural response previously recorded in fear-conditioning studies of fish, and is commonly associated with predator avoidance (47–50).

It is considered a stress response which, when increasing in frequency/intensity, may indicate impaired welfare (47, 48, 51). Feed intake is also generally considered a reliable indicator within health and welfare assessments of farmed fish (52). A loss in appetite is potentially a sign of impaired welfare (28, 53). The main aim of this study was therefore to examine QBA's ability to detect differences in the expressive characteristics of Atlantic salmon after exposure to a stressful challenge (i.e., an intrusive sampling event). In addition, this study also aimed to compare these QBA scores against other welfare indicators for salmon; their daily feed intake (as a proxy for appetite) and darting behaviours (i.e., sudden, rapid movements of the salmon).

2. Materials and methods

2.1. Ethical review

Ethical approval for the recording of salmon and QBA work was obtained from the University of Stirling's Animal Welfare & Ethical Review Body (Approval reference no. 2022-6783-5196).

2.2. Experimental set-up

2.2.1. Animals

The juvenile Atlantic salmon used in this study were transferred on November 16th, 2021, from the Niall Bromage Freshwater Research Unit (NBFRU), Denny, to the Marine Environmental Research Laboratory (MERL) in Campbeltown, Argyll and Bute, Scotland. The salmon were around 14 months of age, and weighed on average 285–360 grams. There were ~ 80 smolts in each tank at the start of the recording, with an average stocking density of ~34 kg/m^3.

2.2.2. Husbandry

The salmon were housed in a total of 9 identical flow-through tanks (1.4 m diameter, 750 L volume). Seawater was filtered through a Lacron sand filter (4×100 micron bag filters) before flowing into the tanks to minimise turbidity. Feed was formulated to satisfy the nutrient requirements for Atlantic salmon (54) and contained 46% protein and 24% fat. Automatic belt feeders provided formulated diet salmon pelleted dry feed to all tanks every 20 min between 05:00–09:00 and 16:30–23:30. Dirty water and uneaten feed were flushed out of the tanks through standpipes daily, between 09:00 and 09:15. Any mortalities found during this period were immediately removed. Lights were turned on at exactly 10:30 am each morning.

2.2.3. Treatments (including stressful challenge)

Video clips for this study were recorded around a stressful challenge, conducted on February 18th, 2022. This stressful challenge involved a sampling event which was carried out for another study on these salmon. This required capturing, anaesthetising, and handling each of the salmon out of water for measuring their weight, length, and condition factor. While feed withdrawal was also required 24 h before sampling could be carried out, the recording schedule was designed on the assumption that the main disturbances (i.e., stressful challenge) to the salmon would occur largely as a result of this sampling event. For the purposes of the study that involved the sampling event, a subset of the salmon that were sampled were then euthanised. Fish were euthanised with anaesthetic overdose of Tricaine Methanesulfonate (MS-222) and pithing (Schedule 1) in order to obtain their hepatosomatic index. Following the sampling event, there were approximately 50 salmon left in each tank, with an average stocking density of ~21 kg/m^3.

2.2.4. Camera and tanks set-up

Cameras were installed in the tanks to record video clips for the QBA and behavioural assessments. To do this, every morning at 9 am, GoPro Hero9 Black© cameras were installed at 1 m depth using a fixed metal pole, which was positioned flush against the inside of each tank to ensure the same angle and field of view (FOV) for recordings. This was carried out 90 min before lights went on to allow time for salmon to habituate to the cameras. These cameras were also installed each morning for 2 days before recording commenced to allow the salmon to further habituate to these novel objects. To minimise any additional disturbances, cameras were turned on before being submerged with recording controlled remotely through the GoPro Quik© mobile application. Connectivity from mobile phone to each underwater camera was achieved through the use of coaxial cables taped to each device. Coaxial cables conduct electrical signals (including Wi-Fi) through an insulated shield, extending network connections to a submerged device (e.g., camera). Recordings for each tank were taken on a strict daily schedule, after lights went on, to ensure consistency. A minimum of 15 min were recorded for each tank once lights went on. All personnel on-site strictly avoided carrying out any procedures around the tanks during filming.

2.2.5. Recording schedule

A 7-day period of video recording was scheduled to gather footage for all behavioural analysis (i.e., QBA and darting behaviours), with the stressful challenge (i.e., sampling) conducted during the middle of this period. Sampling was carried out on all 9 tanks of salmon on February 18th, 2022. To obtain a 'baseline' and account for any potential day to day variation in behaviour, 3 consecutive days were recorded before the stressful challenge occurred. A further 3 consecutive 'post-sampling' days were required for recording the salmon's recovery from this stressful challenge. Figure 1 provides a summary of the recording schedule. The ability for QBA to reflect any impacts on salmon behavioural expressions, as a result of these disturbances, could then be assessed from these recordings (Section 2.3.1.1 outlines how the video clips were prepared for QBA).

2.3. Qualitative Behavioural Assessment

The QBA process consisted of two main stages. Stage 1 involved 12 observers in the generation of the QBA terms for describing the salmon's expressive characteristics and stage 2 involved 5 different observers scoring the QBA terms for each of the video clips.

2.3.1. Stage 1 – term generation

Twelve professionals employed in the Scottish salmon farming industry were recruited for the term generation stage, which involved two separate meetings. All participants had at least 1 year of experience working directly with farmed salmon, with a number of participants in senior/management roles. During term generation, various video clips were used which were taken from

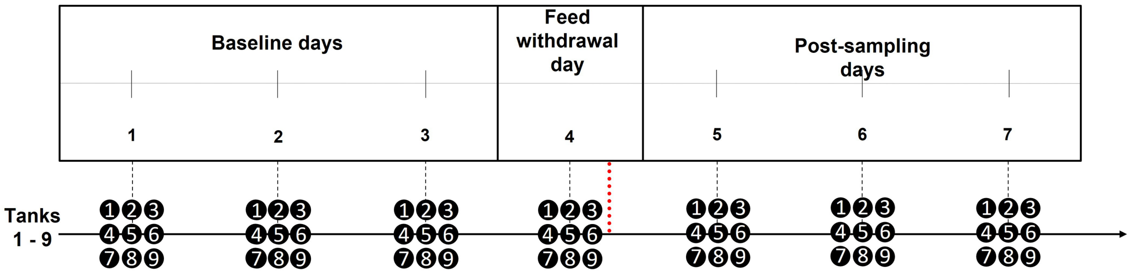

FIGURE 1
Recording schedule and timeline for experiment. Black dots represent each time a tank was recorded for the day, and the dashed red line (after day 4) illustrates when the stressful challenge (sampling event) occurred.

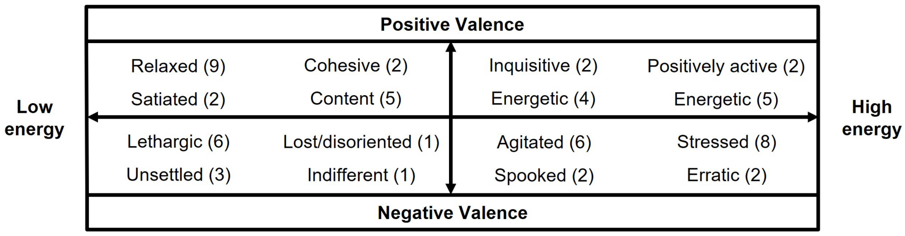

FIGURE 2
Final list of QBA terms generated from stage 1. Valence (positive/negative) and energy (high/low) were used to help describe and discuss terms across the 4 quadrants. Numbers in brackets indicate the total number of participants who brought each term to the initial meeting.

different farm sites under different contexts (e.g., during the middle of the day or during feeding, after treatments/ transportation etc.). In this study, we define 'expressive characteristics' as the extent to which qualitative characteristics of salmon behaviour (e.g., relaxed, purposeful, lethargic, agitated) are expressed. The video clips were selected to represent all 4 aspects, or 'quadrants', of behavioural expression (high to low energy, positive to negative valence) as outlined by Mendl et al. (26) (see Figure 2).

Before terms were generated by participants, the theory and practice of QBA was explained to them and they were provided with guidance on how to generate appropriate terms. To avoid bias, examples of terms from terrestrial farming systems were used. After the first meeting, participants were asked to individually watch the video clips in advance of the second meeting and generate their own personal list of terms. During the second meeting the participants discussed these terms, including how they should be divided between the 4 quadrants of behavioural expression (high to low energy, positive to negative valence). Participants were then asked to select a maximum of 20 terms which were balanced across the 4 quadrants, and best described the range of salmon behavioural expression. By the end of the meeting, the group had agreed on 16 terms. These included the terms "diving deep" and "flighty," which were excluded by the experimenters from the final list used in the second stage. QBA requires terms that convey some aspect of emotional behaviour and the term "deep diving" did not. Other terms (e.g., spooked, erratic, unsettled, agitated) already covered aspects of the term "flighty." The final QBA term list therefore had 16 terms, with 4 in each quadrant of behavioural expression (Figure 2). These terms were then used in the QBA scoring stage.

2.3.1.1. Video preparation before stage 2

For use in the QBA scoring stage, shorter video clips were extracted from each of the 63, 15-min videos. These clips were the first full minute that the salmon remained clearly in view, starting from 30 s after the lights were turned on. This excluded the initial "noise" from the salmon's startle responses to the lights. Video clips were first randomised with respect to their chronological order and their occurrence before or after the sampling treatment. To facilitate observer concentration and motivation, they were then arranged so that clips showing contrasting expressive characteristics (e.g., primarily high energy, negative valence vs. low energy, positive valence) were distributed evenly throughout the scoring sessions. Unknown to observers, 4 of the original 63 video clips were duplicated to allow for an assessment of intra-observer reliability (the degree to which participants showed agreement within their own scoring sessions). This resulted in a total of 67 video clips being scored by each observer.

2.3.2. Stage 2 – QBA training and scoring session

Scoring sessions for the QBA were carried out with a new group of 5 observers. These 5 observers consisted of 2 Post-doctoral fish welfare researchers from the University of Stirling, and 3 industry professionals all with higher degree education and between 3 and 20 years of aquaculture industry experience. These observers consequently had a varied level of experience in working with and observing salmon. All but 1 observer had hands-on experience in salmon husbandry in a commercial setting.

Observers were given online training in QBA. A brief introduction was given on the principles of QBA and the general purpose of this study (i.e., exploring the use of QBA within fish). Observers were kept blind to treatment (i.e., the stressful sampling challenge), and were instead only informed about the general context behind the video clips (location of filming, number of tanks and days involved in the recording). It was explained to observers that qualitative descriptors overlap and complement each other in characterising expressive patterns and are not mutually exclusive in the way ethogram categories are. To capture subtle differences when scoring it is important to consider the meaning of each individual descriptor in its own right. Associations between different terms are complex; fish could for example appear stressed and spooked but not too agitated, or a bit unsettled and agitated but not too stressed. To support such use of the descriptors it is important that everyone's understanding of the terms is aligned as much as possible. To this end, an open discussion of the meaning of terms was conducted, aided by a sheet with brief characterisations of each term created by the experimenters (Table 1). The meaning of each term was discussed and adjusted where required, and observers were invited to raise any questions about terms which required clarification. General instructions were given on how to assess whole animal expressivity and how to use the Visual Analogue Scales (VAS) to score the prevalence of each term within a video clip. A VAS is a measurement instrument that allows for the scoring of characteristics (such as those of behavioural expressions) that are believed to range across a continuum of values (55). Observers were reminded that terms must be scored independently from each other, so that in situations where there were contrasting expressive characteristics among different salmon (e.g., some appearing agitated and others relaxed), those contrasting terms could both receive high scores for the same clip.

All QBA scoring was carried out on scoring sheets developed on SurveyMonkey®. For each term, a horizontal line with a 100-step scale was presented as a VAS, along which a single mark could be made. The distance from the left end of the scale would correspond to the participant's assessment of the intensity for each term observed. The left end of the scale represented complete absence of an expressive characteristic described by a term, whereas the right end represented the maximum expression for the term (e.g., the salmon could not be more erratic). While scoring, observers would not be aware of any quantitative values associated with the VASs. They were encouraged to use the entire scale when judging the intensity of each expressive characteristic. Video clips were labelled according to their order in the scoring sheets and transferred electronically to the group. Due to the large number of clips, observers were instructed to avoid scoring them all in a single session, but also to carry out their scoring sessions with minimal delay between each other (i.e., within the same week) to minimise potential variation introduced by scoring on different days.

TABLE 1 QBA term list with term characterisations.

Term	Term characterisation
Relaxed	Salmon are moving at ease, free from tension or agitation. Does not just apply to resting - animals can be relaxed also while active.
Agitated	Salmon are restless, excessively moving around, over-responding to unexpected stimuli.
Inquisitive	Salmon show an interest/curiosity towards their surroundings - explorative, investigating features (novel or familiar) of their environment.
Unsettled	Salmon are uncertain, ill at ease, twitchy, vigilant.
Cohesive	Salmon are moving together in synchrony/unison; the shoal appears to behave as one organism.
Spooked/ skittish	Salmon are easily scared en-masse (even by small disturbances), abruptly changing behaviour/direction of travel, and avoiding rather than investigating.
Positive active	Salmon are absorbed in activity in a relaxed way, interacting in a positive manner with their environment.
Indifferent	Salmon are unfocused, moving around without much engagement, lacklustre. Not dull or lethargic.
Purposeful	Salmon are self-motivated, focused, determined. Carrying out their actions without hesitation.
Erratic	Salmon movements are un-coordinated, randomly (over)reacting, disorganised. Erratic is more a more vigorous expression than being unsettled.
Energetic	Salmon move in a vigorous, lively way; appearing bright & animated.
Lost/ disoriented	Salmon do not know what to do, appearing aimless, confused, worried, and searching on their own.
Satiated	Salmon appear to be satisfied in their physical needs.
Lethargic	Salmon are dull, morose, unresponsive, slow-moving and without any vigour. Looking unwell.
Stressed	Salmon are troubled, tense, not behaving normally. Something is not right.
Content	Salmon are healthy, calm, satisfied, looking well. At ease but could lack positive engagement/purpose.

Terms are listed in the order in which they were presented for scoring to observers.

2.4. Additional welfare measures – feed intake and darting events

2.4.1. Feed intake

Feed input and feed waste were recorded for each tank daily alongside the 7 days of QBA recordings. The experimental feeds were given by auto-feeders (Arvo-tec TD2000) twice a day from 05:00–09:00 and 16:30–23:30 with uneaten feed collected to measure daily feed intake and calculate apparent feed intake through standpipes daily, between 09:00 and 09:15. Recovered feeds were placed in a 300 μm mesh sieve and rinsed in fresh water in order to remove salt and faeces. The leaching of the feeds was calculated through a nutrient dissolution factor. Briefly, 10 g of each feed was incubated in system water in duplicates for 6 h before drying (24 h, 110°C) and weighing. Feed waste daily collected was weighed wet and converted to dry weight using the nutrient dissolution factor.

2.4.2. Darting behaviour

For the purpose of this study, darting behaviours were defined as a "rapid, burst of movement clearly distinct from the salmons' regular swimming behaviours; this includes sudden changes in direction, acceleration, and/or positioning of the salmon in the tank." A scan sampling method (56) was created to record 'darting events' in the same 63, 1-min video clips used for the QBA. Since any of these darting events would have also been observable during the QBA, and thus potentially affected the scoring of certain QBA terms, another second set of video clips were also investigated. This second, separate set involved an additional 63, 1-min video clips that were taken immediately after the QBA clips.

To allow multiple darting events to be recorded in one clip, any darting behaviour must have stopped before the next event could be recorded. The number of salmon involved in each darting event was first recorded and categorised by the proportion to the total number of salmon in the tank (Table 2). Weighted scores were then assigned to each of these categories, relative to their proportions (Table 2). A final score was then calculated for each clip, based on the sum of weighted scores from all darting events recorded. Video playback speed was altered to ensure the number of salmon involved were counted correctly. Where the number of salmon darting was too high to allow for counting, the event was then categorised as involving more than 15% of the fish in the tank.

2.5. Statistical analyses

2.5.1. Data handling of QBA scores

For each QBA score, the distance of each observers' marks from the zero point of the scales was automatically measured and recorded by SurveyMonkey. The complete dataset of these raw QBA scores were then imported from SurveyMonkey into Microsoft Excel (Version 2,301). Data was organised into a matrix, with QBA terms listed horizontally in the first row and video clip numbers and labels in the first few columns. Unless otherwise stated, all statistical analyses were run in R Studio (version 4.2.2). The threshold of significance for any statistical test was $p < 0.05$. For the Linear Mixed Effects Model (LMEM) analyses conducted later in the study, the package "nlme" was applied.

2.5.2. Principal Component Analysis

A Principal Component Analysis (PCA) was carried out, using a correlation matrix on the entire dataset of QBA scores to reduce the dimensionality of the QBA terms. PCA allows for the 16 terms scored within each video clip to be summarised by a numerical value for each Principal Component (i.e., the PC "score"). No post-processing step of 'rotation' was carried out, as the only goal of the PCA was to reduce the dimensionality of terms.

The highest positively and negatively loaded terms for each component were identified which, together, represented the larger pattern of expressive characteristics illustrated within each PC. To determine whether PCs were eligible for further analysis, a combination of criteria was used. Following the "Kaiser criterion," which states that the number of factors to retain should correspond to the number of eigenvalues greater than one, only PCs with eigenvalues >1 were considered (57). Within each component, there also had to be good inter-observer reliability in the PC scores (Section 2.5.3). There also needed to be a coherent biological interpretation of the terms that had the highest positive and negative loadings within each component. For example, a higher score for PC1 suggested that salmon were more unsettled/stressed, whereas a lower score suggested that salmon were more relaxed/content.

For the complete set of PC scores obtained, Q-Q plots, histogram symmetry, skewness and kurtosis values, sphericity, and Leven's test were inspected to ensure all assumptions required for carrying out further parametric tests were met (including normality of data). The scree plot and proportion of variance for each PC were also used as additional guidance for determining the inclusion of PCs in further analysis.

TABLE 2 Categories of darting events by the proportion of salmon from the tank involved, as well as their corresponding weighted scores.

Proportion of salmon in tank involved in each darting event	Weighted score
Less than 4%	1
Less than 8%	2
Less than 15%	3
More than 15%	4

2.5.3. Inter/intra-observer reliability

Kendall's coefficient of concordance (W) was used to calculate the level of agreement between the 5 participants' PC scores in the combined data set, for each of the PCs. Any value of W less than 0.4 was considered to reflect unacceptable inter-observer variability. This analysis was carried out using IBM SPSS Statistics 28 (58). The degree to which observers showed agreement between their scores of the duplicated video clips was, given normal distribution of the scores, determined using Pearson's correlation, performed on each of the relevant PC scores.

2.5.4. Comparing pre vs. post disturbances

QBA scores of the salmon before and after the stressful challenge were analysed by applying separate Linear Mixed Effects Models (LMEM) to each of the relevant PCs (PC1, PC2, PC3 and PC4). For each LMEM, the PC score was the dependent variable, 'Pre vs. post disturbance' and 'Observer' were fixed factors, and tank number was a random factor. Before the LMEMs were applied, ANCOVAs were first carried out (with day number as a covariate) to ensure that there were no significant time trends within each subset of days 1–3 and 5–7. Since no additional time trends were present within these subset of days, day number was also included in the LMEMs as a random factor.

Although Kendall's coefficient determines whether there is good agreement between observers for PC1, PC2, and PC4, the actual "treatment" effect of observers still needed to be accounted for, hence the inclusion of 'Observer' as a fixed factor.

2.5.5. Comparing feed intake and darting events with QBA scores

For each tank every day, feed intake and two separate sets of darting scores were recorded (2 x separate sets of 63 video clips). Similar LMEMs were applied, with tank and day number as random factors, to first determine whether 'Pre vs. post disturbance' had a significant impact on each of these additional measures. Spearman correlation tests were then carried out to compare feed intake and the two separate sets of darting scores against the corresponding mean PC scores of the 63 clips used in the QBA. Mean PC scores were derived by averaging the PC scores from the 5 observers.

3. Results

3.1. Qualitative Behavioural Analysis

3.1.1. Principal Component Analysis

PC1, PC2, PC3, and PC4 had eigen values >1. PC1 explained the greatest percentage of variation at 37%, with the first four components collectively explaining 74.5% of the variation in the data (Table 3).

As outlined in Table 4, PC1 ranged from relaxed/content/positive active to unsettled/stressed/spooked/skittish/agitated. For PC2, the only positively loading term was relaxed, with the main negatively loading terms being energetic/purposeful/inquisitive. Figure 3 illustrates the relationship that the QBA terms have with both PC1 and PC2. For example, a more negative PC1 score indicates salmon that were more relaxed, content, and positive active.

PC1, PC2, and PC4 demonstrated acceptable inter-observer reliability for their PC scores (PC1: $W = 0.63$, $X^2 = 207.57$, $p < 0.001$; PC2: $W = 0.46$, $X^2 = 152.19$, $p < 0.001$; PC4: $W = 0.56$, $X^2 = 184.94$, $p < 0.001$). All four PCs showed acceptable intra-observer reliability between PC scores of video clips that were duplicated (PC1: $r = 0.716$, $p < 0.001$; PC2: $r = 0.755$, $p < 0.001$; PC3: $r = 0.552$, $p < 0.05$; PC4: $r = 0.581$, $p < 0.01$). PC3 had a W value below 0.4, which was considered unacceptable and therefore not included in further analysis. PC1, PC2, and PC4 were retained for further analysis.

3.1.2. Effect of the stressful challenge (intrusive sampling) on PC scores

There was a significant difference between PC1 scores when comparing days before and after the stressful challenge ($p = 0.03$, Figure 4). PC1 scores (averaged between the 5 observers for each video clip) ranged from −4.97 to 6.04. The mean difference between PC1 scores for pre vs. post-disturbance days was +0.82 (Pre = −0.239, Post = 0.584). Overall, all five observers scored PC1 higher for post-disturbance days. 7 out of 9 tanks received a higher average PC1 score for post-disturbance days. Figure 5 illustrates the comparative likelihood of a PC1 score being higher or lower for video clips that were recorded either before or after the sampling event. No significant differences were found for PC2 and PC4 scores ($p > 0.05$). For PC1, PC2, and PC4, there was a significant effect for observers as a fixed effect ($p < 0.001$).

3.2. Feed intake, darting behaviours, and their association with QBA

A significant difference was found in the feed intake of salmon from tanks before and after the stressful challenge ($p = 0.02$). Mean daily feed intakes were 166 g for pre-disturbance (SEM = 4.72) and 79 g for post-disturbance (SEM = 7.5), resulting in an average 87 g reduction in daily feed intake post-disturbance. However, there was no significant association found between mean PC1 scores and feed intake ($r = -0.19$, $p > 0.05$).

No significant difference was found between darting scores before and after the stressful challenge, in either set of 63 video clips used (same clips as QBA: $p > 0.05$; 1-min post QBA clips: $p > 0.05$). However, PC1 scores showed a moderate positive correlation with the darting scores taken from either set of video clips (same clips as QBA:

TABLE 3 Eigen analysis of PC1, PC2, PC3, and PC4.

Value	PC1	PC2	PC3	PC4
Eigen value	5.88	2.82	1.95	1.27
% of variation explained	36.7%	17.7%	12.2%	7.9%
Cumulative %	36.7%	54.4%	66.6%	74.5%

TABLE 4 QBA term loading values for each principal component.

Term	PC1	PC2	PC3	PC4
Relaxed	**−0.359**	**0.074**	−0.003	0.073
Agitated	**0.309**	−0.272	−0.090	−0.109
Inquisitive	−0.197	**−0.366**	−0.151	0.185
Unsettled	**0.358**	−0.185	−0.073	−0.049
Cohesive	0.039	−0.153	0.297	**−0.491**
Spooked/skittish	**0.327**	−0.199	−0.024	−0.112
Positive active	**−0.286**	−0.286	**−0.168**	0.110
Indifferent	−0.148	−0.254	**0.477**	−0.067
Purposeful	−0.145	**−0.395**	**−0.284**	−0.104
Erratic	0.226	−0.226	−0.038	**0.431**
Energetic	−0.156	**−0.459**	**−0.202**	−0.029
Lost/disoriented	0.103	−0.165	**0.356**	**0.585**
Satiated	−0.224	−0.186	0.232	**−0.290**
Lethargic	0.025	−0.178	**0.560**	0.066
Stressed	**0.332**	−0.171	−0.056	−0.211
Content	**−0.358**	−0.042	0.009	−0.050

The highest negatively and positively loaded terms for each PC are in bold.

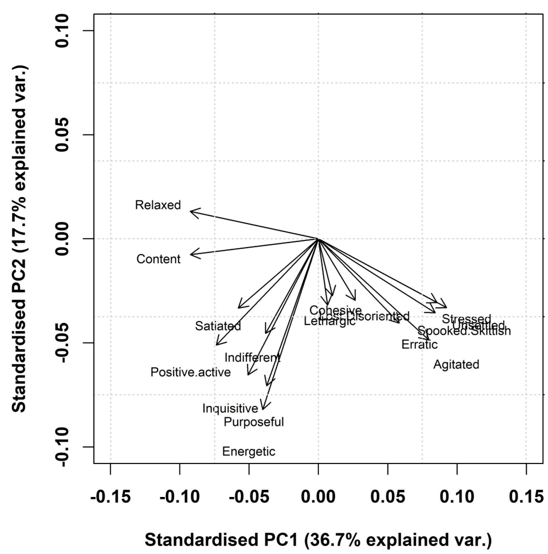

FIGURE 3
Loading plot of all 16 QBA terms used in this study for PC1 and PC2. Axes represent standardised eigen vectors for which the QBA terms load onto the two main Principal Components of the analysis. A more negative value for PC1 indicates an overall higher score for relaxed, content, and positive active.

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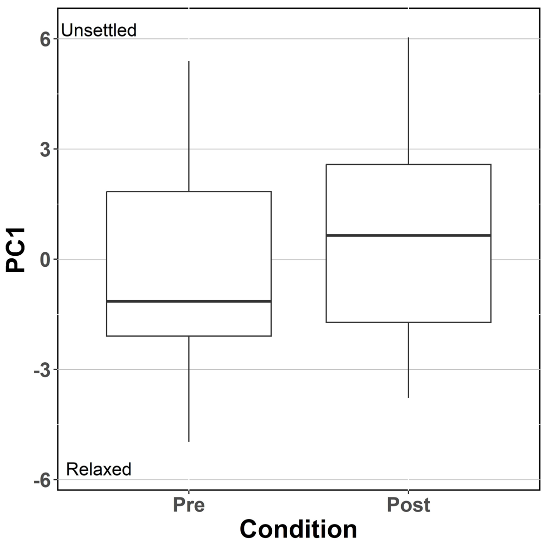

FIGURE 4
Box plot to compare differences in spread of PC1 scores before and after feed withdrawal and sampling events.

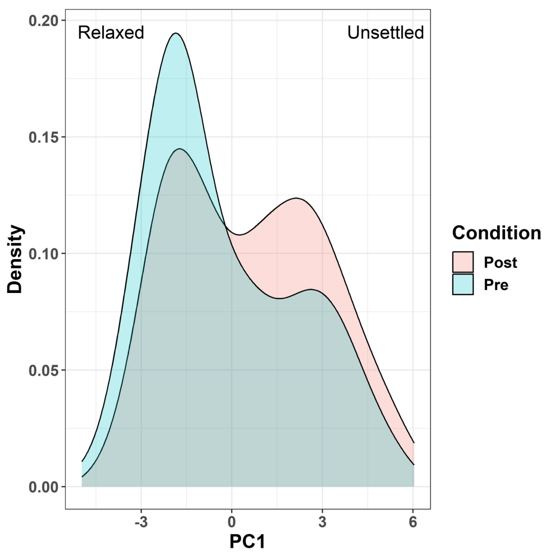

FIGURE 5
Layered density plot comparing different probabilities of various PC1 scores occurring, depending on whether they were taken pre vs. post disturbance.

$r = 0.42$, $p < 0.001$; 1-min post QBA clips: $r = 0.33$, $p < 0.01$, see Figure 6).

4. Discussion

Integrating indicators of the emotional state of animals within welfare assessments has previously proven to be problematic for many reasons. This study's aim was to determine QBA's ability to detect the effects of a stressful event on Atlantic salmon. We applied QBA to

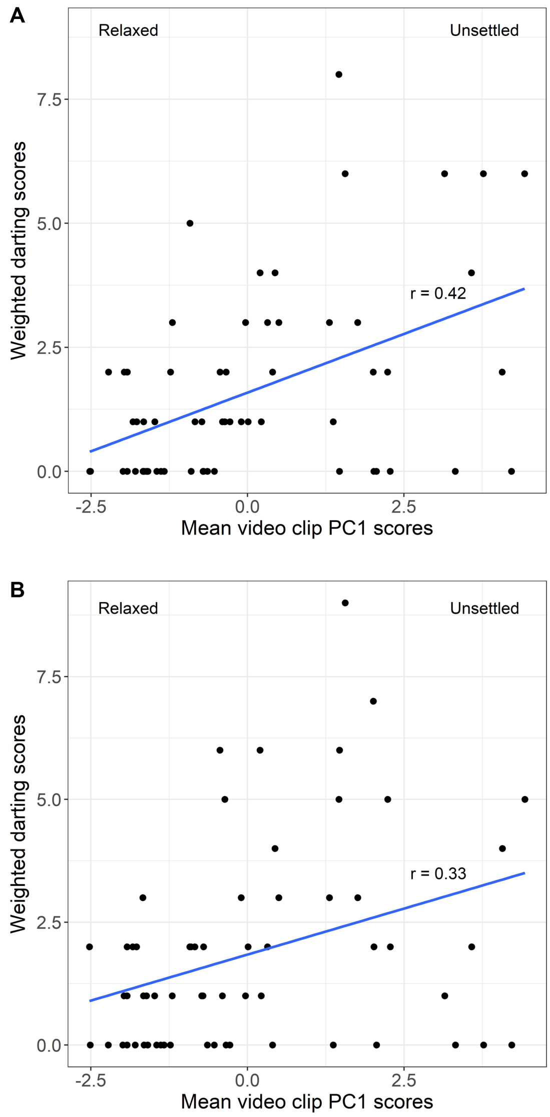

FIGURE 6
Scatterplot of mean PC1 scores (Relaxed – Unsettled) for video clips vs. **(A)** weighted darting scores calculated from the same clips used for the QBA, and **(B)** weighted darting scores calculated from video clips taken 1-min after QBA clips. Line of best fit and r values from spearman correlation tests included.

quantify and evaluate the expressive characteristics of Atlantic salmon before and after exposure to a putatively stressful challenge. While feed withdrawal was required before sampling could be carried out, the sampling event was the focal point as the experimental treatment of this study. The process of capturing, anaesthetising, and handling salmon out of water for sampling has been described as intrusive, stressful, and detrimental for welfare (59–61). Previous studies that have assessed how salmon recover from stressful events (e.g., handling/anaesthesia/invasive sampling) often monitored the recovery over a 24-72 h period (59, 62, 63). Thus, a 3-day period for both the baseline and 'recovery' stage was considered to be sufficient for the purpose of this study.

There was acceptable agreement between the five observers in this study, who were blind to the treatment and had varied experience in monitoring fish behaviour/welfare. There was one main dimension of QBA that proved effective in capturing changes in the emotional state of the salmon within this study; relaxed/content/positive active – unsettled/stressed/spooked/skittish/agitated (PC1). PC1 explained the largest proportion of variation in expressive characteristics of the salmon (36.7%). There were significant differences between PC1 scores before and after the stressful challenge (sampling), with salmon being scored as more unsettled/stressed/spooked/skittish/agitated after sampling. This reflected a shift from low energy, positive valence to high energy, negative valence after sampling, a contrast that was consistently recorded by all observers and in the majority of tanks. In addition, the single recording that was perceived the most "positively" (i.e., the most relaxed/content/positive active) was taken before any potential impacts from sampling had occurred. These results are in agreement with numerous papers that have previously used QBA to assess the emotional state of terrestrial farmed animals (e.g., for cattle, horses, pigs, and hens), with PC1 typically being characterised by terms such as relaxed and content vs. agitated (64–68). Furthermore, these past studies have used similar descriptors to describe the other main terms used in PC1 for this study; unsettled (uneasy), stressed (nervous), spooked/skittish (scared/fearful/nervous), and stressed (tense).

With lights being switched on at precisely 10:30 am every morning, this was considered a routine event that could be methodically recorded and expected to help stimulate activity in the fish. This would potentially maximise what expressive characteristics could be captured without causing additional stress to the salmon. The initial 30 s were cut out to exclude the salmon's startle responses to the lights, which may have otherwise drowned out any potential differences reflected by the QBA scores.

The LMEM determined that there was significant variation between observers in the mean scores they attributed to the 63 video clips on each PC. This suggests that observers may have been interpreting and using the ranges within the VASs differently, while still agreeing on the direction in which the scores should change from one video to another. Such an occurrence is not uncommon when multiple individuals use the same continuous scales (69). In most QBA studies, the directionality of scores, as indicated by Kendall's W, is taken as the most important indicator for inter-observer agreement (17, 44, 70). However, crucial to the aims of this study, the observer effect was accounted for by the LMEM when analysing the treatment effect, and thus a significant difference between PC1 scores was found before and after the stressful challenge.

Previous studies have suggested that significant associations between QBA and other welfare measures help support the validity of QBA as a welfare assessment tool (17, 36, 44, 68). However, as noted by (37), the purpose of QBA is to examine subtle expressive aspects of an animal's demeanour in ways that would be otherwise difficult to quantify for other measures of behaviour. It is important to be reminded of the multi-faceted nature of welfare (16, 71, 72), and that QBA should be regarded as a complementary addition to an integrated approach involving various welfare indicators. QBA is thus used with the intention of gaining unique insights into an animal's emotional state in a way that is complementary to other indicators, allowing for a more comprehensive evaluation of animal welfare (17, 37). Welfare assessments should also aim to minimise redundancies and include measures that are, at least to some degree, independent from each other (73). Feed intake, on average, more than halved following the stressful challenge. Similar reductions in feed intake have been reported in a number of studies exposing fish to stressful challenges (28, 53, 74). As there were significant differences in both PC1 scores and feed intake before and after the stressful challenge, and yet they were not correlated with each other, these results should further support the notion of QBA being a unique welfare assessment tool. In somewhat of a contrast to this, darting scores showed a moderately positive correlation to PC1 scores. Put simply, as the salmon were observed to be more unsettled, stressed, spooked/skittish, and agitated, there was a corresponding increase in the frequency and/or intensity of darting events. However, the darting scores alone showed no treatment effect from the stressful challenge. While these two measures were not entirely independent from one another, QBA was capable of capturing a significant treatment effect when the darting scores could not. This finding highlights the sensitivity of QBA, indicating that the PC1 scores were more capable of capturing the effects of the stressful challenge on the salmon's welfare than the darting scores.

PC2 and PC4 showed acceptable inter-observer reliability, explaining proportions of variation that were comparable to other studies applying QBA to terrestrial animals (36, 44, 75, 76). For PC2, the only positively loading QBA term was relaxed, with the main negatively loading terms being energetic, purposeful, and inquisitive. This meant that PC2 mainly reflected the salmon's degree of relaxation against 'high energy'; lower PC2 scores reflected more lively, energetic salmon. PC4 was characterised by terms that reflected a shift in how "harmonious" or "consistent" the behaviour of the salmon was as a collective (i.e., cohesive vs. lost/disoriented). PC3 explained one third of the proportion of variation explained by PC1, with poor inter-observer reliability. The terms most heavily loaded for this dimension (indifferent and purposeful) may help partially explain this inconsistency between observers. Such terms could have been more difficult to perceive and assess in salmon, in comparison to the terms used within PC1.

There was no statistically significant difference between the pre- and post- sampling event stages in PC2 or PC4. Sampling was specifically chosen as a presumably intrusive, stressful event, with the intentions of then assessing QBA's ability to detect the putative impacts of such an event on the salmons' emotional state. Considering the terms used to characterise PC2 and PC4, these dimensions may not be so relevant to addressing the effects of stress, but may be very relevant to assess fish welfare in other contexts and treatments. For example, the potential benefits of environmental enrichment or the impacts of transportation/transfer to new enclosures. Considering that the most relevant dimension in the context of this study (PC1) reflects a combined shift in both valence (positive – negative) and energy (low – high), this dimension could be of significant use for on-farm welfare assessments of Atlantic salmon. Additional research is needed to further explore and validate the relevance of other dimensions found in this study (i.e., PC2 and PC4), under different experimental treatments, to expand the potential applications of QBA for salmon welfare assessments.

Integrating QBA into future welfare assessments (for research or farming) will first require appropriate training in the observing, scoring, and understanding of terms involved (70, 77). While this may require a significant initial investment towards developing the observers' assessment capabilities, doing so will help ensure acceptable inter-observer reliability and, over the long term, help with the integration of a unique and efficient welfare assessment tool (17). Welfare assessments that include QBA have the advantage of evaluating emotional states of the animals, and the consequent monitoring of positively valenced terms (e.g., content, relaxed, inquisitive, cohesive, purposeful, energetic etc.) also allows for the consideration of positive aspects of fish welfare.

The various ways in which sampling can cause stress and impair fish welfare demonstrates another advantage with implementing QBA; as a non-intrusive method of welfare assessment. QBA avoids any negative impacts from its measurement, an issue that is inherent in many animal-based measures. A large proportion of animal-based measures of welfare are also retrospective, only identifying problems long after they have occurred (72). Analyses of behavioural expression could help minimise this delay, perhaps even to the point of providing early warning signs for pre-clinical health issues (13). QBA has for example been used successfully to detect early clinical signs of mastitis in dairy cows (78). Through virtue of being able to assess behavioural expressions through video monitoring, QBA is also capable of being carried out remotely. Considering the remote locations in which these salmon are often kept (79), as well as issues surrounding monitoring when site access is limited, this feature provides a significant advantage. The need for such welfare monitoring tools was highlighted to the Scottish salmon farming sector when farm staff were restricted from accessing their sites during the 2020 COVID-19 pandemic, and in-person audits for welfare certification schemes had to be replaced with virtual assessments for 2 months (80, 81). During a recent industry survey carried out within the salmon farming sector, various professionals employed in the production process ranked the development of remote, non-intrusive welfare indicators as one of the highest research priorities for farmed salmon welfare (32). The effective implementation of QBA on-site would help meet this demand.

5. Conclusion

This is the first study to demonstrate QBA's ability to capture changes in the expressive characteristics of Atlantic salmon following exposure to putatively stressful events. Five observers from various professional backgrounds achieved acceptable inter- and intra-observer reliability in 3 dimensions of QBA scores. PC1 showed a significant treatment effect, with salmon becoming more unsettled, stressed, spooked/skittish, and agitated after the stressful challenge. Both PC1 scores and feed intake recorded a significant difference before and after the stressful challenge, but were not correlated to each other. PC1 scores showed a moderate positive correlation with darting scores, however the darting scores did not show a significant treatment effect, indicating the QBA scores to be more sensitive to the stressful challenge. These results support QBA's ability to provide unique insights that are relevant to the evaluation of farmed salmon welfare. Future experiments should explore the other dimensions found within QBA (e.g., PC2 and PC4) under different treatment conditions, and across other species of fish, to further investigate QBA's applicability within aquaculture. The results from this study demonstrate that QBA is a promising welfare indicator that, with further research, could act as a time-efficient and complimentary tool for on-farm welfare assessments.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Author contributions

TW: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Visualization, Writing – original draft, Writing – review & editing. SR: Conceptualization, Supervision, Validation, Visualization, Writing – review & editing. MB: Methodology, Validation, Writing – review & editing, Data curation, Resources. MH: Conceptualization, Funding acquisition, Methodology, Supervision, Validation, Visualization, Writing – review & editing. SJ: Validation, Writing – review & editing, Conceptualization, Funding acquisition, Methodology, Supervision, Visualization. AD: Validation, Writing – review & editing. FW: Conceptualization, Validation, Writing – review & editing, Methodology. JT: Conceptualization, Funding acquisition, Supervision, Validation, Visualization, Writing – review & editing.

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Conflict of interest

AD was employed by Aquascot Ltd.

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EDITED BY
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REVIEWED BY
Morris Villarroel,
Polytechnic University of Madrid, Spain
Daniel Díaz Plascencia,
Autonomous University of Chihuahua, Mexico

*CORRESPONDENCE
Gianfilippo Alessio Clemente
g.clemente@izsler.it

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Farmed fish welfare during slaughter in Italy: survey on stunning and killing methods and indicators of unconsciousness

Gianfilippo Alessio Clemente[1]*, Clara Tolini[2], Andrea Boscarino[2], Valentina Lorenzi[1], Tania Lidia Dal Lago[3], Daniele Benedetti[3], Fabio Bellucci[3], Amedeo Manfrin[4], Angela Trocino[5,6] and Sara Rota Nodari[1]

[1]Italian National Reference Center for Animal Welfare, Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna 'Bruno Ubertini', Brescia, Italy, [2]Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna 'Bruno Ubertini', Brescia, Italy, [3]Italian Ministry of Health, Rome, Italy, [4]National Reference Laboratory for Crustacean Diseases, Istituto Zooprofilattico Sperimentale della Venezie, Legnaro, Italy, [5]Department of Agronomy, Food Natural Resources Animals Environment, University of Padua, Legnaro, Italy, [6]Department of Comparative Biomedicine and Food Science, University of Padua, Legnaro, Italy

Information on slaughter procedures for farmed fish in aquaculture is limited, both in Europe and in Italy, due to a general lack of field data. The aim of this study was to gather information on the procedures used to slaughter fish in Italy and to discuss them considering the WOAH and EFSA recommendations on fish welfare. Using a questionnaire survey, data were collected by official veterinarians in 64 slaughtering facilities where 20 different species of fish were slaughtered. The main species slaughtered were rainbow trout (*Oncorhynchus mykiss*; 29/64), followed by European sea bass (*Dicentrarchus labrax*; 21/64), sea bream (*Sparus aurata*; 21/64), Arctic char (*Salvelinus alpinus*; 14/64), European eel (*Anguilla anguilla*; 11/64), sturgeon (*Acipenser* spp; 11/64), common carp (*Cyprinus carpio*; 6/64), and brown trout (*Salmo trutta fario* L.; 5/64). The most applied stunning/killing methods were "asphyxia in ice/thermal shock" and "electric in water bath," followed by "percussion," "asphyxia in air," and "electric dry system." After the application of the method, the assessment of the fish level of unconsciousness was practiced in 72% of the facilities using more than one indicator, with "breathing" and "coordinated movements" the most practiced. The collected data showed a discrepancy between the available recommendations about the welfare of fish at slaughter and what is practiced in many production sites, but for many species precise recommendations are still not available.

KEYWORDS
aquaculture, fish unconsciousness, thermal shock, electrical stunning, trout, seabream, seabass, sturgeon

1. Introduction

Aquaculture production has experienced significant growth over the past two decades (1–3), reaching 87.5 million tons in 2020 at a global level (2). This rapid expansion has led to considerable interest in welfare issues by scientists, consumers and policy makers. The number of scientific studies claiming that fish are sentient animals, able to perceive emotions and thus of experiencing fear, psychological stress, and pain is increasing, as recently reviewed (4). Consumers demand information about the origins of food products and the conditions under which farmed animals are kept (5). Animalist groups claim more than half of the consumers are aware that fish are capable of experiencing pain and that the current protection of fish welfare is insufficient if compared to the other farmed species (6). In fact, despite that the number of farmed and slaughtered fish for human consumption is largely higher than that of farmed mammals (approximately 2.5-fold) (3, 7), the welfare of farmed fish is inadequately protected by the European legislation (3, 6). Specifically for the protection of animals at the time of killing (EC Reg. 1099/2009) (8) fish are included only within the general framework of basic principles. Only the Article 3 (1) of the general provision is applicable for fish, according to which animals for slaughter must be protected from avoidable pain, distress or suffering. Unlike for other species, the methods for fish stunning and killing are not defined. Actually, in the absence of scientific updates and EU rules, Article 27 (1) allows Member States to maintain or adopt national rules regarding the protection of fish at the time of killing (8). However, only a few Member States have implemented national laws which refer only to a few species (e.g., salmon) (9). In order to cope with these legislative gaps, the Farm to Fork strategy of the European Green Deal commits to review and update European animal welfare legislation (10) and for the slaughter of fish, in particular, it is intended to add more guidelines for the main farmed species and to revise welfare assessment criteria that are often inapplicable in the field (10).

In Europe, approximately 50 species of fish are reared for human consumption purpose. The most farmed species is rainbow trout (*Oncorhynchus mykiss*), followed by European seabass (*Dicentrarchus labrax*), gilthead seabream (*Sparus aurata*), common carp (*Cyprinus carpio*), and atlantic salmon (*Salmo salar*) (2, 11). Currently, farmed fish can be subjected to a range of different stunning/killing methods at slaughter. Many of these methods present relevant welfare problems, since they expose fish to prolonged suffering and pain before death (12–19).

Within such a heterogeneous framework of farmed species and stunning and killing methods, both the World Organization for Animal Health (WOAH) and the European Food Safety Authority (EFSA) have provided recommendations and opinions, in order to safeguard the welfare of fish at the time of killing (12–20). Considering the main farmed species, WOAH suggested the following stunning/killing methods as humane: percussion stunning for carp and salmonids; spiking or coring for tuna; and electrical stunning for carp, eel, and salmonids (20). According to EFSA, new methods of stunning/killing are needed, since most of those commonly practiced in Europe do not allow many fish species to be humanely slaughtered (12–19).

In order to be considered humane, a method must involve killing the animal in a state of unconsciousness and insensibility. For this reason, major improvements are necessary regarding the evaluation of unconsciousness or death after the application of the method (12). This control is very difficult under field conditions, as demonstrations of stress or pain in fish are not obvious and complicated to detect. In fact, the best method for recognizing fish unconsciousness is the electroencephalogram (EEG) in a laboratory condition, with the observation of visual evoked responses (VERs) (12, 21–24), which is difficult to be used in the field. According to WOAH and EFSA, spontaneous behavior, responses to stimuli and reflexes, such as the loss of body and respiratory movement and the loss of the vestibulo-ocular reflex should be assessed. However, this assessment presents several technical and practical problems in the field due to the large number of slaughtered species and the lack of standard procedures to follow. Fish are slaughtered in groups while most of the indicators refer to an individual fish (25). The indicators have only been validated in the laboratory for a few species (12) and due to physiological and morphological differences among species, they are not always applicable in the same way. For the assessment of unconsciousness, a specific and adequate training of the operators, likewise Standard Operating Procedures (SOPs) to clarify how to perform it (e.g., number of fish checked/cage) are required.

Italy plays an important role on the European panorama, representing the third largest country in terms of aquaculture fish production (11) with around 55 thousand tonnes in the last 10 years (11, 26). Italy is the largest European producer of sturgeon (*Acipenser* spp.), the second of rainbow trout and catfish (*Ictalurus punctatus* and *Ameiurus melas*) and the third of European seabass, gilthead seabream, and European eel (*Anguilla anguilla*) (11, 26, 27). The farms currently operating to rear fish for human consumption are 558, mainly located in the north: Veneto (114 farms), Piedmont (70), Friuli-Venezia Giulia (69), Trentino-South Tyrol (59), and Lombardy (56),(28).

Currently, there is very little information on the practices used in Italy to protect the welfare of fish at the time of killing as there is no a specific national legislation for the protection of the fish at farm and at slaughter (9). The only available data is that collected in 2018 by the European Commission about the methods used to kill three farmed species (9). It is also not known if the effectiveness of the methods applied are evaluated, and what indicators are used. There are not national guidelines on fish welfare at slaughter but guidelines for fish welfare at slaughtering were recently issued by associations of fish farmers. However, they are generic, not mandatory and do not provide any SOPs on stunning or on the assessment of fish unconsciousness.

In this context, where the information available on what happens in the field is limited, it is of utmost importance to have data on the different realities present in Europe in order to produce applicable legislation without an excessive economic impact on the producers.

For these reasons, the aim of this study was to provide a detailed presentation of the current situation regarding fish welfare at the time of slaughtering in Europe's third largest aquaculture producer, Italy. Using a questionnaire, we collected information on the fish-slaughter facilities currently operating on the Italian territory and on the practices used at the key moments of slaughter: the methods used to stun and kill fish and the indicators used to assess the unconsciousness. The assessment of the conformity of the procedures reported by the facilities was carried out in accordance with WOAH guidelines and EFSA recommendations on the protection of fish at slaughter (12–20).

2. Materials and methods

2.1. Data collection

The data were collected with the support of the Italian National Health System. The Italian Ministry of Health entrusted the Regional Competent Authorities to select an Official Veterinarian from the Regional and Local Health Units (LHU—ASL) responsible for safety and hygiene of food of animal origin for each Region. The selection was made based on their background and their expertise on slaughter as well as on their knowledge of the reality of aquaculture in their territory. The data collection was made through a questionnaire to fill with the information of the fish-slaughter facilities operating within their district. The completed questionnaires have been gathered in different moments as it has not been easy to register or verify the necessary data in all facilities simultaneously. The first questionnaire was collected in August 2022, while the last one was delivered by March 2023.

Official veterinarians were instructed on how to collect the data by means of an explanatory document prepared and disseminated by the Italian Ministry of Health. Support to the official veterinarians was given by the authors belonging to the CReNBA (Italian Reference Center for Animal Welfare—Istituto Zooprofilattico della Lombardia e dell'Emilia Romagna, Brescia) throughout the entire data collection period in order to clarify any possible doubt regarding how to fill in the questionnaire. The completed questionnaires were sent via email by each Italian region to the Italian Ministry of Health, which subsequently sent the questionnaire to the CReNBA for the analysis.

2.2. Development of the questionnaire

The questionnaire was developed by the CReNBA taking into account the structure and technique used in a veterinary questionnaire survey on welfare issues (29); the questions asked in a recent similar survey carried out in Brazil (30); and the generic recommendation of the European Regulation 1099/2009 on the protection of animals at time of killing. The targets of the questionnaire were the Italian Regional Competent Authorities, which sent it to their selected Official Veterinarians.

The questionnaire consists of an Excel document containing six open and closed questions, with the aim of collecting information about fish-slaughter facilities (Table 1). The first two questions related to the identification of the facilities, like their physical location (question 1) and their registration details (question 2). The third question was an open question on the annual processed fish volumes (question 3). This question was fundamental to be able to assess the volumes processed by the surveyed facilities in order to understand their relevance to the overall Italian aquaculture fish production. The following questionnaire question focused on the slaughtered species (question 4), with the possibility to select from seven fish species representing the main species farmed in Italy considering the most recent censuses of FEAP and FAO (26, 43). The last two questions regarded the stunning and/or killing methods (question 4) and indicators of unconsciousness applied (question 5), which had to be reported for each slaughtered species. The list of possible answers was built considering the main methods and indicators reported in literature as indicated in Table 1. The last three questions also allowing the veterinarians to add other possible options (open answer to fill in if necessary) in order to have the most accurate overview of the applied procedures.

2.3. Data analysis

The results of the questionnaire survey were subjected to descriptive statistical analysis in order to provide the geographical distribution of the slaughtering facilities, the stunning/killing practices, and the indicators used for the unconsciousness assessment during slaughter. For a clearer presentation of the results, slaughtered fish species were divided into two groups: main species (i.e., fish species slaughtered in more than five facilities) and minor species (i.e., species slaughtered in less than five facilities).

TABLE 1 Areas and type of information requested in the questionnaire used for data collection in fish-slaughter facilities in Italy.

Questionnaire area	Type of information
Geographic area	Italian region
	Local Competent Authority (veterinary public health system)
Identification of the slaughter facility	Company name
	Address
	Official registration number (approval number)
What is the annual processing volume (tonnes)?	Open Answer
Which species are slaughtered?	Rainbow Trout (*Oncorhynchus mykiss*)
	European seabass (*Dicentrarchus labrax*)
	Gilthead seabream (*Sparus aurata*)
	Arctic char (*Salvelinus alpinus*)
	Common carp (*Cyprinus carpio*)
	European eel (*Anguilla anguilla*)
	Sturgeon (*Acipenser* spp.)
	Other species, please specify
What is the stunning/killing method used? (13–21, 31–37)	Percussion
	Electric in water bath
	Electric dry system
	Carbon monoxide (CO)
	Carbon dioxide (CO_2)
	Asphyxia in ice/Thermal shock
	Asphyxia in air
	Other method, please specify
Which indicators are used to assess fish unconsciousness? (13–20, 24, 32, 38–42)	Breathing
	Eye movements
	Coordinated movement
	Response to stimuli
	Righting ability
	Other indicator, please specify

3. Results

Fourteen Italian regions were confirmed to practice fish slaughtering in a total of 67 facilities: Veneto, Piedmont, Apulia, Lombardy, Friuli Venezia Giulia, Tuscany, Sardinia, Marche, Trentino-South Tyrol, Sicily, Liguria, Emilia Romagna, Lazio, and Campania. The Competent Authorities of the remaining six regions communicated the absence of fish-slaughter facilities on their territory.

Out of the 67 facilities, two slaughter facilities of rainbow trout (one in Veneto and one in Lombardy) and one of European seabass and gilthead seabream (in Sicily) were excluded from the analysis because they did not slaughter fish in the period used for collection of data. Therefore, the number of the facilities included for the data analysis on the fish stunning/killing methods in Italy was 64, where a total volume of 22,229 tonnes of fish was processed. The regions of Veneto, Piedmont, Apulia, Lombardy, and Friuli Venezia Giulia were the only ones which reported more than five fish-slaughter facilities in their territory (Figure 1). Marche, Veneto, Tuscany, Lombardy, and Lazio were the regions processing more than 1,500 tonnes of fish each (Table 2).

Fish slaughtering in Italian facilities involved 20 different species, and the 64% of the facilities processed more than one species. The main slaughtered fish species in Italy were rainbow trout (29/64), followed by European seabass and gilthead seabream (21/64), Arctic char (*Salvelinus alpinus*; 14/64), European eel (12/64), sturgeon (11/64), common carp (6/64), and brown trout (*Salmo trutta fario* L.; 5/64). The minor slaughtered fish species: meager (*Argyrosomus regius*), hybrid striped bass common called "persico-spigola" (*Morone chrysops* × *Morone saxatilis*), trout perch (*Micropterus salmoides*), gray mullet (*Mugil cephalus*), catfish (channel catfish—*Ictalurus punctatus* and black bullhead—*Ameiurus melas*), lavaret (*Coregonus lavaretus*), Danube salmon (*Hucho hucho*), pike-perch (*Sander lucioperca*), royal perch (*Perca fluviatilis*), turbot (*Psetta maxima*), sargo (*Diplodus sargus*), tench (*Tinca tinca*), and greater amberjack (*Seriola dumerili*; Table 2).

The majority of facilities (86%) practiced only one method, while a few used to practice more than one (14%). The most common methods applied were "asphyxia in ice/thermal shock" and "electric in water bath," which were used in 30 and 24 of the facilities respectively, followed by "percussion" (13), "asphyxia in air" (5), and "electric dry system" (3) as shown in Figure 2. Two facilities did not perform any stunning or killing and sold live carps and eels directly to the consumer.

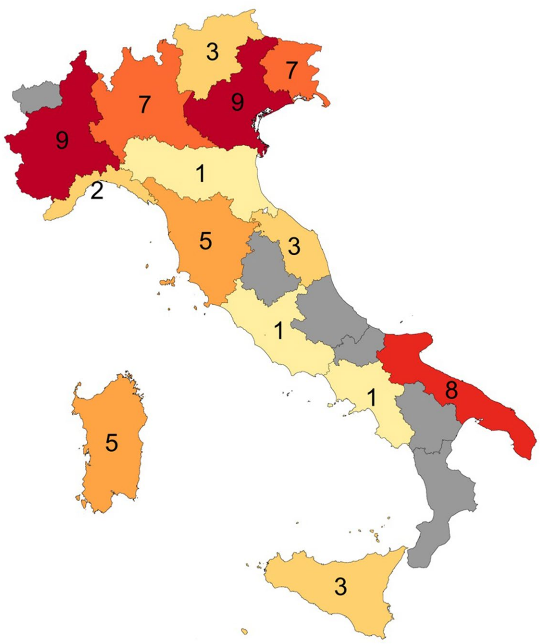

FIGURE 1
Distribution of the 64 fish-slaughter facilities in the Italian regions. In gray, the regions with no facilities.

The different stunning/killing methods and the frequently used indicators to assess the effectiveness of the reported methods for the slaughtered species are shown in Tables 3, 4, respectively.

Looking in detail at the main species, data showed that thermal shock method was used in 95% of the cases for slaughtering of European seabass and gilthead seabream; the electric in water bath method was utilized in more than half of the facilities for rainbow trout, Arctic char, European eel, common carp, and brown trout; percussive method was practiced for sturgeon in more than half of the facilities. Also for minor species, the most used method is thermal shock while the electric in water bath, percussion and asphyxia were less practiced. Overall, the assessment of unconsciousness or death was carried out routinely in 45 of the surveyed facilities (72%). In details, for the main species, more than 90% of the facilities assessed unconsciousness or death, with the exception of European seabass and gilthead seabream which were assessed in only 28% of facilities. For minor species, the assessment was performed in 66% of facilities. The 50% of all facilities assessed the unconsciousness using more than one indicator (Figure 3). "Breathing" and "coordinated movements" were the indicators most frequently used (34 facilities), followed by "response to stimuli" (18), "righting ability" (15), and "eye movements" (14).

"Breathing" and "coordinated movements" were the most frequently assessed indicators for rainbow trout (24/29 and 19/29 respectively), Arctic char (13/14 and 10/14), European seabass and gilthead seabream (4/21 and 6/21), and brown trout (5/5 and 3/5). The indicator "response to external stimuli" is also assessed in about half of the facilities that slaughtered European eel (6/12) and common carp (4/6). For sturgeon "righting ability" is also assessed (6/11). Relating the stunning/killing methods to the assessment of unconsciousness, the electric and percussion methods are those for which the assessment of unconsciousness is practiced in more than 90% of cases in contrast to the ice and air asphyxia methods, for which the assessment is practiced around the 55% of the times (Figure 2). The distribution of the considered indicators used to assess fish unconsciousness differentiated by the methods used, is shown in Figure 4.

4. Discussion

4.1. General consideration on slaughter procedures

4.1.1. Stunning/killing methods

The 14 Italian regions that participated in the survey and confirmed to practice fish slaughtering correspond to the regions with the highest presence of fish farms for human consumption in Italy (95% of all farms). In particular, the top five regions in terms of number of fish farms are Veneto, Piedmont, Friuli-Venezia Giulia, Trentino-South Tyrol, and Lombardy (28). Therefore, we can consider the collected data a significant representation of the current Italian situation of fish farmed and slaughtered for human

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TABLE 2 Distribution of the different fish species slaughtered in the 64 Italian slaughtering facilities surveyed for data collection (*n*=number of fish-slaughter facilities in the region).

	Italian region														
	Veneto (*n* = 9)	Piedmont (*n* = 9)	Apulia (*n* = 8)	Lombardy (*n* = 7)	Friuli V.G. (*n* = 7)	Tuscany (*n* = 5)	Sardinia (*n* = 5)	Marche (*n* = 3)	Trentino ST (*n* = 3)	Sicily (*n* = 3)	Liguria (*n* = 2)	E. Romagna (*n* = 1)	Lazio (*n* = 1)	Campania (*n* = 1)	Total (*n* = 64)
	5,559 t.	*62 t.*	*1,266 t.*	*2,114 t.*	*1,121 t.*	*2,663 t.*	*752 t.*	*6,249 t.*	*61 t.*	*55 t.*	*438 t.*	*38 t.*	*1849 t.*	*2 t.*	*22,229 t.*
Rainbow trout	6	5	-	4	4	1	1	3	3	2	-	-	-	-	**29**
Seabass-seabream	-	-	7	-	3	3	4	-	-	-	2	-	1	1	**21**
Arctic char	2	4	-	1	2	-	-	2	3	-	-	-	-	-	**14**
European Eel	4	3	1	1	-	1	1	-	-	-	-	1	-	-	**12**
Sturgeon	3	3	-	4	-	-	-	-	-	-	-	1	-	-	**11**
Common carp	4	1	-	1	-	-	-	-	-	-	-	-	-	-	**6**
Brown trout	-	-	-	1	1	-	-	2	1	-	-	-	-	-	**5**
Meager	-	-	2	-	-	-	-	-	-	-	-	-	1	-	**4**
Trout perch	-	1	-	-	-	-	-	-	-	1	-	1	-	-	**3**
Hybrid strip. Bass	-	1	-	-	-	-	-	-	-	1	-	1	-	-	**3**
Royal perch	1	-	-	-	-	-	-	-	-	-	-	1	-	-	**2**
Gray mullet	-	-	-	-	-	-	2	-	-	-	-	-	-	-	**2**
Catfish	-	1	-	-	-	-	-	-	-	-	-	1	-	-	**2**
Lavaret	-	-	-	-	-	-	-	-	1	-	-	-	-	-	**1**
Danube salmon	-	-	-	-	-	-	-	-	1	-	-	-	-	-	**1**
Pikeperch	1	-	-	-	-	-	-	-	-	-	-	-	-	-	**1**
Tench	-	1	-	-	-	-	-	-	-	-	-	-	-	-	**1**
Greater amb.	-	-	-	-	-	1	-	-	-	-	-	-	-	-	**1**
Sargo	-	-	-	-	-	-	1	-	-	-	-	-	-	-	**1**
Turbot	-	-	-	-	-	-	-	-	-	-	-	-	-	1	**1**

The annual volume of fish processed for human consumption is reported by each region.

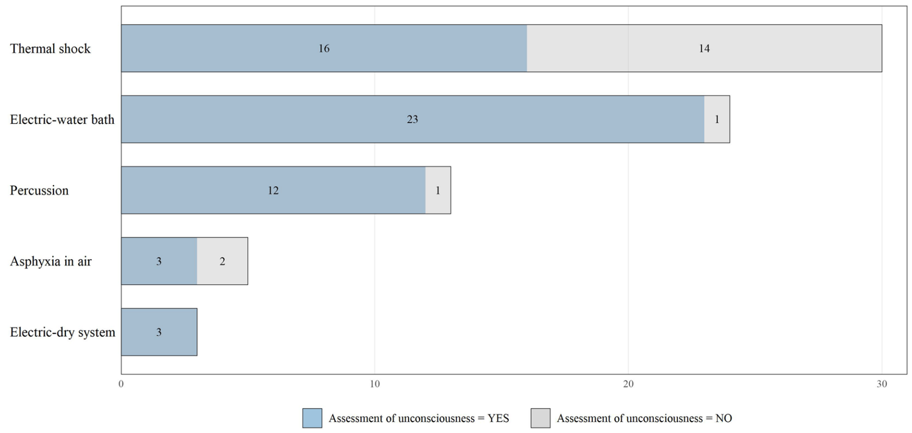

FIGURE 2
Distribution of different stunning/killing methods reported by the 64 fish-slaughter facilities surveyed, in association with the practice of assessing fish unconsciousness.

consumption. As a matter of fact, considering the volumes processed by the 64 facilities, the results show that the data in this survey refer to the 41% of the total volume processed in Italy in 2022 (53,900 t.) (44). Consistent with production data (43), the results of the survey show that rainbow trout is the species most commonly slaughtered, followed by European seabass, gilthead seabream.

The survey also showed that the several facilities practiced slaughtering of more than one species, which could be a critical point for fish welfare, since WOAH (20) recommends that stun and slaughter facilities should be designed and constructed for one specific species or group of species in order not to compromise their welfare and not to cause injuries or stress.

A wide variety of methods were used in the surveyed facilities for the slaughtering of rainbow trout, Arctic char and sturgeon, whereas mainly one method was used for European seabass and gilthead seabream. According to the European legislation on the slaughter of farmed species (EC Reg. 1099/2009) and the recommendations of the WOAH and EFSA, animals must be subjected to a stunning process before being slaughtered in order to become unconscious and insensible. However, based on the results of the survey, in Italy, more than half of the fish-slaughter facilities (35/64) practiced the asphyxia in air or the thermal shock method. These procedures are considered non-humane methods of slaughtering fish by WOAH and EFSA. In fact, the asphyxia in air is considered only as a ‘killing’ method; the thermal shock does not stun the fish but produces only sedation, leading the conscious animal to death by asphyxia (12, 20). The remaining methods used in the Italian fish-slaughter facilities are generally recommended by the WOAH and EFSA as acceptable methods. They are namely the electrical methods and the percussion method, followed by gill-cutting, which, if carried out correctly, can induce a state of instantaneous unconsciousness before slaughtering (12). Specifically, the electrical method induces unconsciousness, but its efficacy can vary considerably according to the parameters set (mainly V, A, Hz and time of application) (12, 20). The details for these parameters were not collected in our study and need further investigations.

4.1.2. Assessment of unconsciousness or death

One of the key aspects of evaluating the welfare of fish slaughtered in the field is to assess their state of unconsciousness or death after the application of the stunning/killing method. In our survey, there were no facilities that reported the assessment of unconsciousness or death by EEC, due to evident practical problems, whereas the majority of the surveyed facilities (72%) used the evaluation of the reflexes, spontaneous behavior or response to external stimuli, as recommended by EFSA and WOAH (12, 20). Indeed, there was a big difference in the frequency of this assessment, depending on the fish species slaughtered and method used (Figure 4). The low number of facilities (28%) assessing unconsciousness or death on European seabass and gilthead seabream was actually striking. This data negatively influences the overall result, since unconsciousness was assessed in almost all facilities for all the other main species. Probably, this result is related to the problem of the assessment of the individual fish unconsciousness, which is particularly difficult when the production volumes are high. For this reason, it is recommended to have group indicators available, as suggested by Noble et al. in their handbooks (45, 46).

Most Italian facilities carried out this assessment using the indicators “breathing” and “coordinated movements” which are probably more easily to check compared to eye movement assessment on an individual fish. However, assessment by breathing and body movements can be ineffective or unreliable indicators (47). In fact, as previously reported, asphyxia in ice and incorrectly performed electrical methods are recognized to paralyze fish, thus making it unable to behaviorally express pain (12). There are currently no behavioral indicators that can fully differentiate paralysis from unconsciousness or death (12). In the absence of alternatives, the assessment of a combination of different indicators can improve the evaluation (48, 49), as it occurred in half of the surveyed facilities (Figure 3).

4.2. Species-specific consideration on slaughter procedures

Since one of the objectives of the survey was to relate the collected slaughtered practices with the available species-specific

TABLE 3 Stunning/killing methods and indicators of consciousness used for the main slaughtered fish species in the surveyed fish-slaughter facilities.

	Stunning/killing methods						Indicators of unconsciousness				
	Thermal shock	*Electric water bath*	*Percussion*	*Asphyxia in air*	*Electric dry system*	*Sold alive**	*Breathing*	*Eye movements*	*Coord. movements*	*Response to stimuli*	*Righting ability*
Rainbow trout (*n* = 29)	7	17	7	4	1	-	24	10	19	10	10
Seabass-seabream (*n* = 21)	20	-	-	1	-	-	4	1	6	2	1
Arctic char (*n* = 14)	2	10	3	1	-	-	13	6	10	6	7
European eel (*n* = 12)	3	8	-	1	-	2	5	1	9	6	2
Sturgeon (*n* = 11)	3	3	6	-	2		7	4	6	5	6
Common carp (*n* = 6)	2	4	-	1	-	1	5	1	2	4	2
Brown trout (*n* = 5)	-	4	1	-	-	-	5	2	3	2	1

*The practice "sold alive" is also reported.

recommendations (Table 5) among the species slaughtered in Italy, only those covered by these recommendations were taken into account for this purpose, i.e., rainbow trout, common carp, European eel, turbot, European seabass, gilthead seabream and, in some aspects, also the other species belonging to the *Salmonidae* family (Arctic char, brown trout, Danube salmon and lavaret). For the other eleven slaughtered species, the general recommendations on welfare at slaughter of the WOAH and EFSA (12, 20) were used.

4.2.1. Rainbow trout

Based on the results of the present survey, trout was the species slaughtered with the highest variety of slaughtering methods in Italian facilities, which is consistent with available data about the other major European producers of trout (France and Denmark) and more generally whole of Europe (9, 16). The most commonly used stunning/killing method was the electric one, like in Denmark (9), which, if carried out correctly, satisfies the recommendations of both WOAH and EFSA (16, 20). This result confirms the use of the electric practice on trout in Italy, as previously reported (9). The second most practiced method was percussion, which is the most used method in France (9). If correctly done within 10 s from the moment the fish is pulled out of the water, percussion is also considered a humane method for trout (16). As in other European countries, asphyxia in ice and air were also practiced, although they should be avoided, since they do not induce effective loss of consciousness (9, 12, 20, 50).

The assessment of trout unconsciousness was mainly carried out using "breathing" and "coordinated movements" as recommended by WOAH (20). According to EFSA (16), however, these indicators are considered acceptable as indicative of unconsciousness but are not really robust or validated in laboratory conditions. As a matter of fact, although some authors have recently stated that loss of respiratory movement could be related to unconsciousness (24), others found no clear relationship between loss of ventilation and brain failure in rainbow trout under laboratory conditions (47). Thus, we can conclude that the assessment of unconsciousness/death for trout in Italy can be considered to be correctly carried out in the majority of the fish slaughter facilities.

4.2.2. European eel and common carp

Different slaughter methods have been reported for eel in our survey in Italy, as in other European countries (15), with the electric method the most widely used. This method is among those recommended by WOAH (20) for this species and has recently been introduced by the first two major European producers, i.e., the Netherlands and Germany (31). Non-humane procedures such as live evisceration or baths in ammonium salts (15), reported to be carried out in other European countries, were not practiced in Italian slaughtering facilities. Nevertheless, both eel and carp can be sold alive to the consumer in Italy (practice previously reported by EFSA in Europe only for carp) (19) which poses possible risks to the welfare of the fish at the time of killing (19). However, this practice was not widespread in Italy compared to northern European countries (19) and was confined only to two regions (Puglia and Veneto). The electric method was the main method practiced in Italy for carp, as in the top two European carp producers, Germany and Poland (9). This method satisfies WOAH recommendations, whereas EFSA highlighted some critical points on the methods and required more investigations in order to be able to express itself more accurately (19). However, recent studies by Daskalova et al. (51) have pointed out that the electrical method can be considered an acceptable method for carp, with a low impact on the welfare of the slaughtered carps. In particular, the use of electrical stunning alone could not make the carp unconscious for a long time, as demonstrated by quick VER recovery after stunning (VER were recorded already at 30 s post stunning) (32). Interestingly, in the field study conducted by Retter et al. (32) in Germany, the majority of farms used a combination of electrical stunning immediately followed by manual percussive stunning (59%). Under this condition, 92.6% of stunned carps displayed no behavioral indicators of consciousness and significantly fewer injuries related to mishits compared to sole percussive stunning. Thus, using a combination of electrical stunning and percussion could be a better option for this species, as the use of the singular methods could not be exhaustive in inducing unconsciousness under field conditions. In our survey, for both eel and carp, killing by ice or air asphyxia were used. However, this can pose risks for fish welfare since carp can survive up to 5 h in apnea (52) and eel, due to its peculiarity of being able to partially breathe with its skin, can survive even for days (12).

The assessment of unconsciousness for carps and eels was carried out mainly using "breathing," "coordinated movements," and "response to stimuli," that are among those recommended by WOAH (20). However, this assessment suggests caution in its application because of the physiological peculiarities of these species: e.g. resistance to breathing out of the water (described above) and as for the eel, the ability to move the body even when

TABLE 4 Stunning/killing methods and indicators of consciousness used for the minor slaughtered fish species in the surveyed fish-slaughter facilities.

	Stunning/killing methods					Indicators of unconsciousness				
	Thermal shock	*Electric water bath*	*Percussion*	*Asphyxia in air*	*Electric dry system*	*Breathing*	*Eye movements*	*Coord. movements*	*Response to stimuli*	*Righting ability*
Meager ($n=4$)	3	-	-	-	-	1	1	1	-	-
Trout perch ($n=3$)	3	-	-	-	-	3	3	2	3	3
Hybrid strip. Bass ($n=3$)	3	-	-	-	-	3	3	2	3	3
Royal perch ($n=2$)	2	1	-	-	-	2	1	1	1	1
Gray mullet ($n=2$)	1	-	-	1	-	-	-	1	-	-
Catfish ($n=2$)	2	-	-	-	-	2	2	1	2	2
Lavaret ($n=1$)	-	-	1	-	-	1	1	1	-	1
Danube salmon ($n=1$)	-	-	1	-	-	1	1	1	-	1
Pikeperch ($n=1$)	1	1	-	-	-	1	-	-	-	-
Tench ($n=1$)	-	1	-	-	-	-	-	1	1	-
Greater amb ($n=1$)	1	-	-	-	-	-	-	-	-	-
Sargo ($n=1$)	1	-	-	-	-	-	-	1	-	-
Turbot ($n=1$)	1	-	-	-	-	1	-	1	1	-

the brain is dead. In fact, for eel, in the surveyed facilities, the assessment was carried out taking into account the fish movements and breathing, which are considered not reliable indicators by EFSA (15).

4.2.3. European seabass, gilthead seabream, and turbot

For seabass, seabream, and turbot, asphyxia ice in was confirmed as the most widely used practice in Italy for slaughtering. This method is not accepted by WOAH (20), although it represents the most common method used also by the other main European producers, Spain and Greece (9). According to EFSA, an alternative slaughter method is requested for these species (14, 17). In fact, thermal shock has been shown to cause immobility and paralysis of fish: seabream remains conscious for up to 5 min after immersion in water and ice (53) and turbot even up to 90 min (54). The use of anesthetics (i.e., clove oil) (55, 56) or the diffusion of gases (i.e., carbon dioxide or nitrogen) (35, 57) in the stunning tank allow fish to reach faster unconsciousness and death than ice slurry application alone. For the same reason, research testing of electrical stunning reached promising results in seabream and seabass. This method, followed by a thermal shock, has recently been introduced in Europe in some seabass and seabream farms on an experimental basis (9). Recent studies have shown that the electrical method can be a valid alternative for the slaughter of turbot also (58). The introduction of this method also in Italian facilities should be considered if experimental data confirm an improvement of the current slaughter conditions for these species.

In the majority of the surveyed facilities, the slaughter of these fish took place without the assessment of unconsciousness or death. When assessed, the main indicators used namely "breathing" and "coordinated movements," have been found in some studies to be not reliable and not robust indicators, even more so in regard to the main method applied, which causes paralysis (12, 17).

4.2.4. Salmonids (different from rainbow trout)

For Arctic char, brown trout, Danube salmon and lavaret, the main methods currently used in the surveyed Italian facilities are among those reported by WOAH specific recommendations on stunning/killing methods for species of the *Salmonidae* family (20), i.e., percussion and electric.

For these species, fish unconsciousness was monitored in almost all facilities, mainly by assessing the respiration and coordinated movements, among the indicators recommended by WOAH (20). To the knowledge of the authors, no scientific study or species-specific opinions/guidelines are available about the reliability of these indicators for these species.

Certain recommendations contained in EFSA's opinions on Atlantic salmon and trout can generally be extended to Arctic char and brown trout and other salmonids. However, caution should be exercised when using these recommendations as there are different species within the family that react differently to stunning and killing methods (e.g., Arctic char has been shown to be strongly resistant to electricity).

4.2.5. Other species

In the surveyed facilities, most of the species in this group were slaughtered using methods considered non-humane, which should be replaced (12, 20). Only asphyxia (in ice and air) was used for meager, trout perch, hybrid striped bass, gray mullet, catfish, greater amberjack, and sargo. For royal perch and pikeperch, electrical stunning was also utilized and should be preferred from a welfare point of view (12, 20).

Sturgeon and tench were the only species of this group to be slaughtered mainly in accordance with the general recommendations of WOAH and EFSA (12, 20) for appropriate slaughter, i.e., with percussion and electric methods. However, it should be highlighted that these are very different species both in terms of size and behavior, thus there is an evident criticality when following the general guidelines for the analysis. For sturgeon, the mechanical or electrical methods perfectly fall within the generically recommended methods by WOAH and EFSA (12, 20). However, stunning procedures can considerably differ from facility to facility. Based on data collected by the survey, some facilities used rubber hammers, others used steel hammers, sometimes inside, some other outside the aquatic environment. In one case, the use of the sheep-specific stunner was also reported. It is evident how these methods have numerous practical critical issues, including dependence on the subjectivity of the operator and on his/her ability, training or state of fatigue. Last, but not least, while slaughtering methods should be species-specific, the use of the same methods and the same working conditions for different species, especially when minor species are involved, is a critical point. For the sturgeon in particular, that is a species so different from the other farmed and slaughtered species, there is a general lack of specific recommendation. Recently, Williot et al. (59) reviewing the existing Siberian Sturgeon farming procedures in relation to their welfare, highlighted that there is no published study focused on slaughter of Sturgeon. Finally, while most of surveyed facilities monitored unconsciousness in fish belonging to this group by the indicators recommended by WOAH (20), species-specific and validated indicators are lacking for these species.

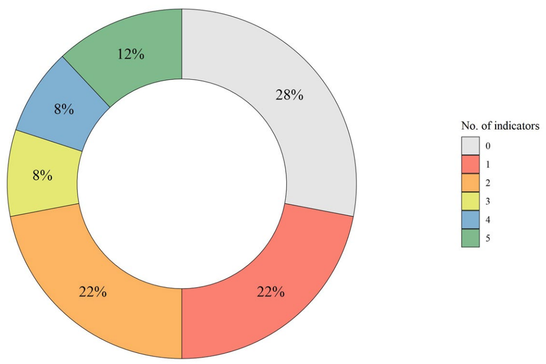

FIGURE 3
Distribution of the number of indicators considered for the assessment of fish unconsciousness during slaughter. The value 0 shows the number of fish-slaughter facilities that do not perform any assessment.

5. Conclusion

This is the first study providing data about the systems used for stunning and killing fish and the methods used to evaluate the unconsciousness in Italian fish-slaughter facilities. In view of the revision of the European legislation about welfare of farmed animals and in the absence of field data about fish, extensive information about the systems currently used for slaughtering is pivotal for all the stakeholders in order to have an overview of fish welfare at slaughter.

Based on the collected information, methods considered non-humane are still widely used for the slaughtering of fish in Italy, especially for sea bass, sea bream, and the less slaughtered species, whereas for the other species, slaughtering mainly follow the WOAH recommendations. The lack of scientific data and validated indicators make it difficult to obtain a clear picture of the welfare condition at slaughter for many species. On the other hand, as for rainbow trout, i.e., the major farmed fish in Italy, both the used stunning/killing methods and the indicators of unconsciousness are consistent with species-specific recommendations about welfare at slaughter.

In conclusion, more studies are necessary to clearly identify the best species-specific methods to protect fish at slaughter and the best indicators to be used to assess unconsciousness and death. Similarly, it is pivotal to gather more data on the feasibility and efficacy of these methods and indicators in field conditions as well as their economic impact.

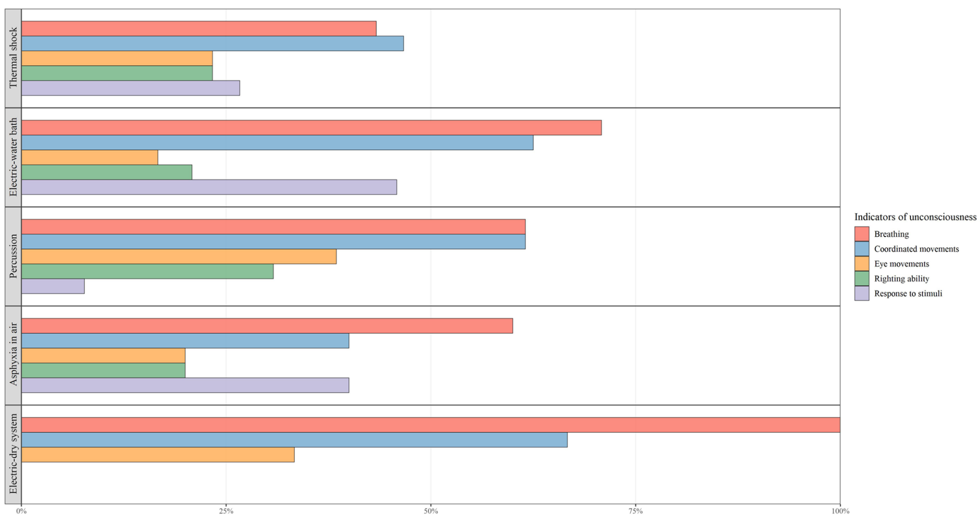

FIGURE 4
Indicators used by fish-slaughter facilities to assess unconsciousness according to the stunning/killing method used.

TABLE 5 General and specific WOAH (20) and EFSA recommendations (12–19) on stunning/killing methods of farmed fish for human consumption.

	Percussion		Electric		Spiking or coring		Asphyxia in air or ice	
					Free bullet		CO2	
	WOAH	*EFSA*	*WOAH*	*EFSA*	*WOAH*	*EFSA*	*WOAH*	*EFSA*
General considerations	✓	✓	✓	✓	✓	✓	✗	✗
Rainbow trout	✓	✓	✓	✓	✗	✗	✗	✗
Eel	✗	*	✓	✓	✗	✗	✗	✗
Carp	✓	*	✓	*	✗	✗	✗	✗
Seabream—Seabass	n/a	n/a	n/a	*	n/a	✗	n/a	✗
Turbot	n/a	*	n/a	*	n/a	✗	n/a	✗
Atlantic Salmon	✓	✓	✓	✓	✗	✗	✗	✗
Other salmonids	✓	✓	✓	✓	✗	✗	✗	✗
Tuna	✗	✗	✗	✗	✓	✓	✗	✗

✓, recommended/acceptable method; ✗, not recommended method; *, recommended/acceptable method which requires further studies on this species; n/a., species-specific recommendations not available.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Author contributions

SR and GC: conceptualization and funding acquisition. SR, GC, AT, TD, DB, FB, and VL: methodology. GC, SR, CT, and AB: formal analysis and investigation. GC, SR, and AT: writing—original draft preparation. GC, SR, AT, AM, CT, and VL: writing—review and editing. SR and AT: supervision. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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EDITED BY
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Shantou University, China
Pablo Almazan Rueda,
National Council of Science and Technology (CONACYT), Mexico
Caroline M. Maia,
FishEthoGroup Association, Portugal

*CORRESPONDENCE
Songming Zhu
zhusm@zju.edu.cn

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The effects of aerator noise on the swimming, feeding, and growth of *Micropterus salmoides*

Yadong Zhang[1], Abubakar Shitu[1], Shengyu Hang[1], Zhangying Ye[1], Hangfang Zhao[2], Wen Xu[2], Jian Zhao[1] and Songming Zhu[1,2*]

[1]College of Bio-systems Engineering and Food Science, Zhejiang University, Hangzhou, China, [2]Ocean Academy, Zhejiang University, Zhoushan, China

Aquaculture systems, replete with equipment noise originating from aerators, pumps, feeders, and filtration systems, are known to exert substantial influence on fish behavior and growth. In this research, the focus was directed towards comprehending the impacts of aerator noise on the swimming, feeding, and growth progression of largemouth bass. In the course of a 50-day experimental period, the bass population was segmented into two groups: aerator noise (90.3dB re μPa RMS^{-1}) and ambient noise (70.4dB re μPa RMS^{-1}). The findings indicated discernible disparities in the swimming behavior and feeding between the two groups. Specifically, bass in the noise group maintained greater average angular distance and physical separation from their nearest counterparts than the bass in the ambient group, which were 43.61 ± 1.89° and 85.47 ± 1.72mm for the ambient group and 48.32 ± 0.49° and 97.01 ± 0.57mm for the noise group. Furthermore, the feeding kinetic energy was markedly lower in the noise group as compared to the ambient group. For the first time, the Shannon-Wiener diversity index was leveraged to gauge the diversity of fish swimming behavior, with the results signifying the diversity index of the noise group was 2.69 ± 0.07 higher than that of the ambient group, which was 2.51 ± 0.02. Lastly, the noise group demonstrated compromised growth performance, with a significantly lower average weight as opposed to the ambient group, along with marked variations in the specific growth rate. These findings offer a telling revelation about the profound impacts of aerator noise on the behavioral and growth of largemouth bass, thereby forming a valuable referential base for future research centered on the effects of noise pollution on aquatic organisms.

KEYWORDS
aerator noise, largemouth bass, swimming behavior, feeding, growth

1 Introduction

As the global populace continues to expand, the imperative of food security and sustainable development is gaining paramount importance (Godfray et al., 2010). Aquaculture, an escalating segment of food production, is playing an increasingly significant role in maintaining global food security and bolstering economic growth (Subasinghe et al., 2009). As per a United Nations Food and Agriculture Organization (FAO) report, nearly half of the world's fishery yield is derived from aquaculture, with the prospect of this fraction growing in the foreseeable future (Canton, 2021). Aquaculture not only offers an abundant supply of animal and plant protein but also contributes to mitigating the overexploitation of marine resources and environmental degradation induced by conventional fishing methods (Naylor et al., 2000; Diana, 2009). In light of technological advances and improved aquaculture management, the industry is progressively adopting more ecofriendly and resource-efficient practices.

For instance, through deploying recirculating aquaculture systems (RAS), multitrophic aquaculture models, and precision feeding strategies, we can alleviate environmental pollution associated with aquaculture while augmenting resource utilization efficiency (Buck et al., 2018; Tang and Liu, 2018; Naylor et al., 2021). Amidst rapid advancements in aquaculture technology, the deployment of aquaculture equipment has become increasingly prevalent. While such equipment is instrumental in enhancing aquaculture productivity, promoting environmental quality, and ensuring product integrity, the escalating prevalence of noise pollution within the industry necessitates heightened attention (Bart et al., 2001; Slabbekoorn et al., 2010; Todd et al., 2021).

Noise emissions from various mechanical activities can have detrimental effects on animal health (Craven et al., 2009; New et al., 2014; Kunc et al., 2016; Slabbekoorn et al., 2019), compromising auditory abilities, inducing physiological stress (Popper, 2003; Halvorsen et al., 2012; Mancera et al., 2017), disrupting communication and behavior (Sarà et al., 2007; Gil et al., 2015; Gendron et al., 2020), and increasing stress-response hormones (Wale et al., 2013a; Hawkins and Chapman, 1975; Smith et al., 2004; Duarte et al., 2015), potentially impacting animal health and survival (Popper, 2003; Purser and Radford, 2011), especially in captive settings like farms and reserves (Popper and Hawkins, 2019; Sherwen and Hemsworth, 2019; Duarte et al., 2023). These noise-induced effects may manifest as reduced feeding behavior due to heightened vigilance (Evans et al., 2018; Giordano et al., 2022), leading to diverted attention (Mendl, 1999; Chan and Blumstein, 2011), potentially causing misjudgments in prey identification and feeding responses (Purser and Radford, 2011; Holles et al., 2013). Additionally, noise could obscure feeding-related acoustic signals (Brumm and Slabbekoorn, 2005; Zhang et al., 2021), resulting in missed feeding opportunities (Schaub et al., 2008; Siemers and Schaub, 2011) and altering food detection and classification during foraging (Lupien and McEwen, 1997; De Kloet et al., 1999).

Noise interference can disrupt animals' ability to accurately discern food quantity and quality (Kight and Swaddle, 2011), potentially leading to reduced foraging efficiency (Schaub et al., 2008), contrary to optimal foraging theory (MacArthur and Pianka, 1966). Alternatively, this could also be attributed to noise-induced stress, suppressing appetite and activity, and consequently reducing foraging behavior (Mendl, 1999; Charmandari et al., 2005; Williams et al., 2015; Zhang et al., 2022). For instance, Gendron et al. (2020) discovered that noise-exposed *Pseudopleuronectes americanus* larvae exhibited significantly shorter feeding durations and smaller stomach capacities than controls. Purser and Radford (2011) observed decreased foraging performance in *Gasterosteus aculeatus* due to increased feeding errors. Likewise, Voellmy et al. (2014) reported on the impact of noise on *Phoxinus phoxinus* feeding success. These investigations collectively suggest that noise pollution can significantly affect animal feeding behavior, consequently influencing their growth and health indices.

Noise disturbances can not only alter fish feeding behavior but also significantly impact their swimming behavior and spatial distribution (De Vincenzi et al., 2015; Sabet et al., 2016; Simpson et al., 2016; Mickle et al., 2019; Hang et al., 2021). For instance, Hanache et al. (2020) reported that *Phoxinus phoxinus*, when exposed to noise, allocated less time to foraging in low food density conditions, exhibited changes in spatial distribution, and demonstrated decreased cohesiveness in their swimming. Similarly, noise-affected tuna schools have been observed to alter their coordinated unidirectional swimming structure, increase vertical movements towards the water surface or bottom, and exhibit uncoordinated swimming behavior in more dispersed groups (Sarà et al., 2007).

Largemouth bass (*Micropterus salmoides*), an economically significant freshwater species known for its fast growth rate, high nutritional value, and strong environmental adaptability (Bai and Li, 2018), holds global economic importance in aquaculture. While previous research has explored the effects of noise on fish feeding and swimming behavior, this study takes approach by introducing the Shannon-Wiener Diversity Index (SWDI) analysis method, extending investigation to encompass group behavior. Specifically, this study will assess the impact of oxygenation equipment noise on feeding and swimming behaviors of largemouth bass school, and quantify the differences in noise impacts on fish school behavior. Through a comprehensive analysis of the findings, this study aims to offer scientific insights to the aquaculture industry, mitigate adverse environmental impacts, and bolster the sustainable development of the industry.

2 Materials and methods

2.1 Fish

In this study, 200 largemouth bass were procured, each with an average body length of 11.17 ± 0.36cm and a weight of 14.37 ± 2.13g, from Hangzhou Jianfeng Agricultural Development Co., Ltd. Before initiating the experiment, the fish were temporarily housed in a RAS for environmental acclimatization. The water parameters in the holding pool were sustained at certain levels to ensure optimal conditions for the fish. Specifically, the temperature was kept steady at around 25°C, within a margin of ±1°C. Similarly, the pH was 7.2 ± 0.8. Additionally, the concentration of dissolved oxygen was ensured to be 6.5 ± 1mg l^{-1}. Monitoring was also carried out on the levels of ammonia nitrogen and nitrite, with both being kept below 0.3mg l^{-1} and 0.5mg l^{-1}, respectively. Lighting for the RAS system was provided by full-spectrum LED lights with a 24-hour photoperiod. The fish were fed with floating feed (Fujian Tianma Science and Technology Group Co., Ltd.) at 8:00 a.m. and 8:00 p.m. daily. These preliminary steps were implemented to ensure optimal environmental and feeding conditions for the smooth progression of the subsequent experiment.

2.2 Experimental system setup

The experimental setup incorporated two sets of RAS (Figure 1), each containing three aquaculture pools made of PVC with an internal diameter of 1 meter and a height of 0.8 meters. The biological filter pool, a 1*0.5*0.8m PVC tank, facilitated ammonia removal, UV sterilization, and solid particle filtration, offering a controlled environment for the fish. The soundscape management equipment included a type 8130 hydrophone (Denmark Brüel & Kjær Co. Ltd.), a player, an AVANT MI-2004 power amplifier, and a US-0150 underwater loudspeaker (Hangzhou ECON Technology Co. Ltd., China), collectively used for noise soundscape collection and provision during the experiment. Additionally, the setup comprised a behavior video capture system (Hikvision, Hangzhou) to record the fish group's feeding and swimming patterns.

The experimental system was established in a quiet laboratory with measures like wrapping the stainless steel frame with blackout cloth, padding the culture pool's bottom with a PVC shock-absorbing tray, employing liquid oxygen for oxygen supply, using a fixed biological bed, and wrapping PVC pipes with sound-absorbing materials to mitigate interference from environmental noise and system operational noise.

2.3 Experimental design

In this study, a noise group was established and subjected to a sound level of 90.3dB re μPa RMS^{-1}, while an ambient control group with a sound level of 70.4dB re μPa RMS^{-1} was also established (Figure 2). The noise sample was collected from the large-scale aquaculture facility of Suzhou Jinchengfu Fisheries Co., Ltd. and effectively represents the acoustic characteristics encountered by fish in a closed RAS. The aeration equipment sampled was a GRB-200A three-lobe roots blower (GSD Industrial Co.,Ltd.) with an air volume capacity of 50m^3 min^{-1} and a pressure of 78.43kPa. To evaluate the acoustic impact of the aeration machine within the fish pond, recordings of the soundscape were obtained when the aeration machine operated exclusively. For the ambient control group, the soundscape was recorded with the complete RAS in the shutdown state.

Each group contained three replicates of the aquaculture pool, each housing 20 fish. The entire experiment spanned 50 days. During the experiment, the recirculating water flow was set at 300L h^{-1}, with a daily water change constituting 15% of the total volume. Fecal waste at the bottom of the fish pond was vacuumed daily. Concurrently, the parameters of the water quality were held in alignment with those established during the acclimation stage, and the fish were fed at 8:00 am and 8:00 pm each day.

2.4 Data collection and analysis

2.4.1 Fish swimming and feeding

To capture the feeding and swimming behavior of the largemouth bass, cameras and 24-hour constant LED lights were installed on the stainless steel frame carrying shade cloth, which was positioned directly above the culture tank. Recordings were made at

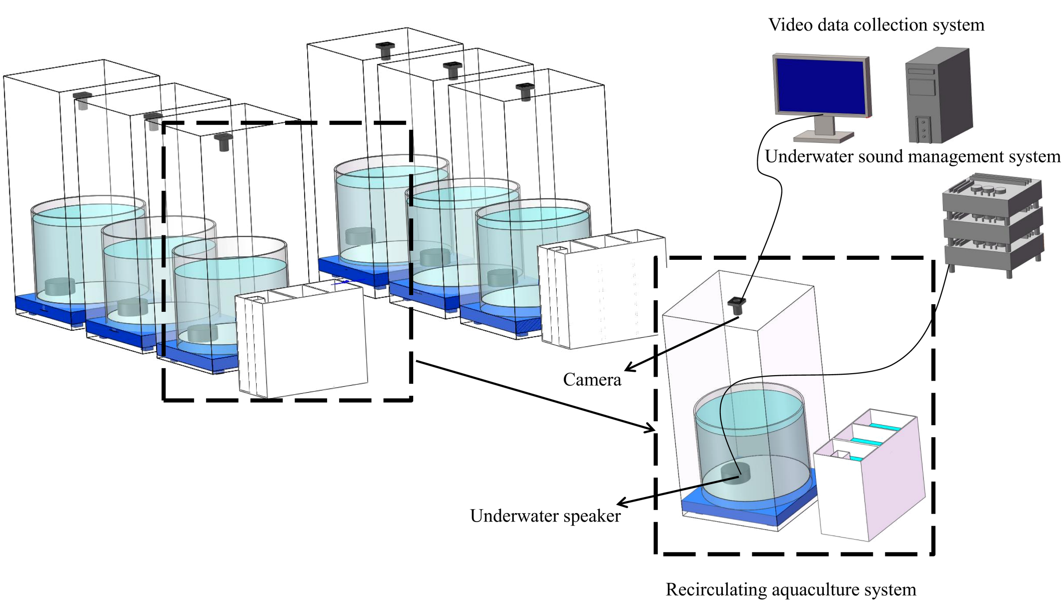

FIGURE 1
Schematic Diagram of the Experimental System.

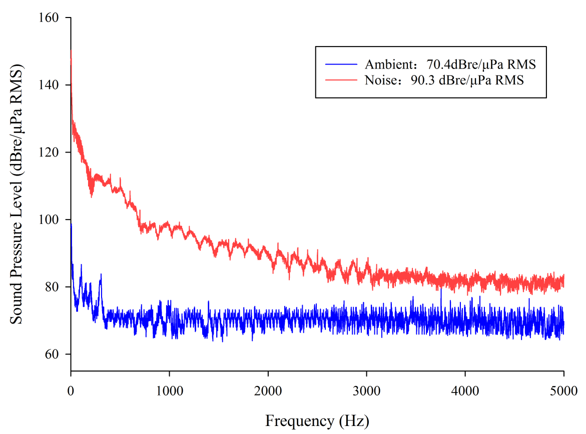

FIGURE 2
Comparison of Frequency Spectrums in Noise and Ambient Groups.

six designated time points (3:00, 6:00, 9:00, 12:00, 15:00, 18:00) each day, with each session lasting ten minutes, to analyze the group swimming behavior. To analyze feeding behavior, video footage was captured every three days at 8:00 in the morning. The cameras were networked to a switch and recorder, facilitating both video storage and real-time monitoring.

For the analysis of group swimming behavior, the Mask RCNN technique was employed (Figure 3). From each video, 60 frames were extracted, and object instance segmentation was conducted on the experimental fish in the frames. This generated a segmentation mask for each experimental fish, enabling their identification and localization (He et al., 2020). To evaluate the cohesion of swimming, the distance between every experimental fish and its two nearest neighbors within the group was computed. A line connecting a fish's head and tail represented its swimming direction. To evaluate the polarity of swimming, the angle formed by the swimming direction of an experimental fish and its two closest neighbors was utilized.

The SWDI, an index commonly used in biodiversity research proposed by Claude Shannon and Norbert Wiener in the fields of information theory and ecology, measures the richness and evenness of species in an ecosystem. This study proposed to use the SWDI to evaluate the diversity of group swimming behavior. The average angle and distance during the experiment were divided into nine angle ranges (0-10°, 10-20°, 20-30°…, >90°) and eight distance ranges (<60mm, 60-70mm, 70-80mm…., >120mm), generating 72 unique angle-distance combinations. The frequency of each angle-distance combination was counted, and the relative frequency calculated. The SWDI for group behavior was then determined using the formula:

$$H = -\Sigma\,(pi * \ln(pi))$$

Where H represents the SWDI, and pi is the relative frequency of the i_{th} angle-distance combination.

In analyzing group feeding behavior, this study implemented an Modified kinetic energy model (MKEM) to quantify the feeding intensity of the group (indicative of the group's appetite). The MKEM model doesn't require tracking or foreground segmentation. It facilitates the extraction and quantification of the spatial behavioral characteristics of the fish group, effectively describing the feeding behavior, independent of light environment and water quality (Wei et al., 2021). Greater intensity of water ripple fluctuations and alterations in the flow field in the reflective area, induced by feeding, result in higher feeding kinetic energy values (Figures 4A, B). Each feeding video clip in this experiment lasted 15 seconds, with a frame rate of 25fps, and the single-time feeding kinetic energy change is shown in Figure 5A. The feeding process was initiated when the feed was introduced. The same quantity of feed was given in each fish tank using a satiety feeding strategy, with leftover feed scooped out, dried, and weighed post-feeding to compute the feed intake quantity.

2.4.2 Growth

Prior to the commencement of the experiment, a random sampling of 20 fish was undertaken to determine initial body length and weight. Over the course of the experiment, fish were weighed every 10 days. At the conclusion of the experiment, eight fish were randomly selected from each cultivation pond for growth evaluation, with the concurrent tally of fish in each group also recorded.

The Specific Growth Rate (SGR, % d^{-1}) and Survival Rate (SR, %) were calculated as follows:

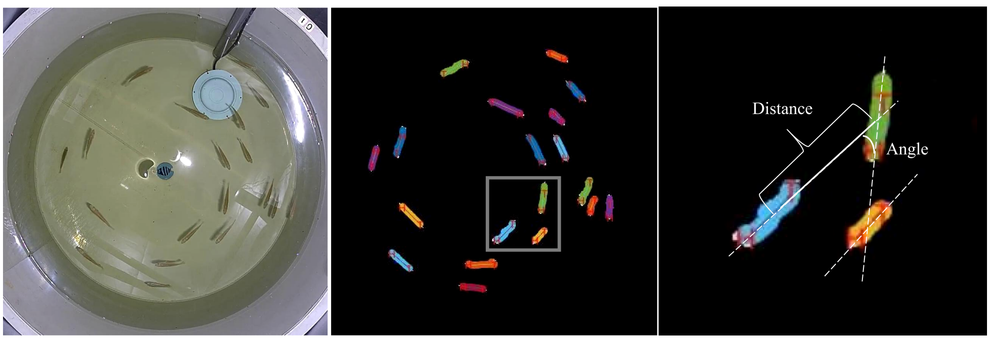

FIGURE 3
Quantitative Measurement of the Angle and Distance Between a Focal Fish and Its Nearest Neighbor.

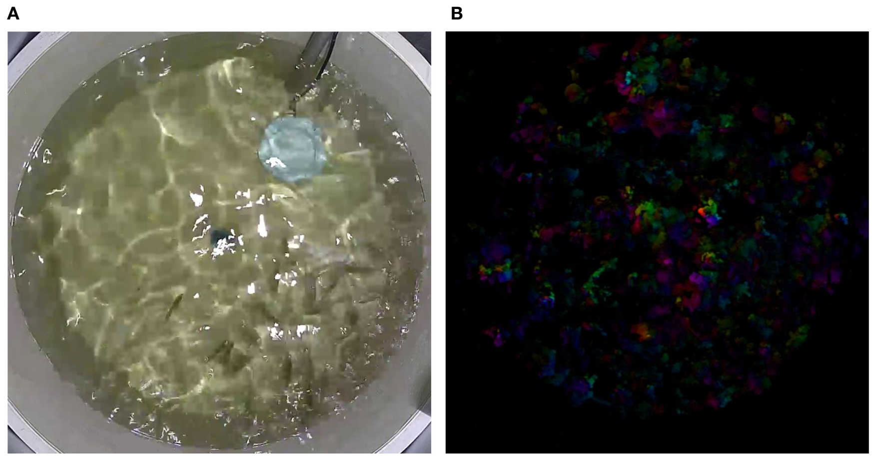

FIGURE 4
Feeding Kinetic Energy Analysis. **(A)** Snapshot of a Feeding Event: Visual depiction of largemouth bass during the feeding process. **(B)** Transformation of Feeding Snapshot into an Optical Flow Image: Conversion of the feeding snapshot into an optical flow image for further analysis.

$$SGR = 100 \times (\ln BW_2 - \ln BW_1) \times (T_2 - T_1)^{-1}$$

$$SR = 100 \times N_f \times N_i^{-1}$$

In these equations, BW_1 and BW_2 represent the initial and final body weights of each fish (in grams), while T_1 and T_2 denote the corresponding time intervals. N_i and N_f stand for the initial and final numbers of fish, respectively.

2.4.3 Data analysis

The data are presented as mean ± standard deviation (SD). A comparative analysis of growth, swimming, and feeding across different treatments was conducted using independent t-tests. For SWDI measured at multiple time points, comparisons were analyzed using repeated measures ANOVA. Prior to analysis, normality of the data distribution was assessed through the Kolmogorov-Smirnov test, and homogeneity of variances was examined using Levene's Test. In cases where the assumptions were met, the threshold for statistical significance was set at $P<0.05$. All statistical analyses were performed using IBM SPSS Statistics version 22.0. Graphical representations were generated using SigmaPlot Version 14.0.

3 Results

3.1 Swimming behavior

The results exhibited substantial disparities in swimming behavior between the largemouth bass in the ambient group and those exposed to noise. Throughout the experiment, the mean angle and distance between a focal fish and their nearest counterparts in the noise group were significantly greater than those in the ambient group (Figure 6), with average values of 48.32 ± 0.49° and 97.01 ± 0.57mm for the noise group, compared to 43.61 ± 1.89° and 85.47 ± 1.72mm for the ambient group ($P<0.05$).

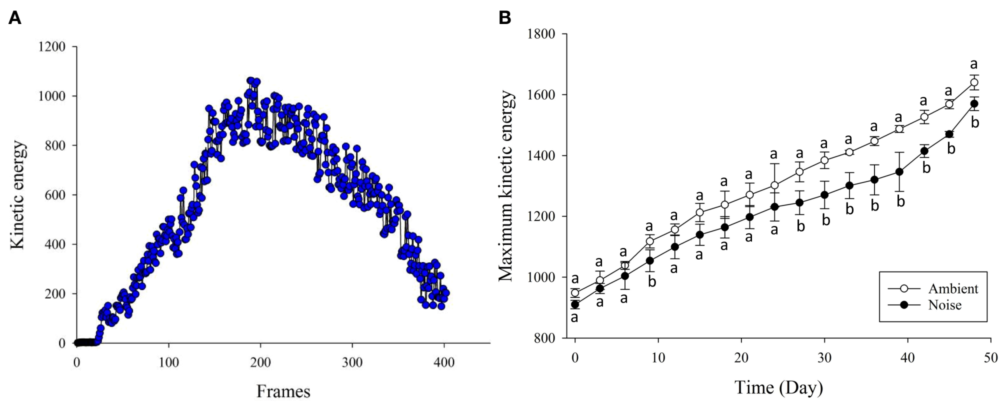

FIGURE 5
Kinetic Energy Changes During Feeding. **(A)** Change in Kinetic Energy During a Single Feeding: Variation in kinetic energy throughout a singular feeding event. **(B)** Peak Feeding Kinetic Energy Changes in Noise and Ambient Groups: Comparison of peak feeding kinetic energy changes in both groups from Day 1 to Day 50 of the experiment.

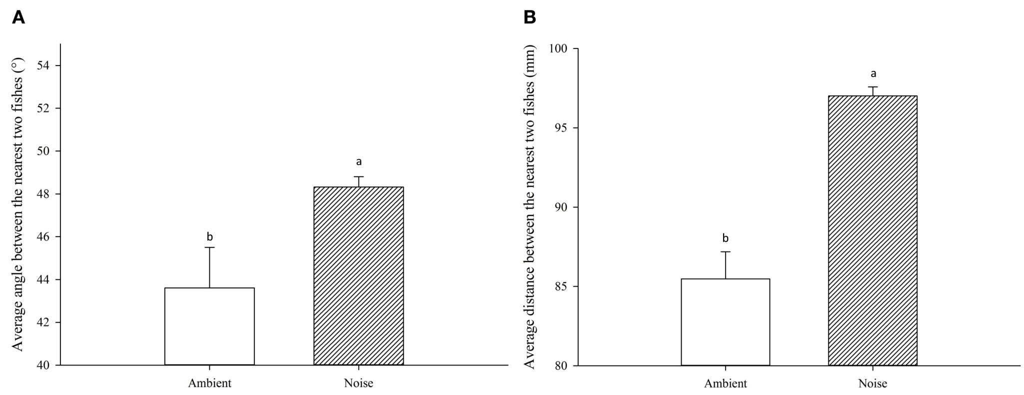

FIGURE 6
Comparative Analysis of Swimming Behavior in the Noise and Ambient Groups. **(A)** The average angle between the nearest two fish in each group. **(B)** The average distance between the nearest two fish in each group.

When considering the diversity of swimming behavior, notable differences were also observed between the ambient and noise groups. An examination of various angle-distance combination categories (Figure 7) reveals that in the ambient group, the combination of an average angle of 30-40° and an average distance of 80-90mm was observed in 12.73% of fish, followed by the combination of 30-40° and 90-100mm, which accounted for 11.23% of observations. Conversely, the fish in the noise group demonstrated more dispersed swimming behavior. The highest proportion of observations in the noise group was in the combination of 30-40° and 100-110mm (9.09%), followed by 30-40° and 90-100mm (8.72%). Swimming behavior in the noise group was characterized by larger average distances.

The mean SWDI also differed significantly between the two groups throughout the experiment (Figure 8A), with the noise group (2.69 ± 0.07) being significantly higher than the ambient group (2.51 ± 0.02) (P<0.05). As depicted in Figure 8B, the SWDI of the fish population in the noise group exhibited an upward trend over time. The two groups started to show significant difference one month after noise intervention, and this disparity persisted until the conclusion of the experiment. The present study further analyzed the average SWDI at various time points (3/6/9/12/15/18 o'clock), revealing diurnal patterns in fish activity (Figure 8C). At 6:00 and 18:00, the diversity of fish swimming behavior was lower (P<0.05), suggesting more stable formations. While similar trends were observed in both the noise and ambient groups, the average SWDI for fish swimming behavior in the ambient group was significantly lower at 3/6/18 o'clock compared to the noise group (P<0.05).

3.2 Feeding

With respect to feeding behavior, the largemouth bass in the ambient group exhibited a more robust feeding inclination, while those in the noise group displayed a diminished appetite (Figure 5B).

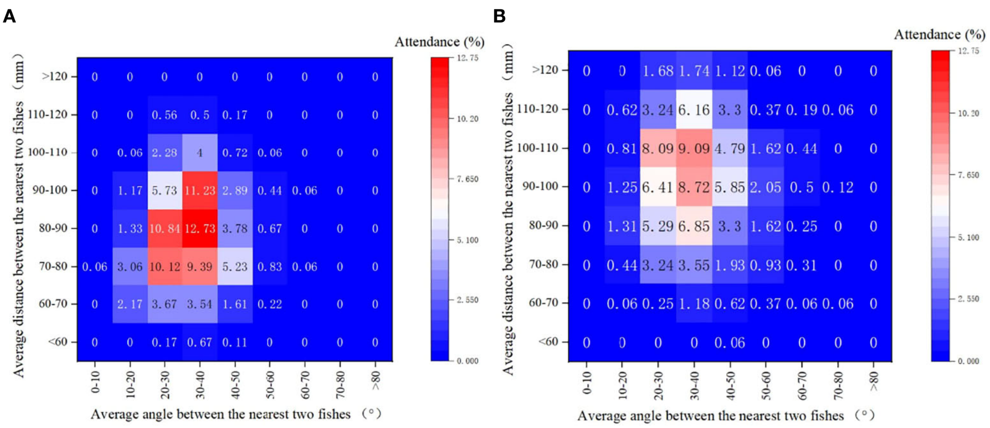

FIGURE 7
Heat Map Depicting the Distribution of Largemouth Bass Swimming Behavior in Both Experimental Tanks: The heat map visualizes the variations in the swimming behavior of largemouth bass in response to the experimental conditions. Both the average angle (horizontal axis) and average distance (vertical axis) between fish are represented. **(A)** Ambient Group: Depicts the swimming behavior distribution under normal conditions. **(B)** Noise Group: Illustrates the altered swimming behavior under the influence of equipment noise.

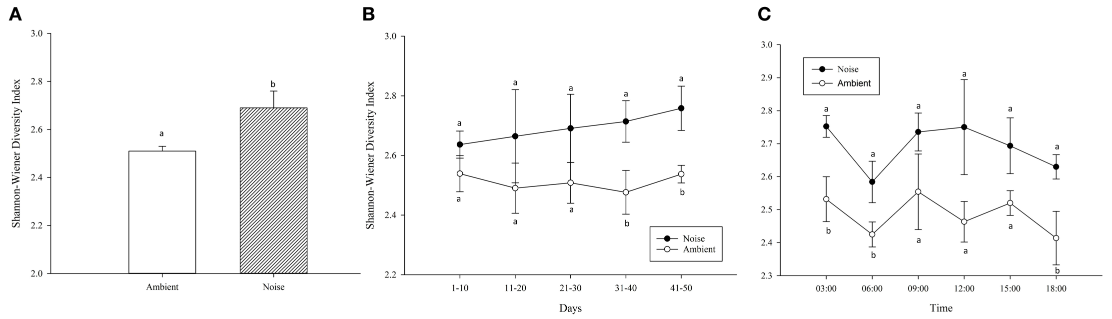

FIGURE 8
Variation in Shannon-Wiener Diversity Index (SWDI) Reflecting Swimming Behavior Diversity in Largemouth Bass Populations Across Noise and Ambient Groups: This figure illustrates the diverse swimming behaviors of largemouth bass in response to both experimental conditions using the SWDI. **(A)** Mean SWDI Throughout the Experiment. **(B)** Mean SWDI for Each 10-Day Period. **(C)** Mean SWDI at Different Time Points of the Day.

Specifically, the feeding kinetic energy in both groups increased as the experiment unfolded, albeit at different rates. During the early stage of the experiment, the feeding kinetic energy of the noise group was lower than the ambient group, though the difference was not statistically significant. However, from the 27th day onwards, the feeding performance of the ambient group consistently outstripped that of the noise group, a trend that persisted until the experiment's conclusion. Measurements taken on the 48th day indicated that the feeding kinetic energy of the ambient group stood at 1639.99 ± 24.30, compared to 1569.97 ± 22.53 for the noise group.

Moreover, the daily average feed intake of the largemouth bass in the ambient group exceeded that of the noise group, with the discrepancy widening over time. For most of the experimental period, the divergence in daily average feed intake between the two groups did not reach statistical significance, except on days 33, 47, 49, and 50 (Figure 9). Nevertheless, a marked contrast in total feed consumption was observed between the two groups (Figure 10), with the noise group's intake (24.95 ± 1.58g) significantly lower than that of the ambient group (29.25 ± 0.86g) (P<0.05).

3.3 Growth

The present study revealed that the SR remained 100% across all groups throughout the experiment, and that the noise generated by aeration equipment significantly impacted the growth performance of largemouth bass. At the experiment's inception, the initial weight of fish across all groups was identical, averaging 11.06 ± 0.68 g. After 50 days of experimental treatment, the mean weight of largemouth bass in the ambient group (30.61 ± 1.51g) was significantly higher than that in the noise group (26.36 ± 1.45g) (P<0.05), indicating that aeration noise impeded growth. Figure 11A illustrates the weights of the fish measured at ten-day intervals throughout the experiment. The data show that during the experiment's first month, there was no significant disparity in weight between the ambient and noise groups. However, from day 40 onwards, the weight of fish in the aerator noise group was persistently and markedly below the level in the ambient group, a trend that endured until the experiment's end. Over the course of the experiment, the weight difference between the ambient and noise groups progressively increased (Figure 11A). The SGR of

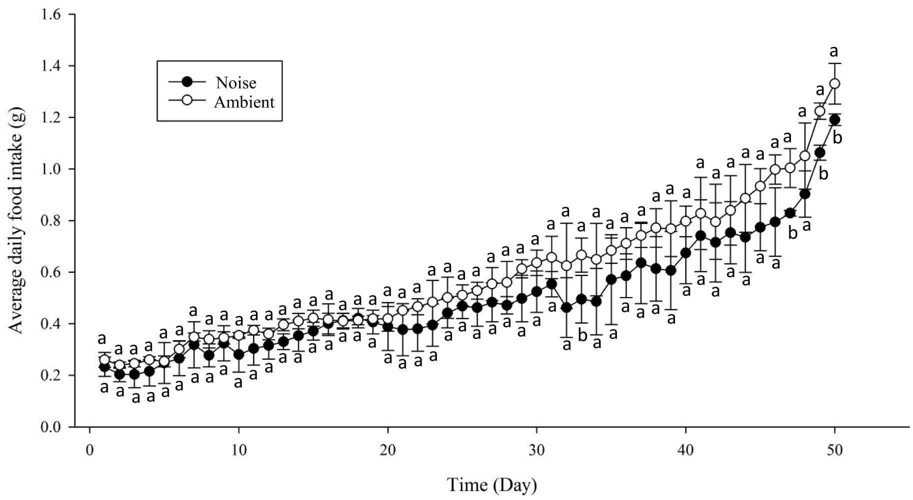

FIGURE 9
Comparison of Daily Feed Intake: This graph depicts the change in the average daily feed intake for both the Noise and Ambient groups over the course of the 50-day experimental period. Differences marked with different lowercase letters are considered significant.

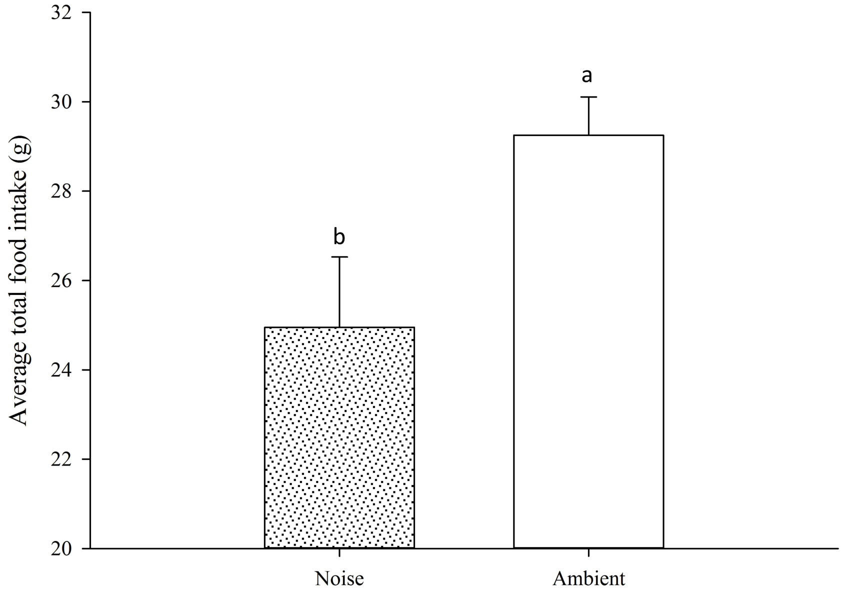

FIGURE 10
Total Food Intake Comparison: This figure illustrates the overall contrast in food consumption between the Noise and Ambient groups throughout the entire experimental duration. Differences marked with different lowercase letters are considered significant.

largemouth bass in the noise group and the ambient group also displayed significant divergence (Figure 11B), with the noise group's SGR (3.38 ± 0.09) markedly lower than that of the ambient group (3.68 ± 0.08) (P<0.05).

4 Discussion

In this investigation, the profound influence of aeration equipment noise on the swimming patterns, feeding habits, and growth of largemouth bass was distinctly observed. Evidently, compared to those in the ambient group, the fish in the noise group exhibited substantial differences in swimming behavior, feeding vigor, and quantity of food consumed. The escalated swimming expenditure and diminished feeding intensity and quantity within a noisy environment adversely affected the growth performance of the largemouth bass in the noise group.

Acoustic signals constitute one of the primary channels through which fish garner information about their environment, with aquatic species relying on auditory data for communication, navigation, orientation, evasion, and foraging (Popper and Hawkins, 2019). Noise acts as a stimulus source that disrupts fish attention and interferes with fish behavior by masking acoustic information (Slabbekoorn et al., 2010). In terms of swimming behavior observed in this study, significant disparities arose between the ambient and aerator noise groups. The average distance and angle between each fish in the noise group and its nearest neighbor were greater, implying that the noise environment

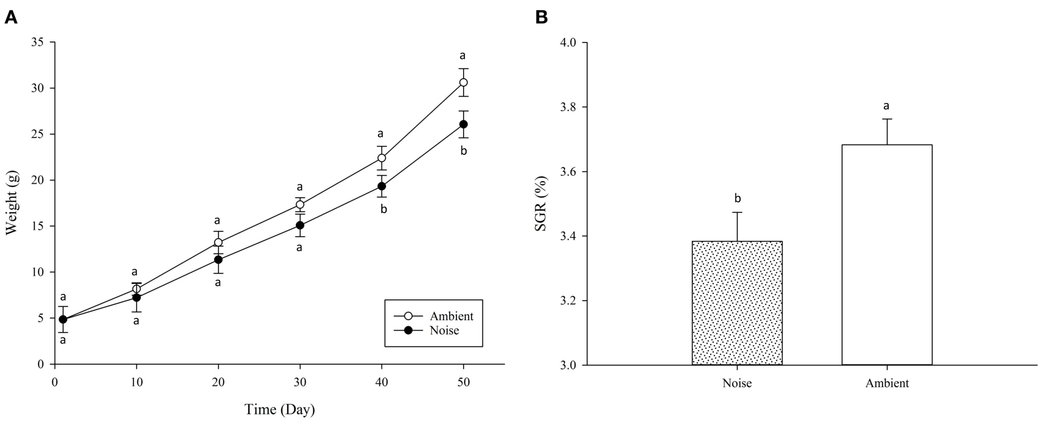

FIGURE 11
Largemouth Bass Growth Comparison in Different Noise Environments. **(A)** Body Weight Change in Body Weight per 10 Days. **(B)** Specific Growth Rate (SGR) Comparison.

might have impeded the orientation navigation information of the largemouth bass or induced discomfort, thus compelling them to maintain a more significant distance and angle from other individuals.

The distance and angle between the closest individuals in a fish school can serve as metrics of the school's clustering strategy, which enhances group collaboration and reduces swimming energy expenditure (Chen et al., 2016; Kent et al., 2019). In this study, the noise group fish had larger average distances and angles, indicative of lower fish school cohesion and polarity. This observation aligns with previous research findings. For instance, Sarà et al. (2007) found that noise could alter the swimming direction of tuna, leading to a relatively dispersed cluster structure and more chaotic swimming behavior in the school. Similarly, De Vincenzi et al. (2015) demonstrated that noise impacted the swimming activity and behavior distribution of the lesser spotted dogfish (*Scyliorhinus canicula*) in aquaculture ponds. McLaughlin and Kunc (2015) reported that noise exposure caused the european minnow (*Phoxinus phoxinus*) to modify its spatial distribution, with marked increases in social interaction and swimming distance. These findings resonate with the present observations: under the influence of the noise environment, the distribution of largemouth bass in the culture pond becomes more dispersed.

However, the research further points out that the noise environment not only affects the clustering behavior of largemouth bass schools but also affects the diversity of their swimming behavior. It was discovered that, compared to the ambient group, the diversity of swimming behavior among the largemouth bass in the noise group was higher. This could be due to the largemouth bass being distracted by the disruption of aeration equipment noise, or the occlusion of orientation and navigation acoustic information by noise. Additionally, this might be a response strategy of the largemouth bass to the noise environment, in which behavioral diversity is increased to adapt to the noisy conditions. A higher SWDI may indicate a more diversified distance and angle between fish in a school and more irregular formations during swimming. In some instances, a greater diversity of swimming behavior may correlate with increased swimming energy expenditure (Webb, 1984). For example, irregular formations that lead to larger distances between individuals could cause fish to expend more energy while adjusting their position and speed during swimming. Maintaining this state of elevated energy expenditure over an extended period could potentially affect individual growth performance within the fish school (Jobling et al., 1993). The application of the SWDI to assess the behavior of largemouth bass schools is a novel contribution of this study. Although this index is often used to evaluate the diversity of biological communities (Spellerberg and Fedor, 2003; Thukral, 2017), it is infrequently applied within behavioral ecology. The study's results indicate that this index effectively captures the diversity of swimming behavior in largemouth bass schools, thereby introducing a novel research tool in behavioral ecology. The reliability of the findings was ensured through meticulous experimental design and data analysis methodologies. For example, the swimming behavior of fish schools was continuously monitored throughout the experiment, encompassing comprehensive classification of angle-distance combinations and rigorous statistical analyses. Additionally, regular inspection and maintenance of experimental equipment and data training models were conducted to ensure the stability of the noise level and the accuracy of the algorithm. As a result, the credibility of the findings is held with a high level of confidence.

While this study primarily aimed to investigate the distinct impact of noise generated by oxygenation equipment on largemouth bass behavior, an additional insight emerged regarding the general behavioral patterns of fish schools. By analyzing the diversity of fish school behavior at different times throughout the day, the present study observed that fish schools maintain a more regular swimming formation prior to feeding times (6:00, 18:00). Post-feeding, fish schools may exhibit a greater diversity of behavior. Once satiated, they might be more inclined to engage in other activities such as reproduction, territorial protection, and social interactions (Ward et al., 2006). At this juncture, the fish school may become more dispersed, reducing the need for foraging, and individuals within the school may have greater freedom of movement. Under conditions of scarcity, the behavior of fish schools may be more uniform. This is due to the need for cooperation in the search for food in the face of reduced availability, causing the fish to form more compact groups. This scenario leads to an increase in fish school cohesion, with the tighter assembly being more effective in food foraging (Keenleyside, 2012). Starving fish schools might rely more heavily on the guidance of a leader (Reebs, 2000), subsequently reducing behavioral diversity.

In this study, it was observed that largemouth bass in the ambient group displayed a stronger feeding inclination, while those in the noise group demonstrated a significantly diminished appetite. This discrepancy might be attributed to noise distractions affecting the largemouth bass's focus, indirectly impacting their feeding desire and resulting in decreased food consumption. These findings align with those of Voellmy et al. (2014), who verified the disruptive influence of sound on fish feeding, showing that significantly more individuals in the control group obtained food, indicating that fish foraging activities were hindered under experimental conditions. Purser and Radford (2011) demonstrated that the stickleback fish, *Gasterosteus aculeatus*, exposed to noise, increased the frequency of attacks on non-food items due to misidentifying them as food, leading to a decline in its foraging performance. Gendron et al. (2020) found that the winter flounder, *Pseudopleuronectes americanus*, preyed less frequently and had a relatively reduced stomach capacity under noise conditions. Wale et al. (2013b) reported that noise affected the feeding behavior and performance of crabs in a tank-based controlled experiment. While the mentioned studies involve different fish species, the present study focus on largemouth bass contributes to understanding of how noise pollution can impact feeding behavior in this particular species.

It's worth noting that this research also observed that the feeding kinetic energy in both groups was initially low, but increased as the experiment progressed with the growth and increased food consumption of the largemouth bass. In the noise group, the increase in feeding kinetic energy may reflect an adaptive response, suggesting that, after a period of acclimation to the noise environment, largemouth bass could adjust their behavior to cope with the noise conditions (Dooling et al., 2015). Nonetheless, despite these adaptive shifts, the feeding performance of the largemouth bass in the noise-exposed group continued to be significantly inferior compared to the ambient group. This diminished performance might be attributed to the persistent interference and suppression induced by the noisy environment on the largemouth bass's feeding conduct and appetite.

This investigation underscores the significant influence of aeration equipment noise on largemouth bass growth performance. Largemouth bass in the noise-exposed group exhibited a considerably lower average weight and slower growth rate compared to the ambient group. This discrepancy may stem from the noise-induced reduction in feeding desire and intake, leading to decreased energy consumption. Additionally, the less cohesive and polarized swimming patterns observed in the noise group could contribute to heightened energy expenditure during swimming activities. These findings align with prior research. Lagardčre (1982) reported elevated noise levels in aquaculture ponds leading to increased metabolic rates in prawns and reduced growth performance. Banner and Hyatt (1973) observed decreased SR and growth performance in carp exposed to noise. Similarly, Wysocki et al. (2007) and Davidson et al. (2009) noted short-term negative effects of noise on rainbow trout growth performance in RAS, with potential adaptability over time. Hang et al. (2021) also explored the negative effects of noise on largemouth bass growth performance in RAS systems, this study uniquely focuses on analyzing the specific impact of aeration equipment noise, which plays a prominent role in the soundscape of intensive aquaculture settings, on the diversity of fish school behavior, providing additional evidence to better assess the impact of noise on fish ecology and welfare. Furthermore, this research expands the understanding by directly linking noise to the total feeding quantity of largemouth bass schools, providing concrete evidence for the reduction in feeding desire and intake caused by noise.

However, the study presents certain limitations. For instance, the sample size employed is relatively modest, potentially lacking comprehensive representation of the entire largemouth bass population. Furthermore, the experiment's duration is comparatively brief, and variations between the experimental conditions and actual production environments may impact the validity of the results. It is also important to acknowledge the influence of the 24-hour photoperiod with full-spectrum LED lights on fish behavior. While this lighting condition was chosen to ensure consistent visibility for our observations, it may have disrupted the natural diurnal rhythms of the tested fish. This is a notable limitation as it could have affected the baseline behavior of largemouth bass, potentially confounding the results. Additionally, the methodologies and instruments implemented for sound environment assessment may inherently possess constraints. While sound pressure was utilized to characterize the noise environment, measurement of the particle motion component remained beyond the scope of this study. Notwithstanding these limitations, the research elucidates the noteworthy impact of noise on the behavior and growth of largemouth bass within the specific experimental framework. Such insights are crucial for understanding the repercussions of noise pollution on fish ecology, contributing to the enhancement of fish welfare and potentially influencing fish production. These findings also provide a foundation for future investigations into the impacts of noise on largemouth bass and other fish species.

5 Conclusion

This investigation has illuminated the influence of aeration equipment noise on the swimming behavior, feeding habits, and growth progression of largemouth bass populations. The results from the experimental trials indicate that largemouth bass residing in noisy environments, when contrasted with the ambient group, display substantial disparities in swimming behavior, characterized by larger average angles and distances from the nearest neighbor fish. Moreover, these bass experience diminished appetite, decreased food consumption, and compromised growth performance. Additionally, the present study has pioneered the use of the SWDI to appraise the diversity of fish swimming behavior, thereby offering a novel evaluation instrument for fish behavior studies. Going forward, it is crucial to delve deeper into the precise mechanisms by which noise impacts largemouth bass and other aquatic organisms, aiming to provide scientific substantiation for the mitigation and regulation of noise pollution. Simultaneously, these research findings assume paramount importance in addressing the challenges posed to fish welfare within aquaculture settings. Efforts to minimize noise pollution within aquacultural settings hold the potential to elevate farming efficiency, foster animal welfare, and establish a robust framework for the sustainable advancement of aquaculture practices.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author.

Ethics statement

The animal study was approved by Experimental Animal Welfare Ethics Committee of Zhejiang University. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

YZ conceived the study, carried out field work, analyzed data, and wrote the majority of the manuscript. AS, SH, HZ carried out filed work. WX, JZ, SZ conceived the study, analyzed data and revised the manuscript All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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EDITED BY
Pablo Almazan Rueda,
National Council of Science and Technology (CONACYT), Mexico

REVIEWED BY
Zonghang Zhang,
Shantou University, China
Ana C. Puello Cruz,
National Council of Science and Technology (CONACYT), Mexico

*CORRESPONDENCE
Liuyi Huang
huangly@ouc.edu.cn

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Experimental study on the effect of sound stimulation on hearing and behavior of juvenile black rockfish (*Sebastes schlegelii*)

Yining Wang[1], Liuyi Huang[1*] and Binbin Xing[2]

[1]Laboratory of Marine Fisheries Technology, Fisheries College, Ocean University of China, Qingdao, China, [2]College of Fisheries and Life Science, Dalian Ocean University, Dalian, China

Assessing the potential impacts of wind farm noise on fish is a crucial aspect of Environmental Impact Assessment (EIA) studies. There is increasing evidence of disturbances and effects on hearing and behavior in animals. The black rockfish (*Sebastes schlegelii*) is a commercially valuable rocky reef fish native to East Asia. However, empirical studies that measure the actual consequences are lacking. In this study, we used auditory evoked potentials (AEP) to assess the effects of dominant frequency noise emitted by offshore wind farms on the auditory sensitivity, hearing threshold, swimming, and feeding behavior of juvenile black rockfish. The experimental findings revealed that the most sensitive sound frequency was 200 Hz, with the lowest hearing threshold recorded at 86.4 ± 3.4 dB re 1 μPa. Following 3 and 7 days of exposure to 200 Hz noise at 110 dB, threshold shifts in black rockfish reached 19.0 dB and 13.3 dB, respectively. During the subsequent recovery phase, these shifts decreased to approximately 9.8 dB after 3 days, respectively. The noise-exposed group exhibited higher swimming duration, moving distance, and caudal fin swing frequency compared to the control group without noise exposure. Furthermore, noise prolonged the feeding rate of black rockfish. Our findings provide the first evidence of noise-induced temporary threshold shift and behavioral disturbances in juvenile black rockfish, implying potential fitness consequences associated with noise pollutant.

KEYWORDS

hearing sensitivity, auditory evoked potential, temporary threshold shift, behavior, fish welfare

1 Introduction

With the increasing energy demand and the pursuit of sustainable development in society, the offshore wind industry has experienced significant growth worldwide in recent decades (Slabbekoorn et al., 2010). Many countries are now focusing on utilizing offshore wind farm (OWF) facilities to operate aquaculture systems, aiming to enhance ocean space utilization and reduce costs. The concept of combining OWFs as fixation points for aquaculture or co-using OWF sites by installing aquaculture farms between wind turbines has garnered considerable attention in recent years (Lindell, 2003; Wever et al., 2015; Tullio et al, 2018). However, the noise generated by OWFs primarily arises during the construction and operation stages (Nedwell et al., 2003; De Jong et al., 2011). Based on extensive measurements reviewed by Madsen et al. (2006), the underwater noise produced by operating wind turbines is predominantly limited to low frequencies (below 1 kHz) and low intensity. For instance, the frequency peak observed at the Vindeby OWF in Denmark and the Gotland OWF in Sweden was 25 Hz and 160 Hz, respectively (Nedwell, Langworthy and Howell, 2004). Similarly, the Horns Rev OWF in Denmark exhibited frequency peaks at 150 Hz and 300 Hz (Betke et al., 2005). Additionally, measurements conducted at three different types of OWFs in Denmark and Gotland (Middelgrunden, Vindeby, and Bockstigen-Valar) revealed a noise frequency range below 500 Hz (Tougaard et al., 2009). Blew et al. (2008) summarized the frequency peaks of four OWFs in Denmark, which were 176 Hz, 150 Hz, 135 Hz, and 134 Hz, respectively, with corresponding sound pressure levels (SPLs) of 114 dB, 117 dB, 110 dB, and 122 dB. In the case of the East China Sea Bridge OWF, the noise frequency range was below 400 Hz, and the SPLs ranged from 81 dB to 99 dB (Zhang et al., 2016). Tougaard et al. (2020) compiled data from 46 measured values obtained from 14 wind farms. Although the distances and wind speeds of the measurements varied, the dominant frequencies were found to be in the range of 10-400 Hz, with equivalent continuous sound levels (LAeq) ranging from 80 dB to 135 dB re 1 μPa.

Previous studies have demonstrated the influence of disturbances on fish behavior, including responses to noise (Stevens and Don, 1979; Russell et al., 2001; Sara et al., 2007; Ladich and Fay, 2013; Andrew et al., 2016; Velasquez et al., 2020; Jones et al., 2020). Andersson et al. (2007) conducted a study on two different fish species, *Rutilus rutilus* L and *Gasterosteus aculeatus*, exposed to single-tone frequencies and sound generated by offshore wind turbines. They observed various swimming patterns in sticklebacks (*Gasterosteus aculeatus*), including forward swimming, twitching, backing, and freezing. Previous studies have indicated that prolonged exposure to noise can lead to damage or loss of hair cells in the fish's inner ear. Severe auditory insults can result in temporary threshold shifts (TTS) or permanent threshold shifts (PTS). In a study by Popper et al (1976), goldfish were exposed to pure tones, and sound pressure levels (SPLs) of 149 dB re 1 μPa caused threshold shifts of approximately 7-9 dB at 500 Hz and 18-27 dB at 800 Hz. Amoser and Ladich (2003) exposed goldfish to white noise at 158 dB re 1 μPa for 24 hours and found the greatest hearing loss at 800 Hz and 1000 Hz. Smith et al. (2004) reported significant threshold shifts (up to 28 dB) at all frequencies in goldfish due to noise exposure, with larger shifts occurring at frequencies where their hearing sensitivity is highest. The negative impact of OWF (OWF) noise on fish has become a matter of concern. Understanding the auditory threshold shift and recovery of fish is crucial (Amoser and Ladich, 2003). Striking a balance between energy development and the preservation of aquatic life is imperative (Lacroix and Sylvain, 2011; Thompson et al., 2020).

In this study, we focused on the black rockfish (*Sebastes schlegelii*) as our research subject. The black rockfish belongs to the *Scorpaenidae* family and is an economically important marine ovoviviparous teleost species. It is widely distributed and cultured in Japan, Korea, and the northeast coast of China (Likang et al., 2018). The objectives of our study were as follows: (i) To determine the sensitive sound frequency and hearing threshold of the black rockfish using the auditory evoked potential (AEP) method. (ii) To assess the degree of hearing loss (temporary threshold shift or TTS) in the black rockfish. (iii) To investigate the impact of noise from OWFs at prominent frequency ranges on fish behavior. The findings of this study are expected to provide valuable insights and serve as a reference for the development of OWFs, taking into consideration the effects on black rockfish and other similar species.

2 Methods

2.1 Study species

Juvenile black rockfish (*n*=80; standard length: 6.99 ± 0.51 cm; wet mass: 19.47 ± 2.93 g) were studied at the Fish Behavior Laboratory at the Dalian Ocean University. Before the experiment, the fish were farmed in a polypropylene water bucket (diameter 52 cm, water depth 46 cm) for 5 days. Water temperature was 21.4-22.8°C and dissolved oxygen was7.89 ± 0.03 mg/L. Fish were fed once daily at noon and 50% of the water was changed after 2 days to clean out excrement. We present the feeding amount based on the experience in the rearing phase. The total quantity fed daily was 1.1% fish body weight (1.7 g/day).

2.2 Auditory evoked potential recordings of black rockfish

The auditory evoked potential (AEP) test conducted in this study was based on the method described by Kenyon et al. (1998). The experimental setup is illustrated in Figure 1. To minimize external noise interference, all equipment was placed inside a soundproof room with an internal sound pressure level measuring 35.2 dB. The testing was carried out in a cylindrical Polyvinyl Chloride (PVC) water bucket with an inner diameter of 0.4 m and a height of 0.4 m. The bucket was positioned on a shockproof table, and an aluminum radiation-proof cloth was applied to the outer wall to prevent noise disturbances. Juvenile black rockfish were first anesthetized and then securely positioned at the center of the tank. The water level was maintained at approximately 3 mm below the fish's skull. Sound stimuli were generated using an underwater speaker (UW-30, China). The generated sound was pre-amplified with a Crown amplifier (D-75A, China) and analyzed in real-time using a Tucker-Davis Technologies (TDT) system (RZ6, USA) equipped with a hydrophone probe mounted near the fish's head. The hydrophone probe was connected to an acoustic acquisition system (Aquafeeler IV, Roland, Japan) used for online measurements. The hydrophone probe had a sensitivity of -190 dB re 1 μPa and a bandwidth of 20 Hz to 200 kHz. Ambient noise and stimulus noise were recorded during the experiments. AEP waveforms were generated using the SigGenRZ software.

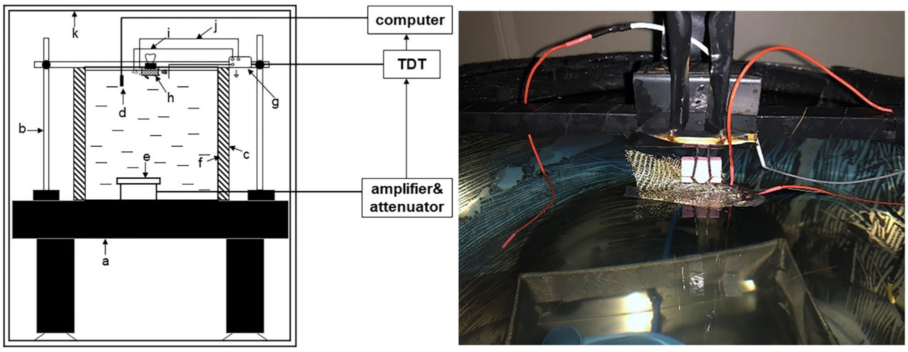

FIGURE 1
Test installation diagram of AEP. a, holographic-table; b, fixed frame; c, acoustic insulation material foam; d, hydrophone; e, underwater speaker; f, water tank; g, preamplifier; h, fish; i, reference electrode; j, recording electrode; k, sound-proof chamber.

Each experimental case in this study involved the use of a single live fish and lasted for a duration of 2 hours. A total of 8 cases were conducted, but unfortunately, one of them resulted in the death of the fish. Prior to any manipulation, the fish were anesthetized by immersing them in water containing 1:14000 tricaine methanesulfonate (MS-222) until their movements ceased, which typically took around 50 seconds. To minimize myogenic noise levels, the fish were temporarily immobilized by injecting gallamine triiodide (Sigma-Hefei Bomeranian Biology) at a dosage of 1.2-1.4 µg/g per body weight. The anesthesia lasted for 4 to 5 hours. For the AEP recordings, two silver needles with a length of 5 mm and a diameter of 0.25 mm were used as electrodes. One needle was inserted through the fish's skull at the medulla region and served as the recording electrode, while a reference electrode was placed subcutaneously between the fish's eyes Codarin and Wysocki (2009). Two additional electrodes were inserted subcutaneously at a depth of 2.5 mm. Shielded electrode leads were connected to the preamplifier's input, and the other end of the shielded electrode was positioned posterior to the fish's body. The reference, ground, and recording electrodes were connected to the amplifier. The sound stimuli used in the experiment were short alternating tone bursts with frequencies of 100 Hz, 200 Hz, 300 Hz, and 500 Hz, which were selected based on the dominant frequency of offshore wind turbine noise. The tone bursts were presented at a repetition rate of 10/s and alternated between 90° and 270° phases. The duration of the sound stimuli ranged from 2 cycles at 100 Hz and 200 Hz to 5 cycles at 300 Hz and 500 Hz. The rise and fall times of the stimuli increased from 1 cycle at 100 Hz and 200 Hz to 2 cycles at 300 Hz and 500 Hz. A total of 1000 stimuli were presented for each polarity. The lowest sound pressure level (SPL) at which a repeatable AEP trace could be obtained was considered the threshold. The SPLs were attenuated in 5-dB steps initially. As the threshold was approached, the SPLs were further attenuated in 1-2 dB steps until the threshold level was determined

2.3 Audiometry test under noise exposure

Sixteen juvenile black rockfish were selected for this study and divided into two groups: a noise-exposed group and a controlled and sheltered group, as depicted in Figure 2. Based on previous research on the dominant noise of single-pile foundation of OWFs, the stimuli used for noise exposure in this study consisted of 200 Hz pure tones at a sound pressure level of 110 dB (Betke et al., 2005; Tougaard et al., 2009; Marmo et al., 2014). The duration of noise exposure for each treatment group was set at 7 days. AEP images were collected separately at two time points: 3 days and 7 days after the initiation of noise exposure for both groups. Additionally, images were recorded 1 and 3 days after the recovery period.

2.4 Behavior test under noise exposure

The noise exposure group of black rockfish was subjected to continuous exposure to a pure tone of 200 Hz at an intensity of 110 dB for a period of 7 days. Throughout this period, video recordings capturing the behavior of the fish from a bird's-eye view were conducted at three specific time points each day: 7:00, 13:00, and 21:00. The duration of each video recording was 5 minutes, and the middle 3 minutes were selected for behavior analysis. Specifically, after the swimming video recording at 13:00 each day, additional videos capturing the feeding behavior of both the noise exposure group and the controlled group were recorded. To minimize anthropogenic disturbances, baits were introduced into the tank using a tube rather than directly by hand. To analyze the potential interaction between tailbeat frequencies (TBF) and swimming time (ST), the analysis of locomotor behavior of the fish was conducted from a side view perspective. Additionally, the feeding time and feed rate of the bottom bait were recorded from each feeding behavior video. The quantity of food provided during the test phase was the same as that during the rearing phase. All the criteria used for recording and analysis are summarized in Table 1.

2.5 Correlation parameter and data processing

The behavioral data collected were analyzed to determine their normal distribution using the Shapiro-Wilk normality test, which was conducted using SPSS Statistics (Version 17.0). Since the data did not follow a normal distribution, non-parametric tests,

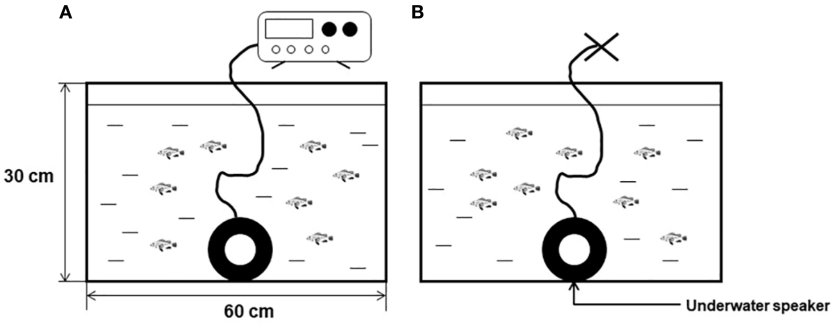

FIGURE 2
Acoustic exposure behavior test diagram **(A)** for the acoustic exposure group, **(B)** for the control group).

specifically the Mann-Whitney test, were performed to compare the differences between the control group and the noise group. The results of the analysis were expressed as the median values with the interquartile range (IQR). To visualize the findings, plots were generated using GraphPad Prism 8 software. The activity behaviors of the black rockfish were tracked and recorded using video footage. The motion trajectory of each individual fish was determined, and the distances traveled were measured using the Digimizer software system. This software facilitated the analysis of the recorded videos to extract relevant behavioral parameters.

2.6 Ethical note

All experimental procedures conducted in this study adhered to the guidelines set forth by the Institutional Animal Care and Use Committee (IACUC). The study protocol, including the ethical considerations and animal welfare practices, was reviewed and approved by the Animal Welfare and Ethical Review Committee of Ocean University of China (IACUC—2105003). Throughout the study, all relevant international, national, and institutional guidelines and regulations regarding the care and use of animals were strictly followed to ensure the ethical treatment and well-being of the black rockfish used in the experiment. These measures were implemented to guarantee the humane and responsible conduct of the research.

TABLE 1 Parameter definition used in the collection of behavioral data from black rockfish exposed or not to sound.

Parameter name	Detailed description
Swimming distance	The horizontal distance from the beginning of a fish's displacement to the end of its displacement
Tailbeat frequency	The movement from one extreme lateral position to the opposite extreme lateral position.
Swimming time	The time from the start of swimming until the time when the fish became stationary.
Feeding time	The time from the first fish starts feeding until the left bait is gone.
Feed rate of bottom bait	The number of tails feeding on the bottom bait divided by the total tails

3 Results

3.1 Sensitive sound frequency and hearing threshold

AEP (auditory evoked potential) waveforms were successfully obtained from 7 black rockfish that were tested in the study. Figure 3 provides an overview of the AEP waveforms specifically generated in response to a 100 Hz sound stimulus. It can be observed that as the sound intensity decreased, the magnitude of the AEP response also declined. Through repeated testing, it was determined that the average number of wave peaks in the AEP waveforms between sound pressure levels (SPLs) of 105 dB and 93 dB was approximately 8. However, when the SPL was further reduced to 92 dB, recognizable and repeatable waveforms could no longer be produced. This suggests that the AEP threshold for the black rockfish at a frequency of 100 Hz is 92 dB, indicating that the fish's auditory response becomes undetectable below this sound pressure level.

Audiograms were recorded using the AEP instrument, and the results are presented in Figure 4. It is important to note that the minimum threshold values obtained from the audiograms were considerably higher than the measured background noise level. This indicates that the audiogram data were not influenced by the background noise. The audiograms reveal a decreasing trend in auditory thresholds within the frequency range of 100-200 Hz, indicating that the black rockfish exhibited relatively lower thresholds for sound stimuli in this frequency range. However, beyond 200 Hz and up to 500 Hz, the thresholds showed a tendency to rise, implying that the fish had higher auditory thresholds in this frequency range. Of particular interest is the lowest threshold observed at 200 Hz, which was measured to be 86.4 ± 3.4 dB.

3.2 Effect of 200 Hz noise on the hearing threshold

The degree of hearing loss was quantified by measuring noise-induced threshold shifts during sound stimulation at 110 dB SPL and 200 Hz, as shown in Figure 5. After 3 days of noise exposure, the average auditory thresholds increased by 19.0 ± 8.4 dB. The maximum threshold shift observed was 27.4 dB at 100 Hz. After 7

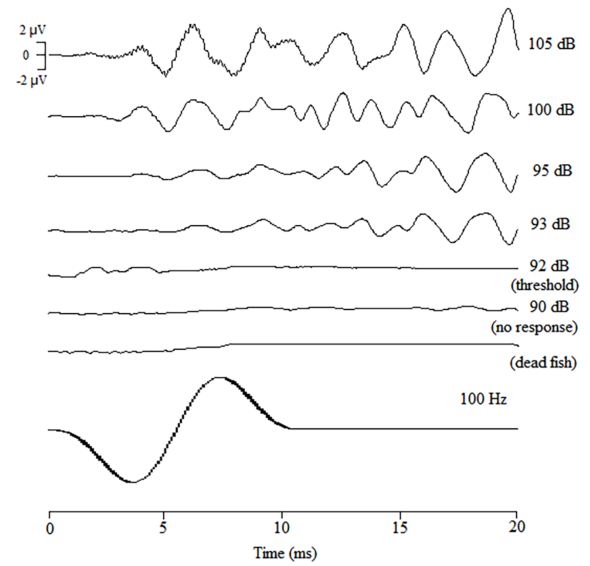

FIGURE 3
AEP waveforms of black rockfish obtained in response to tone bursts of 200 Hz attenuated from 85 to 100 dB (1000 times repeated).

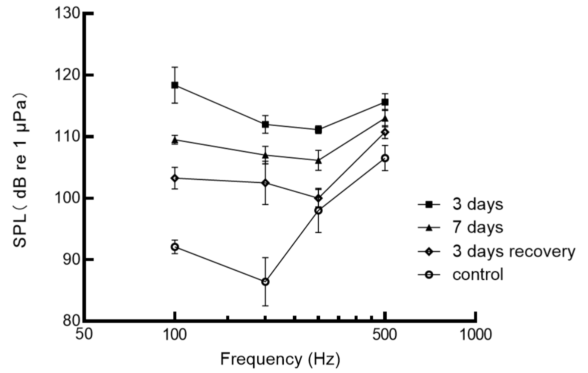

FIGURE 5
Auditory sensitivity of the black rockfish before (control) and after exposure to 200 Hz noise for 3 or 7 days at 110 dB and 3 days of recovery.

days of noise exposure, the average threshold shift was 13.3 ± 8.3 dB, with a maximum shift of 21.6 dB at 200 Hz. During the recovery period, the auditory thresholds gradually improved. After the third day of recovery, the decrease was 9.8 ± 5.6 dB. The maximum threshold shifts during recovery was 18.6 dB at 200 Hz, respectively. Overall, the degree of threshold shift decreased with increasing frequency and increased exposure time. However, except for the 3-day exposure group (p=0.019), there were no significant differences between the control group and the noise-exposed group (p>0.05). The greatest threshold shifts were observed at 100 Hz (27.4 dB) and 200 Hz (26.1 dB) after 3 days of noise exposure. However, hearing thresholds for all frequencies were reduced after 7 days of noise exposure compared to 3 days of exposure. Furthermore, even after 3 days of recovery, the auditory thresholds of the black rockfish did not return to the control levels. These findings suggest that noise exposure can lead to significant threshold shifts in the black rockfish, with the extent of the shifts depending on the frequency and duration of exposure. After 3 days, 7 days of noise exposure, and 3 days of recovery, the best frequency of black rockfish changed from 200 to 300 Hz.

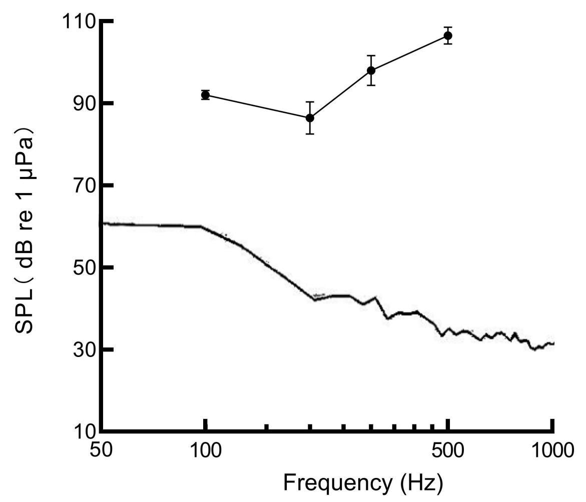

FIGURE 4
AEP audiogram of black rockfish (Solid line is background noise, *n*=7.

3.3 Effects of 200 Hz noise on behavior

3.3.1 Swimming distance

The effect of noise stress on the swimming behavior of black rockfish was examined, and the results are presented in Figure 6. It was observed that the fish in the test group exhibited startle responses and even jumped out of the water during the initial noise stress. The motion tracks of the black rockfish during a 3-minute observation period are depicted. The distribution of the fish was mainly on the sidewalls of the tank, as shown in the figures. The average horizontal movement distance for the test group on day 1 was 2.38 ± 1.00 m, while for the control group it was 1.42 ± 0.16 m. On day 3, the distance for the test group increased to 3.04 ± 0.54 m, and for the control group, it was 2.56 ± 1.18 m. On day 7, the distance for the test group further increased to 3.38 ± 0.53 m, and for the control group, it was 3.56 ± 0.58 m. Initially, the distance moved by the test group was greater than that of the control group, indicating a more active swimming response to the noise stress. However, as the exposure time increased, the gap between the two groups gradually closed. By day 7, the test group's distance reached a constant level similar to that of the control group, and there was no significant difference between the two groups (p>0.05). Based on the visualization of the swimming trend, it can be concluded that black rockfish are capable of habituating to noise over a relatively short period, at least in terms of routine swimming behavior. The average distance moved by both the control and experimental groups became similar after 7 days of noise exposure, suggesting a habituation response to the noise stress.

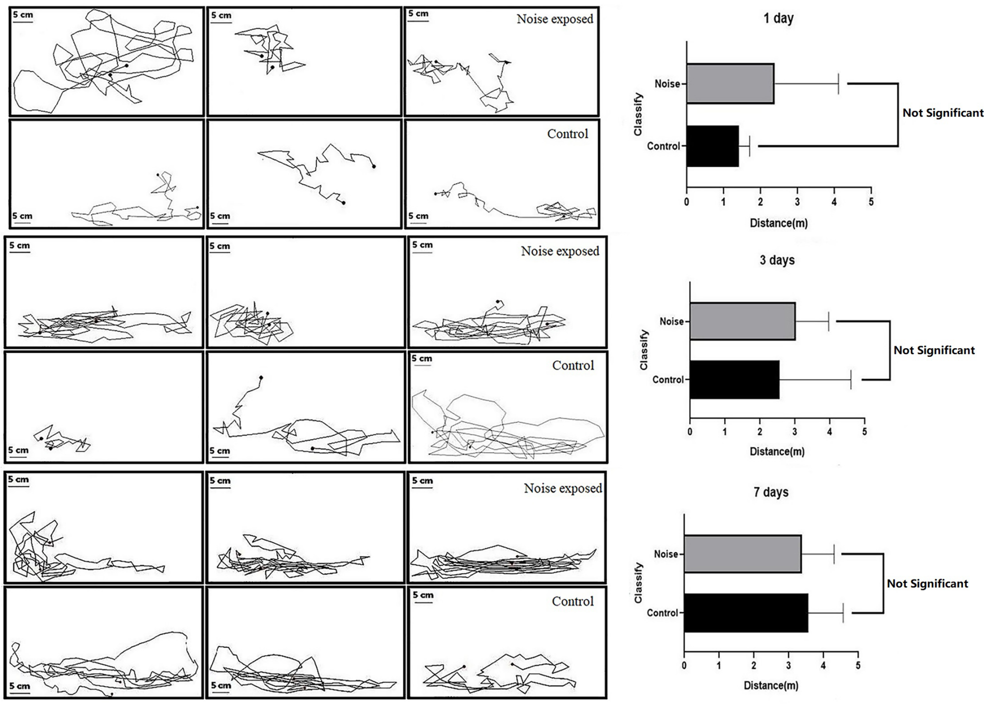

FIGURE 6
Trajectory diagram (left) and movement distance (right) of black rockfish after exposure to 200 Hz noise (110 dB) for 1, 3, and 7 days, 3 min each time (*p*>0.05).

3.3.2 Tailbeat frequencies and swimming time

The average tail beat frequency and swimming duration of the test group and control group are presented in Figure 7 and Table 2. The interquartile range of the tail beat frequency in the test group were 246 times, while in the control group, they were 159 times. The average tail beat frequency in the test group was significantly higher than that in the control group (82 times/min vs 53 times/min, $p=0.001$). There was a significant difference observed between day 4 ($p=0.007$) and day 7 ($p=0.015$) in both the control and test groups. Additionally, the interquartile range of the swimming time in the test group were 60.6 s, while in the control group, they were 45.9 s. The average swimming duration of the test group was higher than that of the control group (33.67% vs 25.50%, $p<0.05$). This difference was found to be significant on day 1, day 2, and day 5 compared to the control group ($p<0.05$). These results indicate that the fish in the test group exhibited increased tail beat frequency and longer swimming durations compared to the control group, indicating a heightened level of activity and possibly stress response to the noise exposure.

3.3.3 Feeding behavior

Based on video observation, it was observed that the time taken for the first fish to locate the bait after feeding was approximately 1-2 seconds in both the control and test groups. However, except for the feeding duration on day 1 and day 7 of noise exposure, the control group exhibited shorter feeding times compared to the test group (Figure 8). The total feeding time in the test group was longer than that in the control group (62 seconds/day vs 43 seconds/day). The noise stimulation had a specific impact on the feeding time of the black rockfish, although the difference was not statistically significant ($p > 0.05$). In the test group, as the bait substrates were not completely consumed, it extended the feeding time significantly. During the recovery stage, only one fish in the experimental group was able to locate the bottom bait on the first day, and from the second day onwards, none of the fish in the experimental group were able to see the bait at the bottom of the tank, resulting in the feeding time being unable to be recorded. In contrast, the control group was able to consume all the bait within 3 days, and there was a significant difference between the control and test groups ($p < 0.01$). These findings suggest that the noise exposure affected the feeding behavior of the black rockfish, leading to prolonged feeding time and difficulties in locating the bait during the recovery period.

4 Discussion

In this study, the best hearing sensitivity of black rockfish was observed at 200 Hz, with a mean auditory threshold of 86.4 ± 3.4 dB re 1μPa. Figure 9 compares three different approaches (behavior, electrocardiogram ECG, auditory evoked potentials AEP) for determining auditory thresholds of black rockfish (Ishizaki et al., 1992; Keiichiro, 1997). The general trends of the three methods were found to be similar. At 100 Hz, the auditory threshold was 92.1 dB, which was 0.1 dB higher than the ECG method and 1.5 dB higher than the behavioral method. At 200 Hz, the auditory

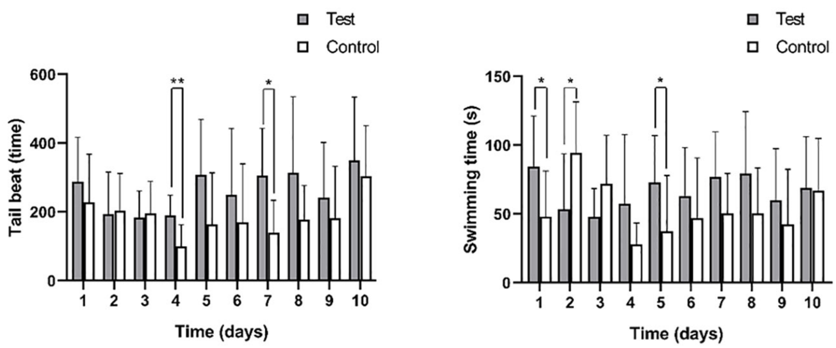

FIGURE 7
The tail beat times (left) and swimming time (right) of black rockfish were measured 3 min each time after exposure to 200 Hz noise (110 dB) for 11 days (7 days acoustic stress+3 days recovery). * p value < 0.05, ** p value < 0.01.

threshold was 86.4 dB, which was 4.1 dB lower than the ECG method and 6 dB lower than the behavioral method. At 300 Hz, the auditory threshold was 98 dB, which was 4 dB lower than the ECG method and 8.4 dB lower than the behavioral method. At 500 Hz, the auditory threshold was 106 dB, which was 14.3 dB lower than the ECG method and 10.6 dB lower than the behavioral method. AEP detects synchronous neural activity in the 8th cranial nerve and brainstem auditory nuclei elicited by sound at the surface of the skull. One advantage of the AEP method over behavioral or ECG methods is that it is not affected by the condition or feeding motivation of the fish. The speed and ease with which AEP audiometry can be performed and the fact that there is no need for lengthy and repetitive subject conditioning, make it applicable to studies of hearing sensitivity in fish (Kojima et al, 2005; Ladich and Fay, 2013).

This study provides the first evidence of Noise-Induced Hearing Loss in black rockfish based on changes in auditory sensitivity. After 3 days of noise exposure, the black rockfish exhibited a significant increase in TTS, indicating a temporary shift in their hearing thresholds. However, after 7 days, this shift started to decrease, suggesting that as the duration of noise exposure extends, black rockfish exhibit a potential gradual adaptation to the deleterious effects of noise. After 3 days of noise exposure, the best frequency of black rockfish changed from 200 to 300 Hz. Similar findings have been reported in other studies. For example, Scholik and Yan (2002) examined the effects of white noise and boat noise on *Pimephales promelas* and found a change in the best frequency from 1000 to 300 Hz. Liu et al. (2013) exposed *Myxocyprinus asiaticus* to ship noise and observed a change in the best frequency from 800 to 200 Hz. These differences in findings may be attributed to variations in noise SPL, bandwidth, and species-specific characteristics. In general, fish species with limited auditory sensitivity (without Weberian apparatus) are less affected by noise. The auditory threshold tends to decrease as the duration of the recovery period increases. Breitzler et al. (2020) measured zebrafish exposed to white noise at different SPLs and found that recovery function occurred within 7 days for fish exposed to 130 dB and 140 dB noise levels, while fish subject to 150 dB only returned to baseline thresholds after 14 days. Further research is needed to determine the time required for the auditory threshold of black rockfish to recover to baseline levels.

Fish behavior research plays a crucial role in noise studies, as fish exhibit various behavioral responses to anthropogenic disturbances in aquatic environments. Changes in swimming activities, tail movements, and foraging behavior have been used as indicators of stress in fish exposed to human-generated disturbances (Mickle and Higgs, 2017; Weilgart, 2018; Faria et al., 2019). Based on the trajectories of black rockfish observed in this study, it was concluded that the fish spent more time irregularly swimming on the sidewalls of the tank, both with and without exposure to 200 Hz noise. Black rockfish are demersal marine fish that typically live in groups on rocky reefs, and their behavior may be influenced by their natural habitat. The onset of underwater sound propagation elicits startled reactions in some juvenile black rockfish, consistent with the findings reported by Spiga et al. (2017). The tail beat frequency (TBF) and swimming time (ST) of the control group were higher than those of the treatment group on days 2 and 3. From the video observations, it appeared that black rockfish tended to remain still and seek cover for extended periods when under constant threat. We hypothesize that fish may undergo a transition from an initial phase to an adaptive phase. During the initial phase, it is plausible that fish would employ strategies to minimize their interactions with noise stimulation, potentially

TABLE 2 Total tail beat times and total swimming time of black rockfish (interquartile range IQR).

Groups	TBF (time)	Z	*p*	ST (s)	Z	*p*
Noise exposure	246(141.3~335.5)	3.328	0.001**	60.6(34.92~91.32)	2.046	0.041*
Control	159(80.5~268.8)			45.9(20.08~82.94)		

* p value < 0.05, ** p value < 0.01.

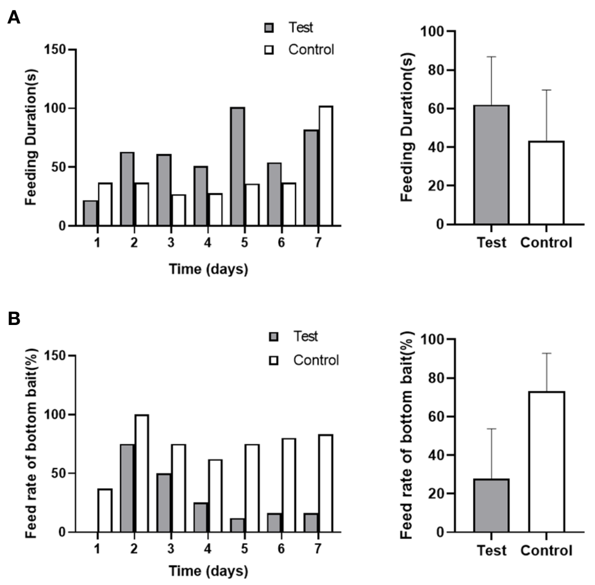

FIGURE 8
The feed duration **(A)** and feeding rate of black rockfish on bottom bait **(B)** after exposure to 200 Hz noise (110 dB) for 7 days (A, $p>0.05$; B, $p<0.01$).

resulting in a decrease in TBF and ST. These strategies may include seeking refuge or escaping from the noise source. However, from the fourth day onwards, the TBF and ST of the treatment group surpassed those of the control group. Significant differences were observed between the treatment and control groups (*p<0.05, **p<0.01). Following prolonged exposure to noise stimulation, it is conceivable that fish could enter an over-adaptation phase. Within this phase, fish may gradually readjust their swimming behavior, leading to an increase in TBF and ST. This phenomenon may arise from the fish attaining a state of equilibrium in their adaptation to the noise or undergoing behavioral modifications to enhance resource utilization or cope with environmental pressures. These behaviors, such as increased tail beat frequency and swimming time, were likely selected as escape strategies. It is worth noting that tail beat consumes energy in fish and can potentially affect their growth rate. Furthermore, in quiet tanks, black rockfish exhibited more frequent and variable gaping behavior compared to fish in loud tanks. During the 7-day experimental period, the number of gaping behavior in the noise-exposed group was recorded as 60, whereas the control group exhibited 74. Both groups displayed a peak occurrence of gaping behavior during the nighttime period, followed by the morning period, and the lowest frequency was observed during the midday interval. Limited observations on gaping behavior in fish in the scientific literature suggest that gaping is associated with low activity levels (Rasa, 1971), which aligns somewhat with the results of this study, indicating less frequent adjustments in animals in quiet tanks (Andersson, 2011). Overall, these findings demonstrate the impact of noise on black rockfish behavior, highlighting the manifestation of stress and anxiety-related responses. The altered swimming distance, increased tail beat frequency, and modified gaping behavior indicate the adaptive strategies employed by the fish in response to the noise stress.

Stressors appear to cause shifts, lapses and narrowing of attention, and can also influence decision speed, which may explain the increase in foraging errors observed in the test group of black rockfish (Mendl, 1999; De Kloet et al., 1999). Interestingly, the black rockfish in the test group displayed rapid reactions to the baits but exhibited specific behaviors such as swimming in the vertical direction and not consuming the baits that fell to the bottom of the tank, especially during the recovery phase. These observations indicate altered feeding behavior and increased avoidance responses in black rockfish under noise conditions. The longer duration of feeding and lower feeding rate observed in the noise-exposed black rockfish align with the findings reported by Mickle and Higgs (2017) and the conclusions drawn by Sabet et al. (2016). Exposure to elevated noise levels can impair foraging behavior through various mechanisms, which are not mutually exclusive. Firstly, noise can trigger stress or fear-related responses in the fish, affecting their feeding behavior. Secondly, noise can act as a distraction, diverting the fish's attention away from the feeding task. Lastly, noise can mask important acoustic information that the fish relies on for successful foraging (Voellmy et al., 2014). The reduced foraging strikes and increased feeding duration observed in black rockfish are consistent with the concept of a defense cascade, where ongoing activities, such as foraging, are interrupted. Such responses are typically associated with stressors and fear-inducing stimuli (Metcalfe, Huntingford, & Thorpe, 1987). These behavioral changes indicate that the black rockfish perceive the noise as a threat and exhibit altered feeding strategies as a defensive response. In summary, the impaired foraging behavior, increased feeding duration, and avoidance responses observed in black rockfish exposed to noise are in line with previous research. The effects of noise on feeding behavior can be attributed to stress-related responses, distraction, and the masking of acoustic cues necessary for successful foraging.

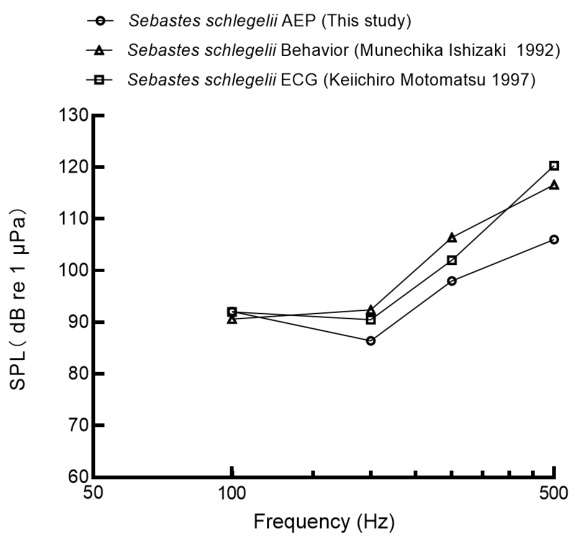

FIGURE 9
The auditory threshold of black rockfish was measured by different publications (Ishizaki et al., 1992; Motomatsu et al., 1997).

Indeed, the research period of this experiment was relatively short, and long-term subsequent studies will provide further insights into the auditory recovery of black rockfish in response to noise. The dominant frequency range of OWF noise falls within the auditory sensitivity range of black rockfish, but it's crucial to recognize that noise levels can vary significantly depending on the turbine power. Therefore, comprehensive field studies are needed to assess the actual impact of OWF noise on fish behavior and its implications for their fitness (Madsen et al., 2006). This research should include realistic noise simulations, considering key factors such as sound pressure levels and particle motion. By conducting such studies and accumulating more data, researchers will be better equipped to provide evidence-based recommendations and guidelines for mitigating the potential impacts of OWF noise on fish.

5 Conclusion

The results of this study emphasize the impact of anthropogenic noise on juvenile black rockfish, particularly in relation to their auditory threshold, swimming distance, and foraging behavior. The findings indicate that the minimum auditory threshold of black rockfish is 200 Hz, with a sound intensity of 86.4 ± 3.4 dB re 1μPa. Following exposure to 200 Hz noise, the most sensitive frequency for black rockfish shifted from 200 Hz to 300 Hz. Furthermore, we observed that continuous noise stimulation increased the swimming distance, swimming time, and tail beat frequency of juvenile black rockfish. Concurrently, the noise also had detrimental effects on their foraging behavior, potentially impacting their ability to effectively search for and capture food. These findings provide mounting evidence that anthropogenic noise can have wide-ranging effects on marine species, including fish.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author.

Ethics statement

The animal studies were approved by Animal Ethical Committee of Ocean University of China. The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent was obtained from the owners for the participation of their animals in this study.

Author contributions

YW: Investigation, Methodology, Writing – original draft, Writing – review & editing. LH: Conceptualization, Formal analysis, Project administration, Supervision, Writing – review & editing. BX: Resources, Writing – review & editing, Data curation.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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EDITED BY
Rosario Martínez-Yáñez,
University of Guanajuato, Mexico

REVIEWED BY
Patricia Mora-Medina,
National Autonomous University of Mexico, Mexico
Jorge Hernández López,
Centro de Investigación Biológica del Noroeste (CIBNOR), Mexico

*CORRESPONDENCE
Erik Höglund
Erik.Hoglund@niva.no

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Does sedation with AQUI-S® mitigate transport stress and post transport mortality in ballan wrasse (*Labrus bergyltae*)?

Sara Calabrese[1], Thor Magne Jonassen[2], Endre Steigum[1], Helga Øen Åsnes[1], Albert Kjartan Dagbjartarson Imsland[2,3], Carolina Serra Saude[4], Truls Wergeland[4] and Erik Höglund[1,5]*

[1]Norwegian Institute for Water Research, Bergen, Norway, [2]Akvaplan-niva AS, Tromsø, Norway, [3]Department of Biological Sciences, University of Bergen, Bergen, Norway, [4]MOWI ASA, Bergen, Norway, [5]University of Agder, Kristiansand, Norway

Ballan wrasse (*Labrus bergylta*) are commonly used as cleaner fish in salmon farms as a biological treatment to mitigate sea lice infestation. Improved welfare for cleaner fish both during production of these fish and when in sea-cages with salmon is crucial for the industry's development. A common operational procedure in ballan wrasse production is transporting juveniles from one land-based farm to another for further on-growing. Episodes of increased mortality have been reported after such transportations. In this study, the relationship between transport stress and post-transport mortality at the on-growing facility was examined. It was also investigated if light sedation with AQUI-S® can mitigate stress during transport. Stress was quantified by measuring cortisol release rate to the tank water during transport. This was investigated in 10 commercial live carrier truck transports (6 without AQUI-S® sedation and 4 with sedation during loading and transport). The total time of transport varied between 12 and 21h. In general, mortality was significantly higher (1.0±0.6% day^{-1}) the first five days post-transport compared to 15–20days post transport (0.5% day^{-1}). There was also a strong relationship between fish weight at transport and post-transport mortality, where higher mean weight at transport reduced mortality. In contrast to what was expected, AQUI-S® treatment during transport procedures increased cortisol excretion rate, suggesting a stimulating effect of AQUI-S® on the stress axis in ballan wrasse. Considering these results, the value of using AQUI-S® to reduce stress during transport of juvenile ballan wrasse might be questioned. However, there was no relationship between cortisol release rate during transport and post-transport mortality. Furthermore, this study emphasizes that water cortisol measurements can be used as a none-invasive tool for monitoring stress and can be integrated into the welfare evaluation during commercial fish transports.

KEYWORDS
delayed mortality, sedation, transport, Cleanerfish, aquaculture, fish welfare

1 Introduction

Sea lice control continues to be the primary challenge for growth and sustainability of the Atlantic salmon farming industry in Norway and elsewhere (1–3). There is currently no effective vaccine against sea-lice. In addition, sea-lice show increased resistance to chemo therapeutics (2). Therefore, non-medicinal solutions for sea-lice control are the most common mitigation strategies. A biological strategy, using cleaner fish to delice salmon is now commonly used and both reduces the need for medicinal treatments and is less stressful for the farmed salmon compared to other delousing methods that require extensive handling (4–6).

In the last 10–15 years commercial production of primarily ballan wrasse, *Labrus bergylta*, has developed to meet the increased demand and to address sustainability and biosecurity issues related to using wild-caught wrasse. In recent years, considerable improvements with regards to delousing efficiency, disease management and welfare of cleaner fish have been made (4, 7). However, there are still large knowledge gaps regarding how common operational procedures affect the welfare of ballan wrasse, emphasized by reports of high losses during production and when used in sea cages (8–10). Transport of cleaner fish, by boat and/or vehicle, is an essential procedure of the production process and often occurs over long distances. Most of the previous studies on welfare issues related to transport of cleaner fish have focused on lumpfish when transported from land-based hatcheries to co-stocking with salmon in sea-cages (8, 9). In ballan wrasse production, transport between land-based hatcheries and on-growing facilities is common, and episodes of increased mortality have been reported after such transport.

Generally, the transport of live fish is one of the most critical operations in aquaculture, in which fish are exposed to multiple handling events and large variations in environmental factors within a short period of time. These are events that can initiate a severe stress response in farmed fish. In response to stress cortisol, the main stress hormone in teleost fishes, is released (11, 12) enabling stress coping mechanisms by making the resources needed to cope with the stressful stimuli available. Energy required for stress coping is redistributed from maintenance functions such as growth and immune reactions (12). In this vein, the sum of the high intense stressor of transport together with the post transport low intensity stressor have been suggested to affect post transport survival (13). Accordingly, stress has been discussed as one of the underlying factors for delayed mortality syndrome or hauling mortality, referring to mortality appearing days or even weeks after transport (13). Factors that have been suggested to affect the post-transport outcome include water quality, confinement, density, holding container design, handling procedures, and agonistic behavior reviewed by Harmon (13–15).

There is a strong relationship between plasma cortisol levels and the release of this hormone from the fish to the water, reviewed by Scott and Ellis (16). Accordingly, the release rate of cortisol to the water has been used as a non-invasive welfare indicator in laboratory studies (17–19) and in commercial aquaculture settings (20). Fish transport in closed tanks in hauling vehicles with or without external water exchange and in well-boats is a common practice in aquaculture. In such closed transports, cortisol will accumulate over time. Therefore, measurements of water cortisol concentrations can be used as an integrative tool for assessing the total stress burden associated with transport. However, its use as a monitoring tool for fish welfare during transport is still very limited.

Using anesthetics prior and during transport is a common practice to lower the metabolism of the fish, thus reducing toxic metabolite production, reviewed in Harmon (13). Anesthetics are also used to lessen the stress response due to handling and transport (13, 21–23). This is achieved by sedating the fish enough to reduce the stress response and the risk of injury, while fish are still able to maintain swimming and an upright position. Therefore the proper dosage is critical and will vary with species and fish size. AQUI-S®, an isoeugenol based sedative, has been used before and during transport to reduce transport stress in several fish species (22, 24, 25). It has proven to be a good sedative for different types of handling procedures, since several fish species go directly into the narcatic (resting) state without a pre excitement phase (26). Although AQUI-S® is a widely used anesthetic the mechanisms of action are not clearly understood. Although Aqui-S has been reported to mitigate stress in several fish species, other studies indicate that the stress mitigating properties of AQUI-S® are species specific (25, 26). If sedation with AQUI-S® affects post transport survival and welfare in ballan wrasse is to our best knowledge unknown.

The objective of this study was to investigate the relationship between transport stress and post transport mortality at a commercial Ballan wrasse on-growing facility. The effect of AQUI-S® sedation on transport stress was quantified by cortisol release rate to the water in the transport tanks, and effects of transport stress on post-transport mortality was investigated by relating cortisol release during transport to mortality rates at the on-growing facility.

2 Materials and methods

2.1 Transports

The study included transports from 2 commercial hatcheries producing ballan wrasse larvae, all transports were delivered to the same receiving land-based farm for further on-growing. Fish were loaded on to transport trucks by lowering the water level in the tank and then vacuum pumping fish into transport tanks. The loading procedure took between 1 and 3 h. The transports were performed in two trucks with 10 or 12 internal tanks, with a volume of 1.2 and 1.0 m^3, respectively. Oxygen saturation (%), temperature (°C) and pH were monitored and logged in the transport tanks. Transport time, fish weight and density, temperature, pH and oxygen saturation during the transport are presented in Table 1. All fish from the same transport were offloaded into one tank at the receiving farm, in which they remained throughout the 25-day study. Dead fish from each tank were counted and removed daily.

To investigate if AQUI-S reduced stress during transport procedures, AQUI-S was used both during loading and transport during four transports. 0.5 h before loading AQUI-S (2.5 μL/L) was added directly to the fish tanks in the hatchery. In the tanks aboard the hauling vehicle the same dose of AQUI-S was premixed in the tank water before the fish were loaded. Six of the followed transports fish were not treated with AQUI-S®, Table 1.

2.2 Water sampling and analysis

2.2.1 Water sampling

One liter water samples were collected from the water supply used to fill up transport tanks at the hatchery (basal levels). Post transport water samples (1 L) were randomly collected from four of the tanks on the transport vehicles, directly when fish were offloaded. The water samples were frozen and stored at −20°C until analysis.

TABLE 1 Conditions during truck transports of juvenile Ballan wrasse between hatcheries and a land based on-growing facility.

	Treatment									
	AQUI-S®					No AQUI-S®				
Transport time (h)	16.25	19.50	17.25	15.25	16.25	20.75	17.50	18.50	15.25	12.50
Fish weight (g)	2.6	1.7	2	2.2	1.8	1.5	2	1.7	2.2	2.3
Tank volume (m^3)	1	1	1	1	1	1	1	1	1,2	1
Fish density ($kg\,m^{-3}$)	29.3	19.8	12.6	19.8	13.4	12.6	19.6	10.8	28.8	21.1
Temp (min-max; °C)	12.1–13.1	11.8–13.1	12.2–13.3	11.8–13.6	11.8–13.3	11.2–12.5	11.4–12.5	11.4–14.0	11.9–13.5	–
pH (min-max)	6.9–7.6	6.9–7.7	6.7–8.1	7.2–7.4	7.1–7.9	7.3–7.9	7.3–7.5	7.3–7.5	7.2–7.5	–
Oxygen (min–max; %)	107–122	107–132	106–132	103–117	105–138	102–121	106–123	92–128	101–128	85–143

2.2.2 Water cortisol analysis

Analysis of accumulated water cortisol during the transport followed a method described by McWhinney et al. (27) with some modifications. Water samples were spiked with 10 ng internal standard (cortisol d4) to correct for matrix effects and for losses in sample extraction, concentration, and analysis. Samples were loaded onto activated Oasis HLB 6 cc (200 mg) solid-phase extraction cartridges (Waters, Milford, MA, United States). After loading 200–300 mL of the samples, the columns were washed with 3 mL milliQ water followed by 3 mL of 20% methanol. Samples were eluted with 5 mL 100% ethyl acetate, dried at 50°C, and reconstituted in 200 µL of 40% methanol with 5 mmol/L ammonium formate and 0.1% formic acid. Separation was achieved on a BEH C8 column (Waters, Milford, MA, United States) using a solvent gradient consisting of 5 mmol/L ammonium formate and 0.1% formic acid in water and methanol. Cortisol content was analyzed with a tandem mass spectrometer (Waters TQ-S, Milford, MA, United States) operated in negative electron spray ionization mode with the following MRM acquisition parameters (precursor and product ions); 407.1 > 331.05, 407.1 > 331.1 for cortisol and 411.1 > 335.05, 411.1 > 335.1 for cortisol d4 (IS).

2.2.2.1 Cortisol release rate

In fish, cortisol in unconjugated form is mainly excreted through the gills to the water and it has previously been shown that free cortisol in the water is directly related to the cortisol concentration in the blood of fish (18). On this basis, the excretion rate of cortisol has been used as a stress indicator in commercial fish farms (20). The excretion rate of cortisol to the water during transport from the hatchery to the receiving on-growing facility, was calculated using the formula:

$$\left(\left[\text{Cortisol transport}_{\text{end}}\right]-\left[\text{Cortisol transport}_{\text{start}}\right]\right)/\left(\text{biomass}_{\text{tank}}{}^{*}\ \text{transport time}\right)$$

[Cortisol transport $_{\text{start}}$] is the cortisol concentration in the water before fish are loaded. [Cortisol transport $_{\text{end}}$] is the cortisol concentration in the tank at the transport end. Biomass $_{\text{tank}}$ is the biomass of the fish in the transport tank and transport time is the time between loading and unloading the fish. [cortisol transport $_{\text{start}}$] was below the detection limit of the analysis method (0.1 µg L-1) and therefore set to 0.

2.3 Statistical analyses

All values are presented as mean ± standard error unless otherwise specified. The effects of AQUI-S® treatment on cortisol excretion rate during transport was investigated by a student's *T*-test. Effect of transport on mortality during five-day intervals following transport was investigated by a repeated measure analysis of variance (ANOVA). Dunnet's *post hoc* test was employed to investigate differences in mortality during the first five days interval after transport compared to the following four five-day intervals. Factors affecting accumulated mortality 25 days after transport was investigated with an analysis of covariance (ANCOVA) with transport treatment (AQUI-S® or no AQUI-S® treatment) as categorical factors and cortisol excretion rate during transport and the mean size of the transported fish as continuous predictors. $p < 0.05$ was set as the threshold for statistical significance.

3 Results

3.1 Water cortisol

Ballan wrasse sedated with AQUI-S® during loading and transport had a significantly higher cortisol excretion rate compared to fish transported without AQUI-S® treatment ($T_{(8)=}$ 5.5, $p < 0.001$) Figure 1.

3.2 Mortality after transport

Mortality changed over time during the first 25 days after transport (Repeated measure ANOVA; $F_{(4,\,36)} = 13$, $p < 0.001$), leading to significantly lower values day 11–15, 16–20, and 20–25 compared to mortality during the first five-day period after transport (day 1–5, $p < 0.01$, $p < 0.001$ and $p < 0.001$ respectively), Figure 2. There was no significant difference in mortality between the first (day 1–5) and the second (day 6–10) period ($p < 0.067$).

The results from the ANCOVA showed a significant relationship between fish weight at transport and accumulated mortality 25 days after transport ($F_{(1,\,6)} = 17$, $p < 0.01$), Figure 3. However, there were no significant effects of AQUI-S treatment ($F_{(1,\,6)} = 0.0001$, $p < 0.98$) on accumulated mortality 25 days after transport. Neither, was there a

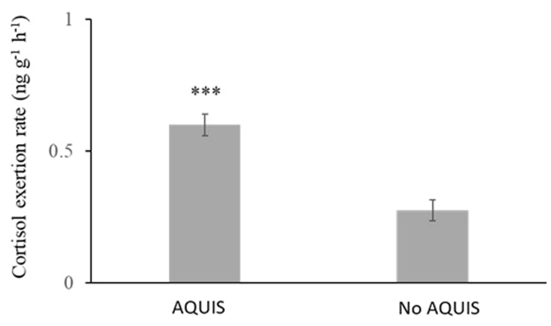

FIGURE 1
Cortisol excretion rate in Ballan wrasse during truck transportation between hatcheries and an on-growing facility. Fish were treated with AQUI-S during loading and transport in four cases and did not receive AQUI-S treatment before and during the transport in six cases. An asterisk (***) marks a significant difference at the level of $p < 0.001$.

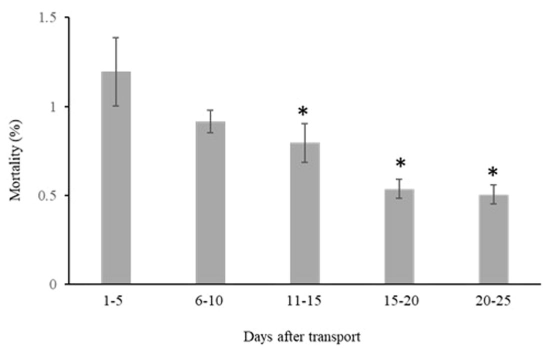

FIGURE 2
Accumulated mortality of Ballan wrasse in five-day periods post-transport at the receiving on-growing facility. Fish from each transport (10 transports in total) were kept in separate tanks at the receiving on-growing facility for 4 weeks. Mortality was recorded daily. For conditions during transport see Table 1. An asterisk (*) marks a significant difference ($p < 0.05$) versus the first 5-day period after transport.

significant relationship between cortisol excretion rate during transport and accumulated mortality 25 days after transport ($F_{(1, 6)} = 0.18$, $p < 0.98$).

4 Discussion

In general transport is one of the most stressful and critical operations in fish farming, including handling, crowding, pumping and potential sub-optimal water quality, and in addition motion in the transport vessel (13, 23). In this study, the average cortisol excretion rate to the water in the transport tanks ranged between 0.15 and 0.6 ng g^{-1} h^{-1}. Laboratory studies with rainbow trout and salmon show that cortisol excretion rate can increase from 0.01 to values up to 0.6–7 ng g^{-1} h^{-1} during stressful events, reviewed in Scott and Ellis (18). The initial process of loading to the transport tanks requires several novel stressors within a short time period, i.e., handling, pumping and a new environment, whereas the long transport time may act as a recovery period if water quality is maintained. The average cortisol extraction rate to the water in the transport tanks can be viewed as the total stress burden during transport. Considering this, and that the cortisol release rate to the transport tanks in this study was just under the values in Scott and Ellis (18), the results might reflect that fish are recovering from an acute-intense stress that is associated with additive stressors during the loading process. However, when comparing cortisol excretion rates, it is important to note that higher baseline levels of plasma cortisol have been reported in ballan wrasse compared to rainbow trout however both species seem to have similar cortisol levels in response to acute intense stress (28). This emphasizes that further studies on stress responsiveness, and the relationship between plasma cortisol and cortisol release rate to the water, are needed to verify the impact of potential stressors on the release rate of cortisol in ballan wrasse.

In this study light sedation with AQUI-S® during loading and transport increased cortisol excretion rate. Similar results have bene reported in Gilthead seabream, showing that AQUI-S® sedation elevated plasma cortisol levels after 6 h simulated transport (25). In a comparable study with Gilthead seabream clove oil, an another isoeugenol based sedative, also increased plasma cortisol levels after transport (29). Based on the elevated post transport plasma cortisol values together with differences in gene expression in the head kidney, the authors suggest that clove oil prolongs the time needed for stress recovery (29). As mentioned above, it is possible that the relatively low cortisol release rate during transport in our study reflects that fish were able recover from the initial intense stress associated with loading during the long transport. Also in line with (28), our findings suggest that AQUI-S® sedated fish take longer to recover and habituate to transport tanks thus have a higher overall cortisol release rate. However, these results are in contrast to studies in salmon that show that AQUI-S® has stress reducing capabilities (22, 30). In Atlantic salmon smolts, sedation just before onloading to the transport truck and again 15 min before offloading resulted in lower post-transport plasma cortisol levels (22). Still, it is important to note that in the studies (22, 30) that demonstrate that AQUI-S® has stress reducing effects during transport procedures, AQUI-S® was not used the whole transport and fish were allowed to recover in water without AQUI-S® before sampled. Thus, it cannot be excluded that the sedation protocol for reducing transport stress in Atlantic salmon smolts (22) might also have stress reducing effects in Ballan wrasse, if only used during handling and onloading operations and not during transport.

In line with the suppressive effects of AQUI-S® on the cortisol response in Atlantic salmon during transport procedures it has also been demonstrated that channel catfish (*Ictalurus punctatus*) exposed to confinement and hypoxia when AQUI-S® sedated have a reduced cortisol response (31). There are also other studies that report no effect of AQUI-S® on the plasma cortisol response such as when used for crowding rainbow trout (*Oncorhynchus mykiss*) (32) or striped bass exposed to low water levels (33). In addition to the mentioned effects of AQUI-S® on cortisol dynamics during stress recovery, species specific effects of the sedative on the stress response might also contribute to discrepancies between studies (26, 34).

Generally, light sedation is considered to mitigate stress related mortality in transported fish reviewed by Harmon (13). Iversen and Eliassen (22) reported that beside the stress reducing effects of AQUI-S® it also resulted in higher post transport survival rates in Atlantic salmon smolts. In our study we could not detect any effects of

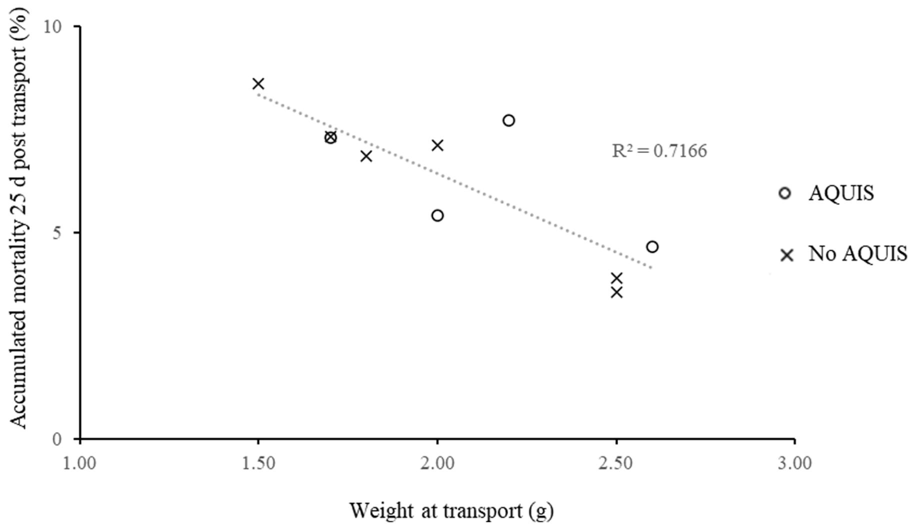

FIGURE 3
Relationship between the weight of Ballan wrasse juveniles at transport and mortality 25 days post-transport, when transported from hatcheries to a land based on-growing facility. The fish from each transport were kept in separate tanks at the on-growing facility. Mortality was recorded daily.

AQUI-S® on post transport mortality. Interestingly, size at transport showed a close negative relationship with accumulated mortality 25 days after transport. Furthermore, in our study, mortality was highest (~1%) the first 5 days after transport to the on-growing facility. After 5 days mortality decreased and 25 days post transport the accumulated mortality was ~4%. In one of the few other studies performed on ballan wrasse transport, Jonassen and Foss (35) showed similar mortality values after transporting larger ballan wrasse from a land-based facility to open sea- cages (by truck and/or well-boat). In that study the cumulative mortality in the sea-cages was 1.1 to 1.3% seven days after transfer and between 4.4 and 5.8% 1 month post -transport. However, in the mentioned study, it is pointed out that the large difference in rearing conditions between the land and sea site and the increased environmental variability at sea has a greater impact on survival than the transport stress. In the present study, fish were reared in similar conditions at the hatchery and at the on-growing facility. Thus, the observed post transport mortality is likely linked to transport conditions and size of the fish. However, it is important to note that data on size related mortality in non-transported fish is needed to verify the actual impact of transport on mortality in farmed ballan wrasse. Still, the strong relationship between fish weight at transport and post-transport mortality in the present studym suggests that farmers should include size at transport as an important factor to consider during planning and establishing best practice protocols for juvenile ballan wrasse transports.

In conclusion, this study shows that slight sedation with AQUI-S® neither had a mitigating effect on cortisol release rate during transport nor reduces post-transport mortality in ballan wrasse. Moreover, somewhat in contrast to what was expected, AQUI-S® treatment during loading and transport increased cortisol excretion rate to the water in the transport tanks, suggesting a stimulating effect of AQUI-S® on the stress axis in ballan wrasse. Furthermore, the cortisol release rate in the present study was rather low in comparison with stressed salmonids. This suggests a species difference in cortisol release rate and/or that during the long transport the ballan wrasse have been recovering from the initial stressful phase of handling and onloading to the transport tanks. The latter is supported by the stimulating effect of AQUI-S® on the cortisol response observed in our study and other studies (25, 29) indicating that using AQUI-S® during transports prolongs the time needed for stress recovery. Overall, this study demonstrates that water cortisol can be used as an integrative tool to assess stress during transport. Still, studies linking cortisol release rate during transport to negative welfare and health effects, and ultimately leading to delayed mortality syndrome or hauling mortality, are required for implying negative transport consequences. Moreover, considering that the negative impact of stress on animal welfare is related to the intensity, duration, and frequency of the stressors (36), further studies linking the dynamics of cortisol release rate to fish health and welfare is needed.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

Ethical approval was not required for the study involving animals in accordance with the local legislation and institutional requirements because we followed commercial fish transport with normal routines. We used water born cortisol as a stress indicator of transported fish. Thus, fish was not exposed extra stress due to sampling.

Author contributions

SC: Investigation, Writing – review & editing. TJ: Methodology, Writing – review & editing. ES: Writing – review & editing. HÅ:

Investigation, Writing – review & editing. AI: Funding acquisition, Project administration, Writing – review & editing. CS: Investigation, Methodology, Writing – original draft, Writing – review & editing. TW: Methodology, Writing – review & editing. EH: Conceptualization, Formal analysis, Investigation, Methodology, Project administration, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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EDITED BY
Pablo Almazan Rueda,
National Council of Science and Technology (CONACYT), Mexico

REVIEWED BY
Amedeo Manfrin,
Experimental Zooprophylactic Institute of the Venezie (IZSVe), Italy

*CORRESPONDENCE
Ren Ryba
ren.springlea@animalask.org

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Humane slaughter in Mediterranean sea bass and bream aquaculture: farm characteristics, stakeholder views, and policy implications

Koen van Pelt, Max Carpendale and Ren Ryba*

Animal Ask, London, United Kingdom

In many countries, increasing concern for animal welfare is driving retailer commitments and government legislation that aim to improve the lives of farmed fish. One aspect of fish welfare involves stunning fish prior to slaughter. The feasibility of stunning depends on the species of fish and physical farm characteristics. In this article, we provide an overview of stunning before slaughter in European sea bass and sea bream aquaculture, one of the largest finfish farming industries in the developed world that does not yet stun most of its production. Sea bass and sea bream stunning necessitates the use of electrical stunning equipment aboard harvest vessels, often a significant distance from the shoreline; this presents an interesting engineering and policy challenge. Together, Türkiye, Greece, Spain, and Italy produced over 400,000 t of sea bass and sea bream in 2020. In Türkiye and Greece, farms are numerous and located very close to the shoreline. In Spain and Italy, farms are few and located far from the shoreline. The highest average production is found in farms from Türkiye (1,000 t) and Spain (1,300 t, and lower average production is found in Greece (300 t) and Italy (350 t). Producer progress towards the installation of electrical stunning appears comparatively well-developed for Türkiye, Spain, and Greece, though we emphasise that producers and other stakeholders require continued support to realise this opportunity. Producers in Italy appear slower to make progress on this aspect of animal welfare and may require additional support.

KEYWORDS
Dicentrarchus labrax, dry stunning, European Union, *Sparus aurata*, Turkey, wet stunning

1 Introduction

The concept of animal welfare is emerging as an increasingly important aspect of sustainable food systems (United Nations Environment Programme, 2019; Coghlan et al., 2021). In many countries, increasing consumer concern for animal welfare is driving retailer commitments and government legislation that aim to improve the lives of animals farmed for food (Alonso et al., 2020; Albalat et al., 2022; Wahltinez et al., 2022; Wickens, 2022). These policies are increasingly targeted at aquatic animals in particular (Ashley, 2007; Stien et al., 2020; Crump et al., 2022). By scale, fish constitute one of the most numerous groups of farmed animals, exceedingly even the number of farmed chickens and pigs and behind only farmed invertebrates (Waldhorn and Autric, 2022; Klaura et al., 2023; Mood et al., 2023). As such, initiatives that aim to improve the lives of farmed fish can cause large overall benefits in the lives of animals.

Fish welfare interventions typically target one of three periods during the lives of farmed fish. Interventions can target the welfare of fish during breeding (the breeding stock or the juveniles) (Grimsrud et al., 2013; Tørud et al., 2019), the welfare of fish on-farm during the grow-out period (Pettersen et al., 2014; Stien et al., 2020), or the welfare of fish at slaughter (Lines and Spence, 2012; European Commission, 2017; Clemente et al., 2023).

One tractable way to improve the welfare of fish at slaughter is to stun fish before slaughter. Stunning involves rendering fish insensible, thus reducing the amount of suffering experienced by the fish when killed (Lines and Spence, 2012). Fish farming industries in many countries have made progress in implementing stunning before slaughter. However, one of the largest finfish farming industries that has not yet made significant progress in implementing stunning before slaughter is the European sea bass and sea bream farming industry.

In Europe, sea bass and sea bream are farmed along the Mediterranean coast. The countries with the highest production of sea bass and sea bream are Türkiye, Greece, Spain, and Italy (Table 1). Türkiye and Greece each have several hundred farms, while Spain and Italy have only 24 farms each. The farms in Türkiye and Spain tend to have larger production volumes per farm, while the industries in Greece and Italy tend to be dispersed with farms having smaller production volumes.

Before turning to the details of humane slaughter, it helps to give a brief overview of the industry structure in these four countries. In Türkiye, 257,000 t of sea bass and sea bream are produced by 237 mostly large-scale sea cage farms and 173 mostly small-scale earthen pond farms (Çoban et al., 2020). In Türkiye, there are fish farms along the western and southern coastlines with the Mediterranean and the northern coastline with the Black Sea. In Greece, 100,000 t of sea bass and sea bream are produced by 347 sea cage farms (European Commission, n.d.). The eastern coastline appears to be more important, with many farms clustered within just a few hundred kilometres of Athens. Sea bass and sea bream farms in Greece are farmed using inshore floating sea cages (Pavlidis and Mylonas, 2011). In Spain and Italy, the farms are located along the length of the countries' coastlines with the Mediterranean. In Spain, 35,000 t of sea bass and sea bream are produced by 24 sea cage farms (Fishcount, 2017; Nielsen et al., 2021). In Italy, 14,000 t of sea bass and sea bream are produced by 24 sea cage farms. This equates to an average production per farm of 250 t (Italy – Eurofish.dk, n.d.; Hofherr et al., 2015; WWF, 2021). Spanish and Italian farms also mostly use floating sea cages, though a minority of production takes place in wetlands/brackish water (Nielsen et al., 2021).

When it comes to distance from shore, these four countries can be divided into two pairs (Figure 1A). Greece and Türkiye tend to have fish farms very close to the shoreline. Half of Greece's fish farms are located within 170 metres of the shoreline, while half of Türkiye's fish farms are located within 180 metres of the coastline (Hofherr et al., 2015). In contrast, Italy and Spain tend to have fish farms much further from the shoreline. Half of all fish farms in Italy are located within 990 metres from the coastline, while half of Spain's fish farms are located within 1,600 metres of the shoreline (Hofherr et al., 2015). The distance from shoreline determines the geographical and wave dynamics of farms, which in turn influences

TABLE 1 Description of the sea bass and sea bream aquaculture industry in the four key countries.

	Türkiye	Greece	Spain	Italy
Annual SBSB slaughter (number of fish)	346 million	235 million	86 million	37 million
Annual SBSB slaughter (weight in tonnes)	257,000 t	100,000 t	35,000 t	14,000 t
Description of farms (% of production)	Sea cages (96%) Earthen ponds (4%)	Sea cages	Sea cages (90%) Wetlands (10%)	Sea cages (78%) Land-based systems (16%) Wetlands (6%)
Number of farms in country	237 (sea cage farms) 173 (earthen pond farms)	347	24	24
Average production per farm	1,040 t (sea cages) 59 t (ponds)	305 t	1,270 t	350 t
Exports (percent of production)	75%	92%	40%	40%

Data summarised by the authors from sources provided in-text.

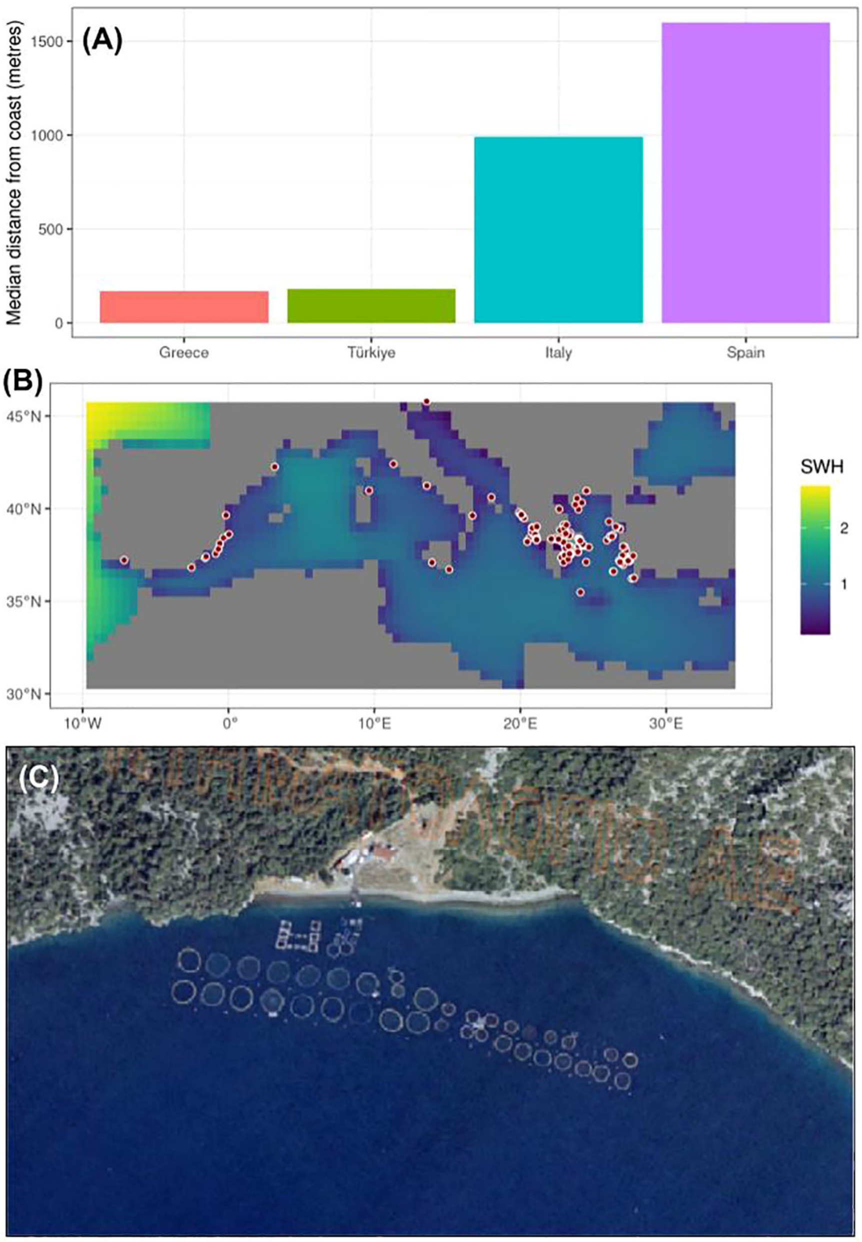

FIGURE 1
Farm physical characteristics. **(A)** median distance from coast by country; **(B)** coordinates of some farms (red points) visualised against mean significant wave height (SWB) throughout 2023 in metres; **(C)** aerial image of a typical fish farm, in this case a near-shore farm in Greece. For **(B)** data are the mean significant wave height 2001 to 2016 for farms with known coordinates (incomplete for Türkiye and Italy). For **(C)** the horizontal extent of the aerial photograph is 0.01 decimal degrees (roughly 800 m). Data in **(A)** from Hofherr et al. (2015); **(B)** from Hersbach et al. (2023), using Copernicus Climate Change Service information and farm coordinates from European Marine Observation and Data Network and Clawson et al. (2022); **(C)** from https://gis.ktimanet.gr/under CC BY 3.0.

the technology and equipment that farmers can adopt. We discuss this point further below.

In the remainder of this article, we examine the technological options for humane slaughter of sea bass and sea bream, before turning to a discussion of the industry perspectives, economic details, and engineering aspects associated with implementing humane slaughter in the European sea bass and sea bream industry.

2 Humane slaughter of sea bass and sea bream

2.1 Electrical stunning before slaughter

Today, almost all sea bass and sea bream are slaughtered by live chilling in ice or ice slurry, followed by a gill cut (European

Commission, 2017). During live chilling, fish generally remain conscious for between 5 and 40 minutes and display signs of suffering, including vigorous escape attempts (Van De Vis et al., 2003; Simitzis et al., 2013; Zampacavallo et al., 2015; de la Rosa et al., 2021). Brain activity continues even after fish become immobile (Lines and Spence, 2012).

During slaughter, animal welfare can be improved by stunning. Electrical stunning involves rendering fish unconscious using an electric current. Studies indicate that electrical stunning causes sea bass and sea bream to immediately become unconscious and lose sensibility (Giuffrida et al., 2007; Lambooij et al., 2007; Zampacavallo et al., 2015) (Panel on Animal Health and Welfare, 2009). It is important that stunning parameters (e.g. current and duration) are carefully selected, especially with reference to the method of electrical stunning, the handling method, and the subsequent killing method. Greater efficacy is generally achieved with higher currents and a longer duration (Robb et al., 2002; Lines et al., 2003; Jung-Schroers et al., 2020).

Electrical stunning can involve one of two methods: in-water and in-air. For in-water electrical stunning, fish are transported to a tank in batches using brailing and then exposed to the electrical field. Alternatively, fish can be captured using a pump and passed along a channel through which an electrical current is generated (Lines et al., 2003). In-air stunning is also possible but introduces the requirement to dewater the fish prior to stunning. Currently, producers of electrical stunning equipment for sea bass and sea bream include the UK-based company Ace Aquatec (in-water stunning), the Norway-based company Optimar (dry stunning), and the Türkiye-based company Smilefish.

The other major category of fish stunning is percussive slaughter. Percussive slaughter is not recommended for small fish, which includes sea bass and sea bream. This is because percussive stunning requires accuracy to be consistently maintained over time, which is difficult for small fish in a commercial setting (de la Rosa et al., 2021).

2.2 Current uptake of humane slaughter

Many Mediterranean sea bass and sea bream producers have expressed an interest in implementing electrical stunning. Currently, progress appears relatively promising in Spain, Greece, and Türkiye, with Italy appearing less promising.

In Spain and Greece, the major producer Avramar intends to implement electrical stunning this decade. Specifically, Avramar has publicly committed to implementing, by 2027, electrical stunning for 100% of its production in Spain and 50% of its production in Greece. Currently, Avramar has electrical stunning installed on two of its farms in Greece (Avramar, 2023). Likewise, the producer Philosofish has begun installing on its harvest vessels electrical stunners produced by Ace Aquatec (The Fish Site, 2023).

In Türkiye, a study conducted by the NGO Future for Fish provides insight into the status of electrical stunning at Turkish fish farms (Future for Fish, 2023). In collaboration with academic advisors, Future for Fish contacted and visited numerous aquaculture companies, accounting for 76% of sea bass and sea bream production in Türkiye. A key finding showed that the majority (90 percent) of Turkish aquaculture companies already have electrical stunning systems. Companies with electrical stunning systems do not always use those systems; only around 40% of companies use electrical stunning for over 95% of the harvesting process. The primary driver of Turkish farmers' adoption of electrical stunning systems is demand from customers (e.g. retailers in the UK and the Netherlands). Turkish fish farmers tend to use electrical stunning systems that are produced and sold in Türkiye, by companies such as Smilefish. Such systems have not been evaluated scientifically for their efficacy in successfully and consistently stunning fish.

In Italy, a survey of 21 sea bass and sea bream farms found that none of the surveyed farms have adopted electrical stunning (Clemente et al., 2023). The slaughter methods used by the surveyed farms were "thermal shock" (20 farms) and "asphyxia in air" (one farm).

3 Industry perspectives and implementation of humane slaughter

3.1 Industry perspectives

The most detailed information about industry perspectives on humane fish slaughter in Türkiye comes from the recent stakeholder survey conducted by Future for Fish (Future for Fish, 2023). The main challenge with electrical stunning systems in Türkiye is the initial installation aboard harvest vessels. The research also found other valuable insights. Implementing electrical stunning in operations leads to a decrease in labour due to "better organisation and occupational safety on harvest ships", better product quality, and possibly a longer shelf life. Fish producers believe that electrical stunning is more challenging for sea bass in particular, due to the fish getting stuck in the fish pumps leading to haemorrhages on their skin. A similar survey was conducted in Italy, but the negligible uptake of electrical stunning in the Italian sea bass and sea bream industry means that the survey did not produce detailed information about farmers' views on those species (Clemente et al., 2023).

3.2 Economic costs and funding

To our knowledge, there are three main manufacturers of electrical stunning equipment for European aquaculture: Ace Aquatec (United Kingdom), Optimar (Norway), and Smilefish (Türkiye). Surveys of manufacturers reveal that installing an in-water electrical stunner aboard a harvest vessel would cost a farmer somewhere in the vicinity of 150,000 €, though stunners produced by Smilefish may cost a different amount (European Commission, 2017). This cost excludes expenses associated with any necessary modification to ships.

When it comes to making the investment necessary to purchase electrical stunning equipment, company size emerges as a critical factor (European Commission, 2017). Larger enterprises, with more resources, may integrate stunning equipment more seamlessly, while smaller companies might face greater challenges, indicating a need for tailored strategies. A stakeholder consultation conducted by the European Parliament's Committee on Fisheries found that European fish farmers most often mention investment funds as the main obstacle to adopting new techniques, followed by justification of need (Pavlidis et al., 2023). Direct funding is mentioned as one of the most useful tools for mitigating potential impacts when transitioning to new fish welfare requirements.

Funding through the European Maritime Fisheries and Aquaculture Fund offers support, with specific budgets allocated for animal health and welfare improvements (e.g. 4M € for Italy, 8M € for Spain, and 560,000 € for Greece). A considerable portion of the funds allocated during the 2014–2020 period for aquaculture innovation remained unused. A lack of transparency in grants hitherto makes it difficult to assess how much money was used on animal welfare improvements. There are however known examples of the previous budget being used for a sea bream and sea bass stunning pilot in Spain. In general, transparency in funding allocation is a concern, with unclear information on recipients and purposes.

3.3 Engineering aspects

The physical, economic, and geographical details of sea bass and sea bream farms vary by country (Figure 1). Therefore, it is reasonable to expect that some countries will have an easier time than others in implementing electrical stunning. Spain in particular appears to have farms located in areas of the ocean with large waves, which could mean that air entering the fish pumps could be an engineering challenge in that country (Figure 1B). Size differences of fish can also cause the design sizes of pumps to be exceeded, likely leading to fish suffering and damaged product (Future for Fish, 2023).

The difficulty of installing new stunning equipment onboard vessels depends on the space available on the vessel, the type of vessel, and the installation requirements for the stunning equipment. Research and data on the types of vessels being used is scarce. Vessel types include platforms, workboats, and larger wellboats. All vessel types have been observed used for harvesting operations. Platforms are floating barges equipped with outboard engines and a crane. Workboats are larger, better equipped vessels ranging from 12 to 30 metres (Paleo et al., 2000). For larger, workboat-type vessels, installation of stunning equipment and fish pumps should not pose large problems. Equipment can be installed on deck or, for in-water stunners beneath deck. For smaller platform vessels, the limited deck space and the lack of below-deck area pose problems for installing a stunner and pump system. The pre-existing power on smaller vessels might not be enough to power both the crane and stunning system.

The study by Future for Fish found that 60% of interviewed farmers mentioned that stunning works faster than live chilling, meaning that stunning equipment may actually offer advantages for the harvest rate and associated labour requirements (Future for Fish, 2023). For some types of systems adding electrical stunning led to a slower harvest. Other technical obstacles include malfunctions in the equipment, long waiting times for spare parts and increased operating costs.

4 Discussion

As consumers develop an appreciation for animal welfare and its role as a component of sustainable food production, the priority placed on animal welfare by producers will only increase. In this report, we have examined the trajectory towards stunning before slaughter in European sea bass and sea bream aquaculture. The current progress towards installing electrical stunners aboard harvest vessels provides optimism, but succeeding in this policy goal will require concerted action by stakeholders throughout the supply chain (McAfee et al., 2019).

Sea bass and sea bream producers have expressed an interest in installing electrical stunners where this is economically attractive, and some producers have begun to do so (Avramar, 2023; Future for Fish, 2023; The Fish Site, 2023). However, success is greatest when there is a clear demand from retailers for stunned fish (Future for Fish, 2023). This mirrors the dynamic in other agricultural sectors like poultry and pork, where demand from retailers drives improvements in on-farm practices (Scrinis et al., 2017; Peacock and Mendez, 2020). As such, retailers have an important responsibility to ensure that their procurement policies account for consumers' increasing demand for animal welfare.

Likewise, producers are likely to respond to clear signals from government, whether at the national or the EU level. The EU is home to some of the world's most progressive pieces of animal welfare legislation, though legislation for the welfare of fish specifically has been lagging behind (McCulloch, 2018; Giménez-Candela et al., 2020). Nevertheless, the EU has expressed interest in legislation that would make stunning before slaughter mandatory for fish farmers (Dullaghan, 2023). The EU is also exploring the possibility of applying EU farm animal welfare legislation to imported meat products, which could have important repercussions for producers in Türkiye who export their product to EU Member States (Dullaghan, 2023). When particular higher-welfare practices become mandatory, this can help ensure that all producers within a country have access to the same markets (Carey et al., 2017, Carey et al., 2020; Department for Environment, Food and Rural Affairs, 2017). It can be useful for governments or other stakeholders to support farmers in the transition to higher-welfare practices by providing funding and other support, a strategy used successfully when installing CCTV in slaughterhouses in Great Britain (Department for Environment, Food and Rural Affairs, 2017; Springlea, 2022). As such, support from stakeholders throughout the supply chain can drive collective action to improve the lives of animals used for food.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

Author contributions

KV: Conceptualization, Funding acquisition, Investigation, Writing – original draft, Writing – review & editing. MC:

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Ana C. Puello Cruz,
National Council of Science and Technology (CONACYT), Mexico

REVIEWED BY
Carlos Alfonso Alvarez-González,
Universidad Juárez Autónoma de Tabasco, Mexico
Pablo Almazan Rueda,
National Council of Science and Technology (CONACYT), Mexico
Xiaoning Yu,
Shandong Technology and Business University, China

*CORRESPONDENCE
Dong An
andong@cau.edu.cn
Yangen Zhou
zhouyg@ouc.edu.cn

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Behavior analysis of juvenile steelhead trout under blue and red light color conditions based on multiple object tracking

Ziyu Li[1,2,3,4], Xueweijie Chen[5], Jinze Huang[1,2,3,4], Dong An[1,2,3,4*] and Yangen Zhou[5*]

[1]National Innovation Center for Digital Fishery, China Agricultural University, Beijing, China, [2]Key Laboratory of Smart Farming Technologies for Aquatic Animals and Livestock, Ministry of Agriculture and Rural Affairs, China Agricultural University, Beijing, China, [3]Beijing Engineering and Technology Research Centre for Internet of Things in Agriculture, Beijing, China, [4]College of Information and Electrical Engineering, China Agricultural University, Beijing, China, [5]Key Laboratory of Mariculture (Ocean University of China), Ministry of Education, Qingdao, China

Introduction: The lighting environment significantly influences fish behavior. This study explores the impact of diverse lighting conditions on the behavior of steelhead trout (Oncorhynchus mykiss) to illuminate the mechanisms underlying their behavioral responses.

Methods: This experiment was set up with six treatments at a constant light intensity of 150 lx: 12h white light + 12h dark (12 W), 12h blue light + 12h dark (12B), 12h red light + 12h dark (12 R), 1.5h blue light + 9h red light + 1.5h blue light + 12h dark (3B9R), 3h blue light + 6h red light + 3h blue light + 12h dark (6B6R), total 12h of blue and red light + 12h dark (T12BR). A multiple object tracking method, YOLOv5 with SORT, was employed to capture the movement trajectory of each fish, quantifying three motion metrics: swimming velocity, swimming angular velocity, and generalized intersection over union.

Results: The results revealed that fish exposed to 12R light environment showed significantly higher activity levels than other groups. The mixed light environments (3B9R, 6B6R) formed significant differences in behavioral metrics with 12R earlier than pure light environments (12B, 12W, T12BR), indicating sudden light color changes should be avoided. Fish in the 3B9R environment exhibited the lowest activity level but highest growth performance, with the highest specific growth rate of 1.91±0.12 d-1, a value significantly surpassing the lowest recorded rate, supported by a p-value of 0.0054, indicating it is suitable for steelhead trout cultivation.

Discuss: Behavioral significant differences were observed as early as week eight, much earlier than physiological differences, which became apparent by week 16. Overall, this paper employs computer vision methods to study the impact of different light colors on fish behavior, found that 3B9R is the optimal lighting condition tested and sudden light color changes should be avoided, offering a new perspective on light conditions and behavior in steelhead trout cultivation.

KEYWORDS

steelhead trout, fish behavior, behavior quantify, aquaculture environment regulation, light color

1 Introduction

In aquaculture, the breeding environment factors, such as light, temperature, and dissolved oxygen, significantly influence fish growth. Light, which consists of light intensity, light color, and photoperiod, serves as a fundamental environmental factor playing a crucial role in the growth process of fish (Villamizar et al., 2011; Zhang et al., 2020). Fish have evolved light-sensing mechanisms throughout their evolution to adapt to environmental light conditions, thereby lighting conditions affect fish behavior (Xu et al., 2019; Noureldin et al., 2021). Existing research has found that light spectrum conditions can influence the growth, immune response, and digestive and metabolic capabilities of steelhead trout (Chen et al., 2022a, 2022b). Light affects fish metabolism, growth, and behavior through visual stimulation, and appropriate lighting conditions are beneficial for improving fish welfare and promoting growth (Ruchin, 2020). Fish behavior reflects their growth and physiological status, and analyzing behavior is conducive to identifying suitable light environments.

Light color (spectrum), as a fundamental factor of light condition (Zhang et al., 2020), has a significant impact on fish physiological traits due to their long-evolutionary adaptations, subsequently affecting fish growth and behavior. Some studies have found that specific light colors can enhance the growth of fish, for instance, yellow light for pearl gourami (*Trichopodus leerii*) (Heydarnejad et al., 2017), green light for barfin flounder (*Verasper moseri*) (Takahashi et al., 2016, 2018; Yamanome et al., 2009), red light for pikeperch (*Sander lucioperca*) (Baekelandt et al., 2019) and yellow perch *(Perca flavescens)* (Head and Malison, 2007), blue light for goldfish (*Carassius auratus*) (Noureldin et al., 2021) and turbot (*Scophthalmus maximus*) (Wu et al., 2021). Additionally, there are previous studies that focus on the influence of light color on fish behavior. For example, juvenile Nile tilapia showed a preference for yellow and red light (Luchiari and Oliveira, 2014). Young grass carp avoid red light and prefer blue (Mu et al., 2019). Juvenile bighead carp (*Aristichthys nobilis*) swim significantly slower under yellow and green light than under red and blue light (Xi et al., 2019). Goldfish (*Carassius auratus*) reared under blue light exhibited a lower ventilation frequency, longer reaction time, and longer swimming duration (Noureldin et al., 2021) in the novel object test, a behavioral assessment where fish are presented with an unfamiliar object to measure their exploratory and stress responses (Norton et al., 2011; Norton and Gutiérrez, 2019). Both behavior and growth are long-term outcomes of fish physiological traits. Analyzing fish behavior is conductive to identify suitable light environments for fish cultivation. However, previous studies mainly focused on the effects of single light color on fish growth and behavior, and there is limited information on the effects of light combination on fish behavior.

With the advancements in deep learning and computer vision in recent years, computer vision based methods are increasingly being employed in the study of fish behavior (Li et al., 2020; Yang et al., 2020; Li, D. et al., 2022). Fish multiple object tracking technology has demonstrated increasingly powerful capabilities, efficiently and accurately capturing fish trajectories (Li, W. et al., 2022; Mandel et al., 2023; Liu et al., 2024). Compared to manual and acoustic-based methods, computer vision-based fish multiple object tracking approaches offer lower costs and higher efficiency, enabling rapid processing of large volumes of videos to obtain fish behavior information for further study. Currently, fish multiple object tracking methods have been widely applied in fish behavior and aquaculture research. For instance, Wang et al. proposed an end-to-end detection and tracking method to identify abnormal behaviors in porphyry seabream (Wang et al., 2022b). Li et al. introduced a detection and tracking method to study the behavior of *Oplegnathus punctatus* in the ammonia nitrogen environment (Li et al., 2023). Additionally, Xiao et al. developed a tracking method based on attention regions to investigate fish behavior for water quality monitoring (Xiao et al., 2016).

The spectral distribution of light in water is greatly influenced by depth (Liu et al., 2016; Hou et al., 2019). Specially, blue light can penetrate up to 200 meters in clear water, while red light is mostly absorbed within the first 20 meters (Sanchez-Vazquez et al., 2019; Ruchin, 2020). Juvenile steelhead trout reside in shallow freshwater streams (Hartman, 1965; Bugert et al., 1991), and the average swimming depth for adult steelhead trout is around 1.6 meters (Ruggerone et al., 1990), where both blue and red light can be perceived by fish. In this study, considering the natural light environment of steelhead trout, a variety of experimental conditions were established with mixed light colors and varying durations, employing computer vision and artificial intelligence techniques to quantify fish behavior in their daily activities. The growth and immunity performance of steelhead trout under different light color combinations has been studied (Chen et al., 2022b) as well as their digestive and anabolic capabilities (Chen et al., 2022a) based on this experiment. The computer vision-based object detection method YOLOv5 (Jocher et al., 2022) and the multiple object tracking method SORT (Bewley et al., 2016) were introduced to capture fish trajectories. The potential relationship between quantified behavioral traits and physiological characteristics was discussed. Therefore, this study demonstrates the feasibility of using behavioral quantification methods to guide the regulation of fish growth environments. The proposed quantification process in this paper can provide guidance for precise environmental control.

2 Materials and methods

2.1 Animals

Triploid steelhead trout eyed eggs were purchased from Troutlodge, Inc. (Washington, USA) and hatched at the Wanzefeng Fishery Company (Rizhao, Shandong, China). Before starting the experiment, the juvenile trout were acclimatized to a brackish saltwater environment (salinity: 14.2 ± 0.7) for 2 weeks. In this stage, the fish were given twice-daily feedings to apparent satiation at 08:00 and 18:30 with a commercial trout feed from Greatseven Inc. (Qingdao, China). The controlled environment was maintained with a constant 24-hour oxygen supply and a 12 light (L):12 dark(D) photoperiod.

2.2 Trial design

Six light color treatments were established with a photoperiod of 12L:12D (light period: 07:30-19:30) and total light intensity of 150 lx: 12 h white light + 12 h dark (12 W, full spectrum); 12 h blue light + 12 h dark (12B, peak at 454.9 nm); 12 h red light + 12 h dark (12 R, peak at 614.8 nm); 1.5 h blue light + 9 h red light + 1.5 h blue light + 12 h dark (3B9R, peaks at 454.9 nm and 614.8 nm, respectively); 3 h blue light + 6 h red light + 3 h blue light + 12 h dark (6B6R, peaks at 454.9 nm and 614.8 nm, respectively); 12 h blue light and red light + 12 h dark (T12BR, peaks at 454.9 nm + 614.8 nm, respectively), as illustrated in Figure 1. The light intensity of each treatment ensured to be consistent by calculating the average from measurements taken at the surface, middle, and bottom layers of the water. This guaranteed that the spectral composition of light was the only varying factor between treatments. By eliminating the influence of fluctuating light intensities, the differential impacts of light spectra on fish behavior can be analysed more accurately. The light was provided by packaged LED (COB) designed and produced by Qingdao Lanchi Technology Company. High-precision electronic adjustment of light intensity is not supported. Instead, light intensity was ensured by adjusting the distance between the light source and the water surface. Light intensity and spectra were measured using a handheld illuminometer (PLA300; Everfine Inc., Hangzhou, China). The 16-week trial was conducted using a completely randomized block design with four replicates per treatment, 20 fish per tank (380 L volume, 0.72 m height × 0.95 m diameter, white background). Each tank was shielded by black light-absorbing fabric, ensuring no light interference occurred between the various treatment groups. Fish were weighed by an electronic balance (Mettler-Toledo International, Inc., Greifensee, Switzerland).

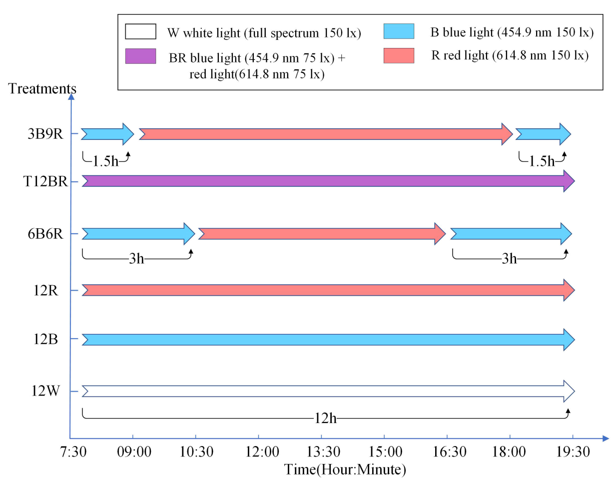

FIGURE 1
setting of light color groups in this experiment. In this experiment, the photoperiod was set to 12L12D, with 12 hours of light (from 7:30 to 19:30) and 12 hours of darkness every day. The naming convention for treatments is derived from the arrangement of light colors within the 12-hour light period, denoted as xCyD: C color light for X hours, D color light for y hours. For instance, '3B9R' denotes blue light for 3 hours and 9 hours of red light, and '12B' denotes blue light for 12 hours.

At the the start and conclusion of the trail, there was a 36-hour halt in feeding to guarantee the digestive tracts of fish were empty. Before beginning the experiment, the fish were submerged in a solution containing 30 mg/L of tricaine methanesulfonate (MS-222; Sigma-Aldrich, USA) for anesthesia. And they were delicately dried using tissue before being weighed. Throughout the cultivation period, each trout, with an initial weight of 34.67 ± 2.69 g, received commercial trout feed daily at 08:00 and 18:30 respectively. Residual unconsumed feed was collected after feeding for 30 min. The calculation of daily feed intake involved evaluating the moisture content of the feed and adjusting for any loss. The water was renewed via a single flow system with a flow rate of 1.15 L/min. Parameters such as water temperature, salinity, dissolved oxygen, and pH were measured three times daily using a YSI ProPlus handheld multiparameter meter (YSI Inc., USA). Sampling of the water occurred every three days, with subsequent analysis involving the Cleverchem 380 automatic chemical analyzer (DeChem-Tech Inc., Hamburg, Germany) to determine levels of total ammonia nitrogen (TAN), phosphate, nitrite nitrogen, and nitrate nitrogen.

Several measures were implemented in order to minimize noise interference in the experiment. Firstly, PVC damping trays (1.1m × 1.1m in width × 10 cm) were placed at the bottom of the rearing tanks. Secondly, the exterior of the rearing tanks and the black shielding fabric were covered with sound-absorbing cotton. Thirdly, nano-aeration discs and liquid oxygen were utilized for aeration. Fourthly, adherence to standardized experimental protocols was strictly maintained, minimizing disturbances during operations. Finally, the PVC pipes for water inflow and outflow were covered with shock-absorbing sound insulation materials.

During the experimental period, the following water quality parameters (mean ± standard deviation) were maintained: water temperature at 16.5 ± 0.2°C, salinity at 14.2 ± 0.7, dissolved oxygen at 8.7 ± 0.3 mg/L, pH value at 7.3 ± 0.1, ammonia nitrogen (TAN) at 0.03 ± 0.03 mg N/L, phosphate at 0.11 ± 0.08 mg P/L, nitrite nitrogen at 0.09 ± 0.05 mg/L, and nitrate nitrogen at 3.43 ± 1.9 mg N/L.

At 4-week intervals, the fish in each tank were anesthetized with 30 mg/L tricaine MS-222, counted, and weighed. The fish behavior was captured using 1080P infrared night vision video webcams (DS-IPC-T12) from Hikvision Digital Technology Co., Ltd. (Hangzhou, China) and saved as videos. The webcams, mounted on a bracket 1.5 m above each tank, recorded videos at a frame rate of 25 frames per second (FPS), capturing the fish swimming behavior from a top-down perspective. Videos were captured daily between 07:30 h and 19:30 h, stored on the hard disk, and each video lasted approximately one hour (resulting in 12 videos for 12 hours per day). The total experimental environment is shown in Figure 2. To prevent the influence of light color on the image-based method, the videos were captured in grayscale. Due to the substantial volume of video data and the limitations in processing and analysis speed, a balance was struck between the representativeness of the sample and analytical efficiency. Segments of the videos that included fish feeding moments (30 minutes each in the morning and evening), were removed to prevent any interference with the swimming behavior analysis. Furthermore, the initial ten minutes of each processed hourly video were selected as a representative segment for that hour, which was then utilized for the subsequent tracking of fish trajectories and behavior analysis. Although the trial lasted 16 weeks, the records from the first 8 weeks were utilized for behavior analysis, as significant behavioral differences became apparent by the eighth week, which was earlier than significant differences in growth.

In this experiment, fish were reared in experimental tanks (380 L volume, 1.0 m height × 0.95 m diameter, with a white background), with 20 fish per tank. Tanks were covered with a back shielding fabric to prevent interference between different treatment groups. Illumination with various light colors was provided by LED sources (packaged LED (COB), Qingdao Lanchi Technology Company), and fish behavior was recorded from a top-down perspective using a camera (DS-IPC-T12, Hikvision Digital Technology Co., Ltd.), with videos stored on computer hard drives.

2.3 Fish behavior analysis

In this experiment, the YOLOv5 with Sort algorithm was employed to perform multiple object tracking on ten-minute video segments of six treatments, with twelve segments recorded each day to correspond with the 12L:12D photoperiod. This process generated frame-by-frame bounding boxes depicting the motion trajectories of individual fish. Subsequently, fish swimming behavior was quantified using three behavioral metrics: Swimming Velocity (SV), Swimming Angular Velocity (SAV), and Generalized Intersection over Union (GIOU). Finally, a statistical analysis was conducted to assess the impact of different light color environments on fish behavior.As for the possible social interactions (hierarchies) among fish, thisstudy primarily focused on investigating the collective behavior of the entire fish population, particularly by measuring average values of metricssuch as SV, SAV,

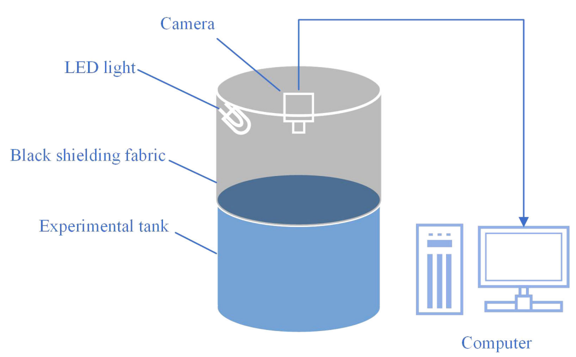

FIGURE 2
Experimental environment.

and GIOU under different light conditions. This approach naturally integrates the possible social interactions among fish, as these averages reflect the collective behavioral performance of all individuals under specific light conditions. By concentrating on the behavioral dynamics at the population level, theanalysis implicitly including the influence of social structures on overall behavioral patterns without the need for direct quantification of specific social interactions among individual fish.

2.3.1 Multiple fish tracking

A subset of fish behavior videos was selected from the experiment to create a fish dataset through annotation. Training and performance comparisons of several popular Multiple Object Tracking (MOT) algorithms, including YOLOv5 with SORT and CenterTrack, were conducted using this dataset, as shown in Table 1 in section 3.1 of the Results. Metrics such as Mean Average Precision (mAP50), Multiple Object Tracking Accuracy (MOTA), and Identity F1 Score (IDF1) were employed for evaluation. YOLOv5 with SORT exhibited superior performance and was chosen in this trial. YOLOv5, an object detection algorithm that has been widely utilized in aquaculture studies (Li, X. et al., 2022; Wang et al., 2022a), can locate fish individuals in each frame with bounding boxes. SORT is an instance association algorithm that uses positional information to associate bounding boxes of the same fish individual across consecutive frames, resulting in fish motion trajectories, as shown in Figure 3. SORT was also widely employed for fish tracking (Wang et al., 2021; Gong et al., 2022; Zhang et al., 2023). In this experiment, YOLOv5 with SORT was employed for multiple object tracking of fish videos recorded, utilizing bounding boxes to represent fish motion trajectories, for subsequent behavioral quantification and analysis. Despite the inevitable presence of some false positives and false negatives, the comprehensive fish dataset, effective behavioral quantification metrics, and scientifically sound statistical methods employed ensure that the results accurately reflect the overall movement trajectories and behavioral states of the fish.

2.3.2 Quantification and calculation of behavior metrics

Using the bounding boxes obtained from tracking fish videos as input, three metrics were utilized to quantify fish behavior for subsequent statistical analysis: Swimming Velocity (SV), Swimming Angular Velocity (SAV), and Generalized Intersection over Union (GIOU). These behavioral metrics can be computed over three consecutive frames, as depicted in Figure 3. A sliding window approach was employed to sequentially select M frames throughout the entire video for calculating metrics, where M represent the window size with a default value of 3.

The Swimming Velocity reflects the displacement per frame, representing the straight swimming of fish, with higher values denoting a relatively higher activity level. The Swimming Angular Velocity is the change rate in the swimming direction of the fish, representing circular swimming behavior in fish, with higher values denoting a relatively lower activity level. Employing angles and distances to study fish behavior is a commonly used method in fish behavior analysis (Hang et al., 2021). GIOU provides a composite measure of the

TABLE 1 Evaluation Metrics for the performance of MOT(Multi Object Tracking) methods.

Method	mAP50 (%)	MOTA (%)	Rcll (%)	IDF1 (%)	IDP (%)	IDR (%)
YOLOv5+SORT	95.7	87.4	92.2	47.7	48.7	46.9
CenterTrack	90.7	64.2	73.4	27.5	30.7	24.9

The mAP50 denotes mean Average Precision under a condition with Intersection over Union (IoU) threshold at 0.5, reflecting the performance of object detection. The MOTA denotes Multiple Object Tracking Accuracy, integrating false positives, misses, and identity switches, and reflects tracking performance. Rcll, the recall of the model, evaluates the ability of model to find all positive samples, with higher values suggesting fewer missed objects. IDF1, IDP, and IDR focus on the continuity and identity preservation in tracking. IDP is the proportion of correctly identified matches by the model that maintain the correct identity, while IDR represents the proportion of all true identity matches correctly identified and maintained by the model. IDF1 is the harmonic mean of IDP and IDR, and these metrics collectively reflect the consistency of object identities throughout the tracking process.

overlap, distance, and dimension differences between two bounding boxes. Since the shape and size of the bounding box corresponds to the pose of the fish, and the displacement of the bounding box corresponds to the movement velocity of the fish, GIOU effectively encapsulates the fish behavioral dynamics. It can serve as an indicator of information representation in various fish poses and can be considered an expression of the stress response capability in fish (Huang et al., 2022).

The left side of Figure 3 shows the multiple object tracking results of a single fish across three consecutive frames, represented by moving bounding boxes that indicate the fish trajectory. On the right side, the figure illustrates the underlying principles and calculation methods for the three behavioral metrics: Swimming Velocity (SV), Swimming Angular Velocity (SAV), and Generalized Intersection over Union (GIOU). These metrics correspond to the following Equations 1, 2 and Algorithm 1.

The notation fu was used to denote the u^{th} fish and the center motion of bounding boxes to represent the movement feature of fish swimming. (xj_{fu}, yj_{fu}) means the center coordinates at j^{th} frame and the swimming velocity (v^{fu}), swimming angular velocity (θ^{fu}) of fish in three consecutive frames were calculated as described in Equation 1 and Algorithm 1.

$$v_j^{fu} = \frac{\sum_{m=1}^{M}\sqrt{\left(x^{fu}_{j+m-1} - x^{fu}_{j+m}\right)^2 + \left(y^{fu}_{j+m-1} - y^{fu}_{j+m}\right)^2}}{M} \quad (1)$$

Input:

x, y: Coordinates of u^{th} fish positions in each frame

M: Window size for computing swimming angular velocity

Output:

θ_j^{fu} Swimming angular velocity of u^{th} fish in j^{th} frame

Algorithm:

1: Initialize $Angles \leftarrow \emptyset$

2: **for** each m from 1 to M **do**

/* *Compute changes in angles of fish swimming velocities* */

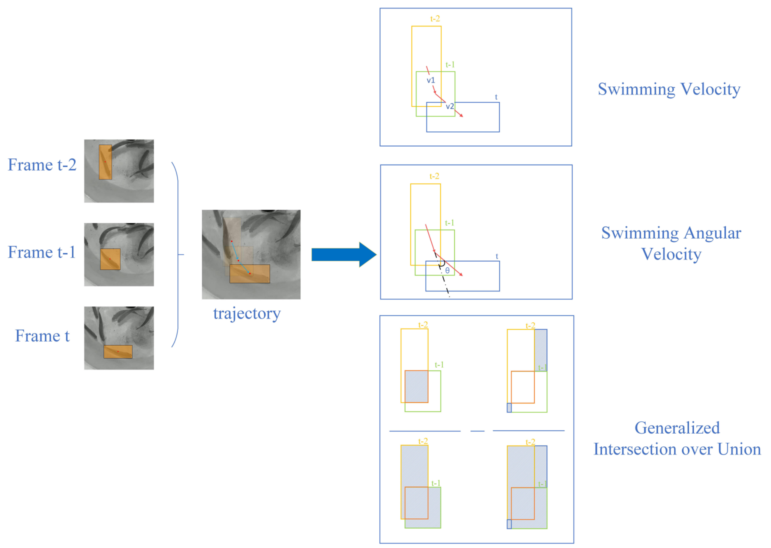

FIGURE 3
Behavioral metrics quantification.

3 : $\Delta x_1 = x_{j+m} - x_{j+m-1}$

4 : $\Delta y_1 = y_{j+m} - y_{j+m-1}$

5 : $\Delta x_2 = x_{j+m-1} - x_{j+m-2}$

6 : $\Delta y_2 = y_{j+m-1} - y_{j+m-2}$

7 : $\angle 1 = \arctan\left(\frac{\Delta y_1}{\Delta x_1}\right)$

8 : $\angle 2 = \arctan\left(\frac{\Delta y_2}{\Delta x_2}\right)$ /* Compute the absolute difference of angles and convert to degrees */

9 : $\Delta angle_m = |\angle 1 - \angle 2| \cdot \frac{180^\circ}{\pi}$ /* Convert the difference of angles less than 180° */

10 : $\Delta angle_m = \min(\Delta angle_m, 360^\circ - \Delta angle)$ /* Accumulate to Angles */

11 : $Angles \leftarrow Angles \cup \{\Delta angle_m\}$

12 : **end for** /* Compute the average angle change */

13 : $\theta_j^{fu} \leftarrow \frac{1}{|Angles|} \sum_{\alpha \in Angles} \alpha$

14 : **Return** θ_j^{fu}

Algorithm 1. Calculate Swimming Angular Velocity of Fish.

$GIOU_j^{fu}$, reflecting the degree of overlap of the fish bounding boxes in consecutive frames, which was calculated as Equation 2. $A_{j_{k=m}}^c$ denotes the area of min box which contains two bounding boxes of the same fish in consecutive two frames, IoU is the area ratio of the intersection and union of the two bounding boxes, U_{j+m} is the union area of the two bounding boxes.

$$GIOU_j^{fu} = \frac{1}{M}\sum_{m=1}^{M}\left(IoU_{j_k=m} - \frac{A_{j_k=m}^c - U_{j_k=m}}{A_{j_k=m}^c}\right) \quad (2)$$

Based on the principle and calculation of these behavioral metrics, it can be inferred that higher swimming speed indicates a higher fish activity level, while increased swimming angular velocity and GIOU values signify a reduced fish activity level. The integration of these indicators provides a comprehensive perspective to systematically evaluate the impact of different light color conditions on fish behavior by comparing the variations among groups based on these three metrics. This methodology enables us to identify the suitable light color conditions for steelhead trout.

2.3.3 Statistical analysis and methods

In the quantification of behavioral metrics, which included twelve values (12 hours per day) for each metric, two parts were conducted. The first part of the study used behavioral metrics from the last five days of the trial, serving as the final behavior effects of the first 8 weeks of the trial, for one-way analysis of variance (ANOVA). The results of the first part are in Sections 3.2 to 3.4 of the Results. The second part utilized daily data on swimming velocity, each comprising 12 hourly values. The 12W group was used as a baseline to identify when significant differences (represented by p-values) between 12W and other groups stabilized. Given the significant differences of the 12R group with other groups, the 12R group was similarly employed as a baseline for conducting the same analysis. This approach allowed for a comprehensive analysis of the effects of different lighting conditions on fish behavior from various perspectives. The results of the second part are in Sections 3.5 of the Results.

The homoscedasticity (P>0.05) of data was evaluated using the Levene test and the normality (P>0.05) of data was assessedthrough the Shapiro-Wilk test. Subsequently, the following stratified approach was adopted to assess inter-group differences: For data that conformed to both normality and homogeneity of variance, ANOVA and LSD tests were utilized; for data that adhered to normality but not to homogeneity of variance, Welch ANOVA and Games-Howell tests were employed; for data that did not meet the normality criterion, Kruskal-Wallis H test and Dunn's Test were conducted. Statistical analyses were performed using Python packages including Scipy version 1.7.1, scikit_posthocs version 0.8.1, pingouin version 0.5.4, and statsmodels version 0.14.1, along with IBM SPSS Statistics version 25.

2.4 Animal and ethics approval

All experimental methods of this study were performed under the Regulations of the Administration of Affairs Concerning Experimental Animals of China, as well as the Regulations of the Administration of Affairs Concerning Experimental Animals of Shandong Province.

3 Results

3.1 Comparison of multiple object tracking methods

The comparative results between two Multiple Object Tracking (MOT) methods, YOLOv5+SORT and CenterTrack, are presented in Table 1. Both methods were trained and tested using a dataset collected and annotated as part of the experiment. The results showed that YOLOv5+SORT outperforms CenterTrack in the context of fish multiple object tracking within this study, evidenced by achieving a Mean Average Precision (mAP50) of 95.7%, a Multiple Object Tracking Accuracy (MOTA) of 87.4%, and an Identity F1 Score (IDF1) of 47.7%. These metrics collectively underscore the exemplary proficiency of YOLOv5+SORT method in target detection and identity association.

3.2 Swimming velocity

The effects of different light conditions on fish behavior metrics are shown in Figure 4. After the first 8 weeks of the trial, the results revealed that there was a significant difference (P< 0.01) in

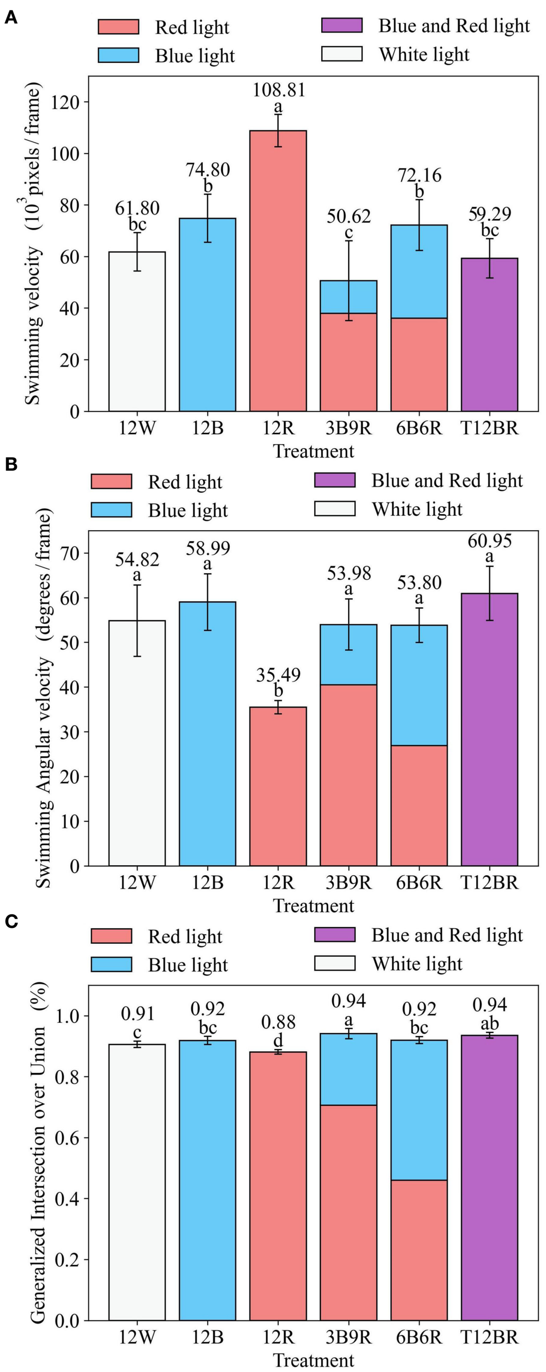

FIGURE 4
Swimming velocity, Swimming Angular velocity, GIOU for juvenile steelhead trout. **(A)** Swimming velocity **(B)** Swimming angular velocity **(C)** Generalized Intersection ove Union.

swimming velocity among the six light color treatments (as shown in Figure 4A). The swimming velocity of the 12R group was the highest ($P < 0.01$), which significantly differed from the other groups. The fish reared under the 3B9R groups had the lowest swimming velocity, which was significantly lower than that of the 12R, 12B, and 6B6R groups.

In this Figure 4, panels (a–c) are statistical analyses of fish behavioral metrics (Swimming Velocity (SV), Swimming Angular Velocity (SAV), and Generalized Intersection over Union (GIOU), respectively) at the end of the trial, reflecting the behavioral influences of fish in the trial. Different letters denote significant differences ($P < 0.05$) between groups based on a one-way analysis of variance (ANOVA) with the Least Significant Difference (LSD) test. Results are expressed as the mean ± standard deviation. The naming convention for treatments is derived from the arrangement of light colors within the 12-hour light period, denoted as xCyD: C color light for X hours, D color light for y hours. For instance, '3B9R' denotes blue light for 3 hours and 9 hours of red light, and '12B' denotes blue light for 12 hours.

3.3 Swimming angular velocity

The results indicated a significant variance in swimming angular velocities among the six light color groups ($P < 0.01$), as illustrated in Figure 4B. Specifically, the fish in the 12R group exhibited the slowest swimming angular velocity ($P < 0.01$), significantly differing from the other groups. The swimming angular velocities of the other groups were relatively similar, with T12BR displaying the highest value.

3.4 Generalized intersection over union

The results revealed significant differences in GIOU among the six light color groups ($P < 0.01$), as depicted in Figure 4C. The fish in the 12R group exhibited the lowest GIOU, showing a significant distinction from the other groups. Conversely, the 3B9R group had the highest GIOU, significantly differing from the 12R, 12B, 12W, and 6B6R groups.

3.5 Dynamics of swimming velocity differences among treatments

Temporal dynamics of significant differences in swimming velocity between treatment groups were demonstrated in Figures 5, 6. Specifically, Figure 5 illustrates the variations in swimming velocity p-values and metrics for the 12W group in contrast with the pure color groups (12B, 12R, and T12BR), while Figure 7 presents these comparisons for the 12W group against the mixed color groups (6B6R and 3B9R). In a parallel fashion, Figure 8 details the changes in swimming velocity p-values and metrics for the 12R group relative to the pure color groups (12B, 12W, and T12BR), and Figure 6 does the same for the 12R group when compared to the mixed color groups (6B6R and 3B9R).

Comparative analysis of the 12W group revealed that stable significant differences were only observed with the 12R group by the conclusion of the trial ($p<0.05$). During the mid-phase of the trial (weeks 3 to 4), the 3B9R and 6B6R groups exhibited transitory significant differences when compared with the 12W group, which were not maintained in the long term.

Comparative analysis of the 12R group revealed that significant differences eventually manifested between this group and all other

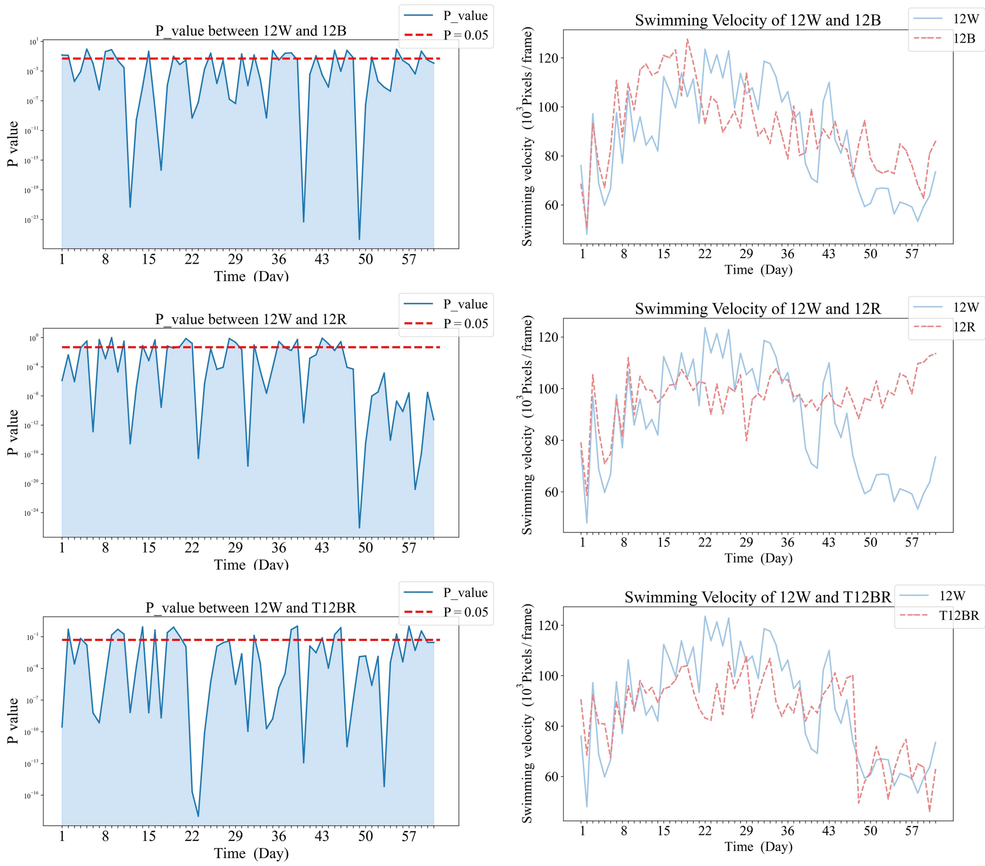

FIGURE 5
Comparative analysis of Swimming Velocity in 12W and pure color groups.

groups. Notably, the 3B9R and 6B6R groups displayed significant differences from the 12R group as early as the sixth week. In contrast, the 12W, 12B, and T12BR groups developed these differences more gradually, becoming apparent by the eighth week.

In Figure 5, panels (a1), (b1), and (c1) on the left display the daily variance in swimming velocity P-values between each pure color group (12B, 12R, T12BR) and the 12W group, over the initial eight-week period. Panels (a2), (b2), and (c2) on the right present

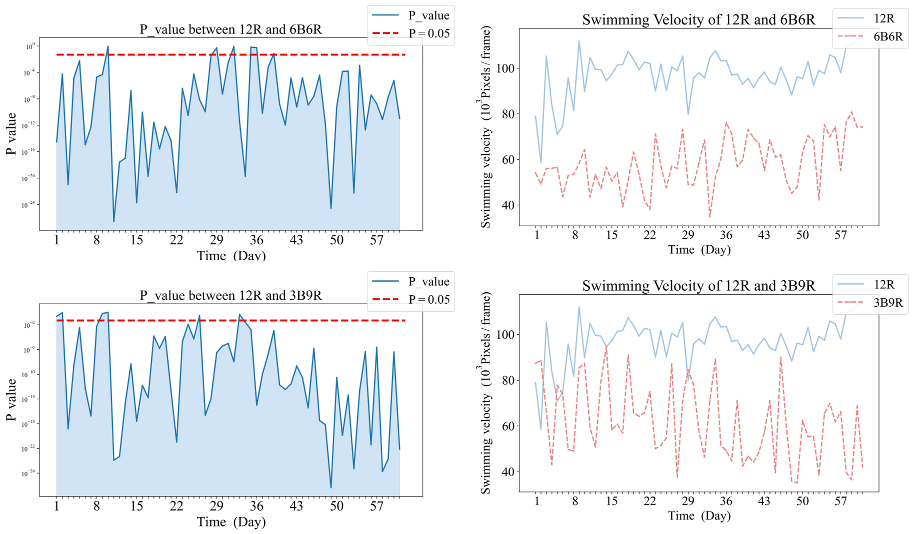

FIGURE 6
Comparative analysis of Swimming Velocity in 12R and mixed color groups.

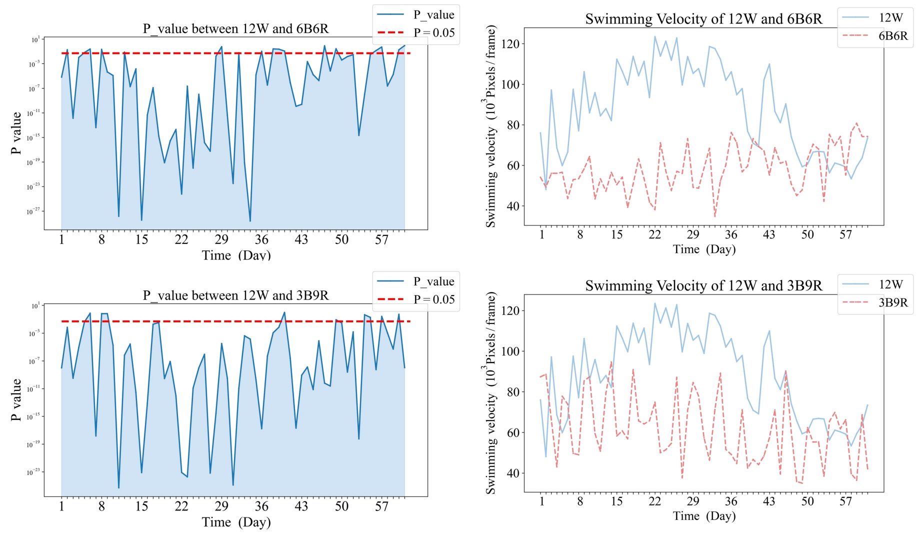

FIGURE 7
Comparative analysis of Swimming Velocity in 12W and mixed color groups.

the corresponding swimming velocity values for these groups compared to the 12W group across the same duration. A P-value below 0.05 is indicative of a statistically significant difference. The naming convention for treatments is derived from the arrangement of light colors within the 12-hour light period, denoted as xCyD: C color light for X hours, D color light for y hours. For instance, '3B9R' denotes blue light for 3 hours and 9 hours of red light, and '12B' denotes blue light for 12 hours.

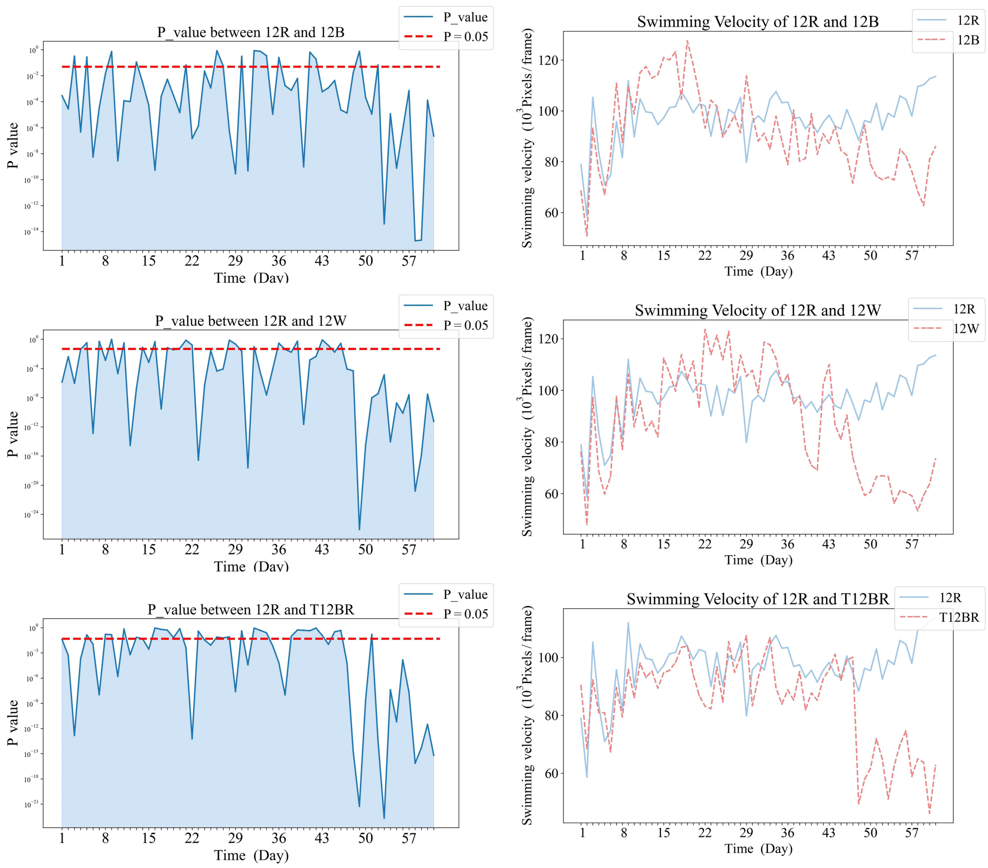

FIGURE 8
Comparative analysis of Swimming Velocity in 12R and pure color groups.

In Figure 7, panels (a1), and (b1) on the left display the daily variance in swimming velocity P-values between each mixed color group (6B6R, 3B9R) and the 12W group, over the initial eight-week period. Panels (a2), and (b2) on the right present the corresponding swimming velocity values for these groups compared to the 12W group across the same duration. A P-value below 0.05 is indicative of a statistically significant difference. The naming convention for treatments is derived from the arrangement of light colors within the 12-hour light period, denoted as xCyD: C color light for X hours, D color light for y hours. For instance, '3B9R' denotes blue light for 3 hours and 9 hours of red light, and '12B' denotes blue light for 12 hours.

In Figure 8, panels (a1), (b1), and (c1) on the left display the daily variance in swimming velocity P-values between each pure color group (12B, 12W, T12BR) and the 12R group, over the initial eight-week period. Panels (a2), (b2), and (c2) on the right present the corresponding swimming velocity values for these groups compared to the 12R group across the same duration. A P-value below 0.05 is indicative of a statistically significant difference. The naming convention for treatments is derived from the arrangement of light colors within the 12-hour light period, denoted as xCyD: C color light for X hours, D color light for y hours. For instance, '3B9R' denotes blue light for 3 hours and 9 hours of red light, and '12B' denotes blue light for 12 hours.

In Figure 6, panels (a1), and (b1) on the left display the daily variance in swimming velocity P-values between each mixed color group (6B6R, 3B9R) and the 12R group, over the initial eight-week period. Panels (a2), and (b2) on the right present the corresponding swimming velocity values for these groups compared to the 12R group across the same duration. A P-value below 0.05 is indicative of a statistically significant difference. The naming convention for treatments is derived from the arrangement of light colors within the 12-hour light period, denoted as xCyD: C color light for X hours, D color light for y hours. For instance, '3B9R' denotes blue light for 3 hours and 9 hours of red light, and '12B' denotes blue light for 12 hours.

4 Discussion

In this study, the behavioral responses of steelhead trout under various light color combinations (12B, 12R, 12W, 3B9R, T12BR, 6B6R) were investigated to identify the most suitable light environment for steelhead trout cultivation. Light color is a significant factor in aquatic environments, with numerous studies investigating its effects on the physiology and behavior of cultured fish species. For example, A study on the impacts of blue, red, green, white, and natural sunlight on goldfish (*Carassius auratus*) found that blue light facilitated better growth and lower ventilation frequencies (Noureldin et al., 2021). It was found that steelhead trout are not recommended to be cultured under blue light, as it was associated with a reduction in liver total lipids and plasma glucose levels, along with an increase in serotonergic and dopaminergic activity in the brain (Karakatsouli et al., 2007). Meanwhile, another study found that steelhead trout exhibited good growth performance under yellow light Heydarnejad et al., 2013, indicating that different light colors have varying effects on steelhead trout. In contrast, this study found that the fish in the 12R group exhibited the highest swimming velocity and the lowest swimming angular velocity and GIOU, with significant differences ($P < 0.05$) from the other groups. This indicates that fish under red light exhibit the highest amount of movement, preferring to swim over a large area rather than slowly wandering in a small radius. In the comparative analysis with the 12W group, it was found that only the 12R group exhibited significant differences with 12W in the end, indicating that red light has a greater behavioral impact on fish, as shown in Figures 5, 7.

Previous research has also discovered that steelhead trout exhibit an avoidance behavior towards red light (Luchiari and Pirhonen, 2008). The unique behavioral responses of steelhead trout under red light environment could potentially be explained by the physiological impacts of red light on fish. Several studies have found that under red light, steelhead trout exhibit hepatic stress, indicated by higher levels of alkaline phosphatase (ALP) ($P < 0.05$), total bilirubin (T-BIL) ($P < 0.05$), alanine aminotransferase (ALT), lysophosphatidic acid (LPA), and malondialdehyde (MDA) (Chen et al., 2022b); and hepatic oxidative stress, indicated by significantly decreased activities of catalase (CAT), glutathione peroxidase (GPx), glucose-6-phosphate dehydrogenase (G6PD), glutathione reductase (GR), glutathione S-transferase (GST), and acetylcholinesterase (AChE) in the liver (Guller et al., 2020).

The linkage between red light exposure and heightened fish activity may be a consequence of hepatic stress. As the liver plays a pivotal role in metabolic processes, its impaired function due to stress or oxidative damage may increase fish activity levels, likely as a stress response. Notably, this pattern of behavior under red light is not unique to steelhead trout. Similar hepatic stress responses have been observed in turbot (*Scophthalmus maximus*) (Wu et al., 2021), with juveniles exhibiting increased levels of Hsp70 mRNA and antioxidant enzymes when exposed to red and orange spectra. Moreover, goldfish (*Carassius auratus*) exposed to red light displayed reduced growth rates and heightened ventilation frequencies (Noureldin et al., 2021). Tiger puffer (*Takifugu rubripes*), too, exhibited avoidance behaviors to long-wavelength red light, with their movement rates correlating directly with light wavelength (Cai et al., 2023), echoing the increased activity levels noted in this study. These collective findings underscore the significant influence of red light on fish behavior and physiology. However, the specific mechanisms underpinning these effects, particularly how red light-induced hepatic stress and oxidative damage translate into altered behavior, warrant further investigation.

Beyond the impact of monochromatic light on fish, some studies have also investigated the effects of combinations of light colors on fish. For instance, a study on the influence of red, blue, green, white light, and three mixed light combinations on Nile tilapia (Elkadom et al., 2023), found that the growth performance under red/blue light was superior, whereas red light exhibited poorer growth performance, causing significant liver inflammation and damage, showing some similarities with this study. In contrast, this research investigates the effects of different light color combinations on the behavior of steelhead trout. It was discovered that the 3B9R group exhibited the lowest swimming velocity and highest GIOU, indicating lower activity level and exercise expenditure than other groups. It has also been found (Chen et al., 2022b) that the 3B9R environment showed the best growth performance and no hepatic stress. This may be attributable to the spectral distribution under 3B9R conditions more closely to the natural light spectrum in trout habitats, thereby avoiding excessive stimulation and consequently resulting in lower activity levels. The spectral distribution of light in water is influenced by climatic conditions, incident angles, depth, and water quality (Liu et al., 2016; Hou et al., 2019). Light penetration in water varies with its wavelength; red light has poorer penetration. Due to longer light paths at dawn and dusk compared to noon (Wetzel, 1983; Ruchin, 2020), blue light dominates at the same depth in the morning and evening, while red light prevails at noon, which is similar to the light color distribution under the 3B9R condition. Thereby, 3B9R was considered a suitable light environment for trout cultivation.

In the comparative analysis based on the 12W and 12R groups, distinct behavioral patterns were observed in the mixed light groups (6B6R, 3B9R) as opposed to the pure light groups (12B, 12W, 12R, T12BR). Specifically, the results reveal that in the comparisons with the 12W group (showed in Figures 5, 7), mixed light groups (6B6R, 3B9R) briefly showed significant differences with 12W during the mid-term phase of the experiment, which subsequently disappeared, unlike the pure light groups (12B, 12R, T12BR). In the comparisons with the 12R group (showed in Figures 8, 6), the mixed color groups (6B6R, 3B9R) established long-term stable significant differences earlier, emerging by the sixth week, whereas the pure color groups (12B, 12W, T12BR) formed these differences at a later stage, becoming apparent by the eighth week, indicating a two-week advancement for the former. The key difference between mixed and pure light groups lies in the fact that 12W, 12B, 12R, and T12BR environments all provided light with constant spectral distribution, that is, a pure color light without spectral change (T12BR offers blue and red light, akin to violet light). In contrast, the 3B9R and 6B6R environments' light colors both have a blue-red-blue change, which might have led to the unique behavioral patterns observed in the mixed light groups. Previous research on the biochemical characteristics (Chen et al., 2022a) has found that fish in 3B9R and 6B6R environments showed myocardial stress with higher levels of lactate dehydrogenase (LDH). This physiological response may be correlated with the unique behavioral patterns of the mixed light groups. It suggests that a gradual light color transition phase is needed to facilitate fish adaptation and environments like 3B9R, and 6B6R should be cautious of abrupt light color changes.

Environmental light impacts fish behavior through the nervous system. Usually, light is detected by the eyes and the signal is send to the brain for processing and interpretation. Some studies have delved into the effects of different environmental light colors on fish brain histology. For example, it was analyzed the brain histology of Nile tilapia under various light color environments and observed that red light exposure led to adverse histological changes in the brain (Elkadom et al., 2023), including significant neuronal degeneration, cerebral hemorrhaging, and pronounced lesions. Conversely, fish exposed to red and blue light showed excellent cytoarchitecture. This may offer a potential explanation from a neurological and histological perspective for the negative results seen in this experiment under the 12R red light environment and the positive effects under the 6B6R and 3B9R red and blue light combinations. Further exploration is needed to deeply understand the mechanisms behind how light color influences fish behavior through the nervous system and brain histology.

This study currently still has several limitations, such as the accuracy of the MOT methods that could be further improved due to the lack of high-quality fish datasets. The behavioral metrics used primarily pertain to swimming behaviors of fish, with a dearth of metrics directly reflecting fish group interactions. Moreover, the internal mechanisms through which light affects histological, physiological, and biochemical features by impacting their neurological systems, leading to behvaioral performance, remain unclear. In future research, more efficient and precise fish multiple object tracking methods could be employed, and additional fish behavior indicators could be proposed to comprehensively reflect both swimming behaviors and group interactions of fish. This would allow for a deeper investigation into the effects of light on the histological, physiological, and biochemical aspects of fish, and the underlying mechanisms of changes in fish behavior traits, thereby enhancing the understanding of fish behavior.

5 Conclusion

In the present study, the effects of various light color combinations on the behavior of juvenile steelhead trout were explored, integrating the analysis of how light color impacts on fish growth, physiology, and histology. Behavioral differences were discovered to emerge significantly earlier than physiological ones, manifesting as early as week eight. Notably, a distinct behavioral change was observed under the red light (12R) environment, characterized by increased swimming activity, which suggested the potential stress and discomfort experienced by trout. Consequently, the 12R environment appeared less favorable for long-term cultivation. Conversely, the mixed color light condition 3B9R, closely mimicked the natural light spectrum preferred by trout in their natural habitats, presenting a suitable light environment for steelhead trout cultivation where gradual light color transitions could help avoid stress. Existing brain histological analyses of fish also support these behavioral findings, particularly the adverse changes in brain histology observed under red light exposure contrasted sharply with more favorable outcomes under red and blue light conditions. Future research should continue to investigate the multifaceted effects of light on aquacultural species, employing more efficient and precise MOT methods for fish trajectory tracking. By utilizing more comprehensive behavioral metrics to quantify fish behavior, further studies can explore the potential relationships between fish behavior, physiology, and histology to identify more suitable aquacultural lighting environments, thereby enhancing fish welfare and productivity.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

All experimental methods of this study were performed under the Regulations of the Administration of Affairs Concerning Experimental Animals of China, as well as the Regulations of the Administration of Affairs Concerning Experimental Animals of Shandong Province. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

ZL: Data curation, Formal analysis, Investigation, Methodology, Software, Visualization, Writing – original draft. XC: Writing – review & editing, Investigation, Methodology. JH: Methodology, Software, Writing – review & editing. DA: Writing – review & editing. YZ: Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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