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DE MURCIA

Escuela
de Doctorado

TESIS DOCTORAL

Mejorando la evaluación del bienestar animal en peces: indicadores de estrés comportamental y ritmos diarios para una acuicultura de precisión

Improving welfare assessment in fish: behavioural stress indicators and daily rhythms for precision aquaculture

AUTOR/A Santiago Pintos

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“Improving welfare assessment in fish: behavioural stress indicators and daily rhythms for precision aquaculture”

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Abstract

In recent years, animal welfare has gained increased attention in both society and scientific research, driven by rising concerns about the ethical implications of farming practices. This concern has led to stricter welfare regulations, higher consumer demand for welfare-certified products, and broader social awareness of the living conditions of farmed animals. While significant progress has been made for terrestrial animals, the welfare of aquatic animals has received comparatively less attention and remains insufficiently addressed. Indeed, despite robust evidence supporting that fish can experience pain, stress, and discomfort — abilities once thought exclusive to mammals and birds — fish welfare is still not adequately ensured across farming practices. Consequently, there is mounting pressure on the aquaculture industry to improve the welfare of farmed fish. This requires a clearer understanding of what welfare entails for each farmed species, how it can be accurately assessed, and the implementation of tailored management practices that enhance animal welfare while ensuring the sustainability and productivity of aquaculture operations.

This doctoral thesis aimed to improve fish welfare assessment by developing non-invasive indicators of stress (i.e., behavioural indicators) and examining daily rhythms in stress responses. Furthermore, this research explored strategies to enhance fish welfare by refining management practices that consider the time of day, developmental stage, social interactions, and the impact of sustainable aquafeeds. Through a series of experiments, this thesis evaluated species-specific stress responses in model and farmed species, including Nile tilapia (*Oreochromis niloticus*), tench (*Tinca tinca*), koi carp (*Cyprinus carpio*), turbot (*Scophthalmus maximus*), gilthead seabream (*Sparus aurata*) and zebrafish (*Danio rerio*), offering novel insights into the distinct welfare needs and indicators of each species.

Chapter 1 focused on identifying behavioural stress indicators in economically important farmed species that have received little attention in welfare research. Our results highlighted species-specific markers of stress and the importance of developing tailored welfare protocols to ensure accurate assessments and improve animal welfare. This chapter also explored the role of conspecifics in reducing stress responses in farmed species. *Chapter 2* investigated the presence of daily rhythms in stress responses in both diurnal and nocturnal fish models and farmed species, revealing that stress responses vary not only with the time of day but also according to species-specific activity patterns. Besides, our results showed that daily modulation of stress depends on other factors such as the age of individuals and social environment. These findings supported the importance of considering the time of the day to minimise stress in fish and to ensure more accurate and precise welfare assessments.

Chapter 3 evaluated the effects of sustainable diets containing alternative feed sources, such as quinoa and spirulina, on Nile tilapia welfare. Although these diets showed potential benefits for stress resilience, issues with palatability and growth performance were also observed, highlighting the need for improved formulations and feeding strategies. *Chapter 4* extended the research to an industrial context by examining daily variations in water quality within a large-scale decoupled aquaponic system. This study suggested that aligning water management with daily water quality fluctuations could optimise resource use and improve system efficiency.

Lastly, the general discussion integrated findings from the experimental and industrial studies, supporting the use of behavioural stress indicators and behavioural tests to assess fish welfare. Moreover, it highlighted the necessity of species-specific management practices to minimise stress and improve fish living conditions. The discussion also emphasised the role of chronobiological, social, ontogenetic and nutritional factors in modulating fish stress responses, suggesting potential strategies to enhance fish welfare. Overall, this thesis offers valuable insights into fish welfare research by improving fish welfare assessments and exploring innovative strategies to enhance animal welfare. Thus, this thesis aims to support the development of more precise and ethically sound aquaculture practices.

Resumen

En los últimos años, el bienestar animal ha recibido mayor atención tanto en la sociedad como en la investigación científica, impulsado por preocupaciones crecientes acerca de las implicaciones éticas en las prácticas de cultivo animal. Esta inquietud ha conllevado a regulaciones más estrictas en materia de bienestar animal, a una mayor demanda por parte de los consumidores por productos certificados y a una conciencia social más amplia acerca de las condiciones de vida en las que los animales son criados en cautiverio. Aunque se han logrado avances significativos en animales terrestres, el bienestar de animales acuáticos ha recibido menor atención y sigue sin ser abordado adecuadamente. De hecho, a pesar de la robusta evidencia que respalda la capacidad de los peces para experimentar dolor, estrés y malestar —habilidades que se pensaban exclusivas de mamíferos y aves—, el bienestar de los peces no está aún garantizado en las prácticas de cultivo. En consecuencia, la acuicultura enfrenta una presión creciente para mejorar el bienestar de los peces cultivados. Esto exige una comprensión más clara de lo que implica el bienestar para cada una de las especies cultivadas, cómo éste debe ser evaluado de forma precisa y la implementación de prácticas de manejo adaptadas que optimicen el bienestar animal sin comprometer la sostenibilidad ni la productividad de las operaciones en acuicultura.

Esta tesis doctoral tuvo como objetivo mejorar la evaluación del bienestar animal en peces mediante el desarrollo de indicadores no invasivos de estrés (por ejemplo, indicadores comportamentales) y el análisis de los ritmos diarios en las respuestas al estrés. Además, se exploraron estrategias para potenciar el bienestar de los peces a través de la optimización de prácticas de manejo que tengan en cuenta la hora del día, la etapa del desarrollo, las interacciones sociales y el impacto de piensos acuícolas sostenibles. Mediante una serie de experimentos, esta tesis evaluó las respuestas especie-específicas

al estrés en especies modelo y de cultivo —incluyendo la tilapia del Nilo (*Oreochromis niloticus),* la tenca (*Tinca tinca*), la carpa koi (*Cyprinus carpio*), el rodaballo (*Scophthalmus maximus*), la dorada (*Sparus aurata*) y el pez cebra (*Danio rerio*)—, proporcionando así nuevos conocimientos sobre las distintas necesidades de bienestar e indicadores particulares de cada especie.

El Capítulo 1 se centró en identificar indicadores comportamentales de estrés en especies de cultivo de importancia económica que han recibido poca atención en la investigación científica sobre bienestar. Los resultados evidenciaron marcadores de estrés específicos para cada especie, resaltando la importancia de desarrollar protocolos de bienestar adaptados que aseguren evaluaciones precisas y mejoren la calidad de vida de los animales. Además, se exploró el rol de los individuos de la misma especie en la reducción de las respuestas al estrés en especies de cultivo. El Capítulo 2 investigó la presencia de ritmos diarios en las respuestas al estrés de especies modelo y de cultivo, tanto diurnas como nocturnas, revelando que estas respuestas varían no solo según la hora del día, sino también de acuerdo con los patrones de actividad particulares de cada especie. Asimismo, los resultados mostraron que la modulación diaria del estrés depende de otros factores, como la edad de los individuos y el entorno social. Estos hallazgos resaltan la importancia de considerar la hora del día a la hora de minimizar el estrés en peces y lograr evaluaciones de bienestar más precisas.

En el Capítulo 3 se evaluaron los efectos de dietas sostenibles que incorporan fuentes alternativas de alimentación, como la quinoa y la espirulina, sobre el bienestar de la tilapia del Nilo. Aunque estas dietas demostraron potenciales beneficios para la resiliencia al estrés, también se observaron dificultades en cuanto a la palatabilidad y el rendimiento en el crecimiento, lo que destaca la necesidad de mejorar las formulaciones y estrategias de alimentación. El Capítulo 4 trasladó la investigación a un contexto industrial al examinar las variaciones diarias en la calidad del agua dentro de un sistema acuapónico desacoplado a gran escala. Este estudio sugirió que alinear el manejo del agua con las fluctuaciones diarias de su calidad podría optimizar el uso de recursos y mejorar la eficiencia del sistema.

Finalmente, la discusión general integró los hallazgos de los estudios experimentales e industriales, apoyando el uso de indicadores comportamentales de estrés y pruebas de comportamiento para evaluar el bienestar de los peces, al tiempo que destacó la necesidad de prácticas de manejo específicas para cada especie, orientadas a minimizar el estrés y mejorar las condiciones de vida animal. Asimismo, se enfatizó el papel de factores cronobiológicos, sociales, ontogenéticos y nutricionales en la modulación de las respuestas al estrés, sugiriendo estrategias potenciales para mejorar el bienestar animal en peces. En conjunto, esta tesis ofrece valiosos conocimientos sobre la investigación del bienestar animal en peces, al mejorar las evaluaciones existentes y explorar estrategias innovadoras para potenciar el bienestar. Así, esta tesis pretende apoyar el desarrollo de prácticas más precisas y éticamente responsables en acuicultura.

Riassunto

Negli ultimi anni, il benessere animale ha ricevuto un'attenzione sempre maggiore sia nella società sia nell’ambito della ricerca scientifica, per via delle crescenti preoccupazioni riguardo le implicazioni etiche delle pratiche di allevamento. Questa si è tradotta in normative più severe riguardo il benessere animale, ma anche in una maggiore richiesta da parte dei consumatori di prodotti certificati in termini di benessere, portando ad una consapevolezza più ampia delle condizioni di vita e mantenimento degli animali in allevamento. Sebbene siano stati compiuti progressi significativi per gli animali terrestri, il benessere degli animali acquatici ha ricevuto meno attenzione e rimane ad oggi non adeguatamente indagato. Infatti, nonostante le numerose prove a sostegno del fatto che i pesci possono provare dolore, stress e disagio - capacità un tempo ritenute esclusive di mammiferi e uccelli - il benessere dei pesci non è ancora adeguatamente garantito nelle pratiche di allevamento. Di conseguenza, si è sviluppata una crescente pressione sull'industria dell'acquacoltura per migliorare il benessere dei pesci in allevamento. Ciò richiede una maggiore comprensione di cosa rappresenti il benessere per ogni specie allevata, di come possa essere valutato accuratamente e dell'implementazione di pratiche di gestione personalizzate che migliorino il benessere degli animali, garantendo al contempo la sostenibilità e la produttività in acquacoltura.

La presente tesi di dottorato mira a migliorare le tecniche di valutazione del benessere dei pesci sviluppando indicatori di stress non invasivi, come quelli comportamentali, ed esaminando i ritmi giornalieri delle risposte allo stress. Inoltre, questa ricerca ha esplorato diverse strategie per migliorare il benessere dei pesci perfezionando le pratiche di gestione tenendo in considerazione l'ora del giorno, lo stadio di sviluppo, le interazioni sociali e l'impatto dei mangimi sostenibili per l'acquacoltura. Tramite la realizzazione di diversi esperimenti, questa tesi ha valutato le risposte specifiche allo stress in specie

modello come lo zebrafish (*Danio rerio*) o di allevamento tra cui il rombo (*Scophthalmus maximus*), la tilapia del Nilo (*Oreochromis niloticus*), l'orata (*Sparus aurata*) e la tinca (*Tinca tinca*), offrendo nuove informazioni su specifici indicatori di benessere per ciascuna specie.

Il *Capitolo 1* si concentra sull'identificazione degli indicatori comportamentali di stress in specie di allevamento economicamente importanti che hanno ricevuto poca attenzione nella ricerca sul benessere. I risultati evidenziano l'esistenza di indicatori specifici di stress per ogni specie, ma anche l'importanza di sviluppare protocolli di benessere su misura per garantire valutazioni accurate e migliorare il benessere animale. Nel *Capitolo 2* si indaga la presenza di ritmi giornalieri nelle risposte allo stress in pesci diurni e notturni ed in specie di allevamento, rivelando che le risposte allo stress variano non solo in base all'ora del giorno, ma anche secondo i pattern di attività delle specie. Questi risultati sottolineano l'importanza di considerare l'ora del giorno per minimizzare lo stress nei pesci, così come per garantire valutazioni del benessere più accurate e precise.

Il *Capitolo 3* valuta gli effetti di diete sostenibili contenenti fonti alimentari alternative, come quinoa e spirulina, sul benessere della tilapia del Nilo. Sebbene queste diete abbiano mostrato potenziali benefici per la resilienza allo stress, sono emerse problematiche legate alla palatabilità e alle prestazioni di crescita, evidenziando la necessità di migliorare le formulazioni e le strategie alimentari. Il *Capitolo 4* estende la ricerca ad un contesto industriale esaminando le variazioni giornaliere della qualità dell'acqua all'interno di un sistema acquaponico disaccoppiato ("decoupled") su larga scala. Questo studio suggerisce che allineare le pratiche giornaliere di gestione dell'acqua con le fluttuazioni della qualità dell'acqua potrebbe ottimizzare l'uso delle risorse e migliorare l'efficienza del sistema.

Infine, la discussione generale integra i risultati degli studi sperimentali e industriali, supportando l'uso di indicatori comportamentali di stress e di test comportamentali per valutare il benessere dei pesci. Viene inoltre evidenziata la necessità di pratiche di gestione specie-specifiche per minimizzare lo stress e migliorare le condizioni di vita dei pesci in allevamento. La discussione sottolinea inoltre il ruolo dei fattori cronobiologici, sociali e nutrizionali nella modulazione delle risposte allo stress dei pesci, suggerendo potenziali strategie per miglioramento del welfare. Complessivamente, questa tesi offre preziosi risultati promuovendo valutazioni più accurate e strategie innovative per migliorare il benessere dei pesci in allevamento. Pertanto, questa tesi mira a sostenere lo sviluppo di pratiche di acquacoltura più precise ed eticamente valide.

INDEX

INDEX

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Preamble

In recent years, animal welfare awareness has gained increasing attention in society, consequently growing in relevance for scientific research and global policy. This concern has been translated into mounting pressure on the animal farming industry, including aquaculture, to prioritise the living conditions of farmed species. This implies providing more suitable environments for captive animals as well as a clear species-specific understanding of what welfare means and how it can be accurately assessed.

This doctoral thesis aimed to improve the welfare assessment of fish by investigating non-invasive indicators of stress, namely behavioural traits. Furthermore, it intended to determine how different management procedures can be optimised to minimise stress in fish. For example, by controlling the time of day at which fish are disturbed or considering the age of individuals and social interactions to design welfare standards.

This thesis starts with a summary of the **Doctoral framework** as part of the "*easyTRAIN: eco-innovative training network*". Then, a **General Introduction** provides an overview of the aquaculture and aquaponic industries along with current concerns regarding animal welfare. The importance of developing non-invasive stress indicators (i.e., behavioural traits) is highlighted, and the species evaluated in this thesis are listed and briefly described. Thereafter, the **Objectives** of the thesis are detailed.

The first experimental chapter (**Chapter 1**) aimed to describe behavioural stress responses of farmed species, such as turbot (*Scophthalmus maximus*), Nile tilapia (*Oreochromis niloticus*), gilthead seabream (*Sparus aurata*), and koi carp (*Cyprinus carpio*), and identify which behavioural traits may act as reliable indicators of stress. Additionally, this chapter examined the influence of social companions on fish stress responses, aiming to explore further strategies to enhance animal welfare. Our results supported different behavioural assays to assess stress responses across farmed species as well as distinct behavioural traits as potential stress indicators. However, our findings generally indicated interspecific variations in those behavioural variables sensitive to stress. Furthermore, our studies suggested the importance of social companions to attenuate stress responses and improve fish welfare.

The objective of **Chapter 2** was to determine whether behavioural stress response follows daily rhythmicity in fish. To this end, stress indicators were analysed in model (zebrafish *Danio rerio*) and farmed species (Nile tilapia *Oreochromis niloticus* and tench *Tinca tinca*) at different times of the day. Novel welfare insights were gained by observing the circadian modulation of behavioural stress responses. Furthermore, our results pointed out species-specific stress indicators that may be valuable to accurately assess stress responses depending on the time of day. Additionally, our findings suggest the importance of considering the age of individuals and the presence of social companions when establishing welfare standards and management practices.

In **Chapter 3**, the impact of sustainable and novel aquafeed diets on fish welfare was evaluated. Our results indicated that alternative ingredients such as spirulina (*Arthrospira platensis*) and quinoa (*Chenopodium quinoa*) improved welfare indicators in Nile tilapia (*Oreochromis niloticus*), generally suggesting reduced stress responses but also impaired growth performances. These findings suggest that while organic feeds can reduce stress responses in fish and support more sustainable aquaculture practices, they also underscore the need for further refinement in diet formulations to balance welfare benefits with optimal growth performance.

The last chapter (**Chapter 4**) was based on my industrial experience in the aquaponic farm Tilamur®. There, the daily variation of water quality parameters was studied in decoupled aquaponic systems. Our results revealed distinct dynamics of nitrites and nitrates between the hydroponic and recirculating aquaculture systems, offering valuable insights for optimising water management in aquaponics and supporting the welfare of farmed species.

The **General discussion** contains the main findings and insights gained from both the experimental and industrial chapters. It emphasises the value of behavioural stress indicators for assessing welfare in farmed species with limited knowledge of welfare indicators. Additionally, it discusses species-specific management strategies that can enhance welfare assessments and improve the living conditions of farmed fish species.

Finally, overall **Conclusions** are detailed in the last few paragraphs of this thesis.

Doctoral framework

The *easyTRAIN eco-innovative training network* is a research programme tailored for industrial doctorate students and founded by the European Union's Horizon 2020 under the Maria Skłodowska-Curie grant agreement Nº 956129. This doctoral framework has focused on promoting the sustainability and profitable development of the fish farming industry through effective cooperation and communication between the academic and private sectors. This project combined the expertise of 2 academic and 4 industrial partners from Spain, Portugal, the Netherlands and Italy, with complementary expertise in the fields of plant and fish farming, feed production, aquaculture engineering and microalgae biotechnology. Six doctoral students with different academic profiles were selected to accomplish different research and industrial objectives.

I was selected as early-stage researcher number five (ESR5), aiming to improve the assessment of fish welfare by developing and evaluating non-invasive indicators of stress. During my doctoral studies, I spent 9 months at the Laboratory of Animal Behaviour and Chronobiology of the University of Ferrara (Ferrara, Italy), 6 months at Sparos (Olhão, Portugal), 12 months at Tilamur (Murcia, Spain) and 9 months at the Laboratory of Animal Physiology of the University of Murcia (Murcia, Spain). These secondments are summarised in Figure 1.

Figure 1: Summary of the secondments completed by the early-stage researcher number five (ESR5). The academic partners were the University of Ferrara (Ferrara, Italy) and the University of Murcia (Murcia, Spain), and the industrial partners were Tilamur (Murcia, Spain) and Sparos (Faro, Portugal).

General introduction

1.1. Fish farming systems

1.1.1. Aquaculture

According to the United Nations, the world population is projected to reach 9.7 billion by 2050 (United Nations, 2019). If this estimation is accurate, this would lead to an unprecedented challenge for our society: feeding 1.7 billion more people than we do today. However, traditional food production methods such as fisheries will likely face numerous difficulties in meeting this future demand. The current decline in wild fish populations, stemming from years of overfishing, habitat degradation, climate change and pollution, has compromised not only the sustainability of conventional fishing practices but also the livelihoods of millions who depend on fisheries for sustenance (Brander *et al.*, 2007; Iudicello *et al.*, 2012, Kroetz *et al.*, 2022). In that context, farming aquatic organisms for commercial purposes has been extensively proposed as a promising solution to address this pressing social and environmental scenario.

Aquaculture can be defined as a fast-growing industry that encompasses breeding, rearing and harvesting seawater or freshwater species for economic purposes. This includes fish, shellfish, aquatic plants or even algae that can be reared in both land-based recirculating systems (Figure 2A) or open ponds and cages outdoors (Figure 2B). While the last FAO report supports that aquaculture already exceeds fisheries production (i.e., 51 % of total aquatic animal production), current projections suggest that this difference will continue to increase in the coming years (Figure 3; FAO, 2024). However, its further expansion requires transformative changes in policy, management, and innovation to cope effectively with the endangered environmental scenario and global demand for protein fish sources (Focardi *et al.*, 2005; FAO, 2022; Mitra and Sikder, 2023; Zhang *et al.*, 2024). This would involve more than simply increasing and scaling up global production, but rather a rigorous transition towards conscious and ethical practices that preserve aquatic ecosystem health and protect biodiversity. This objective can be gradually reached by improving water management, promoting sustainable sourcing of feed ingredients and enhancing welfare standards for captive animals (Boyd *et al.*, 2020; Browning, 2023; FAO, 2024; Tom et al., 2021).

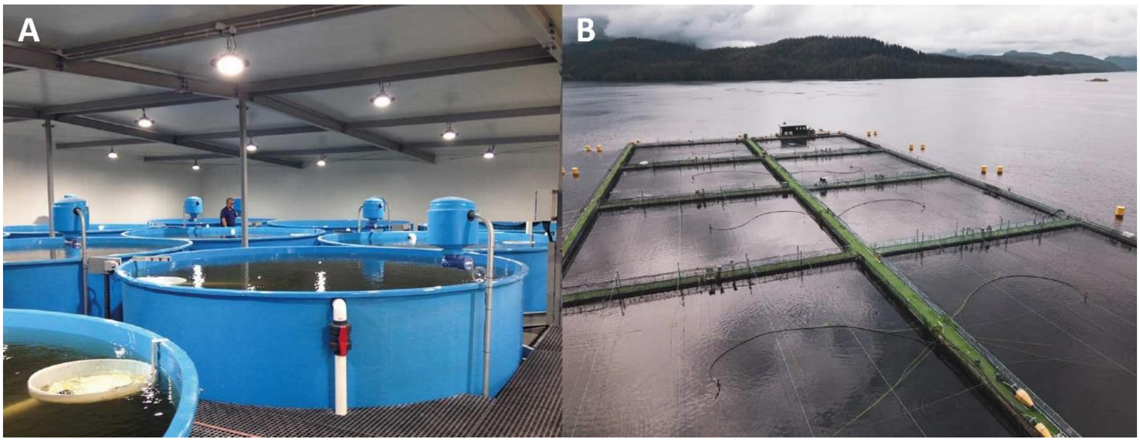

Figure 2: Representative pictures of indoor and outdoor fish farming systems. **A.** Land-based systems generally consist of closed-loop systems with continuous biofiltration and recirculation to maintain optimal water quality, known as recirculated aquaculture systems. Source: http://www.freshbydesing.com.au **B.** Outdoor systems rely on natural environmental factors for water circulation. Water flow and quality are influenced by factors such as wind, temperature and topography. Freshwater outdoor systems often have inflow and outflow channels to regulate water levels and exchange. Source: https://www.globalseafood.org.

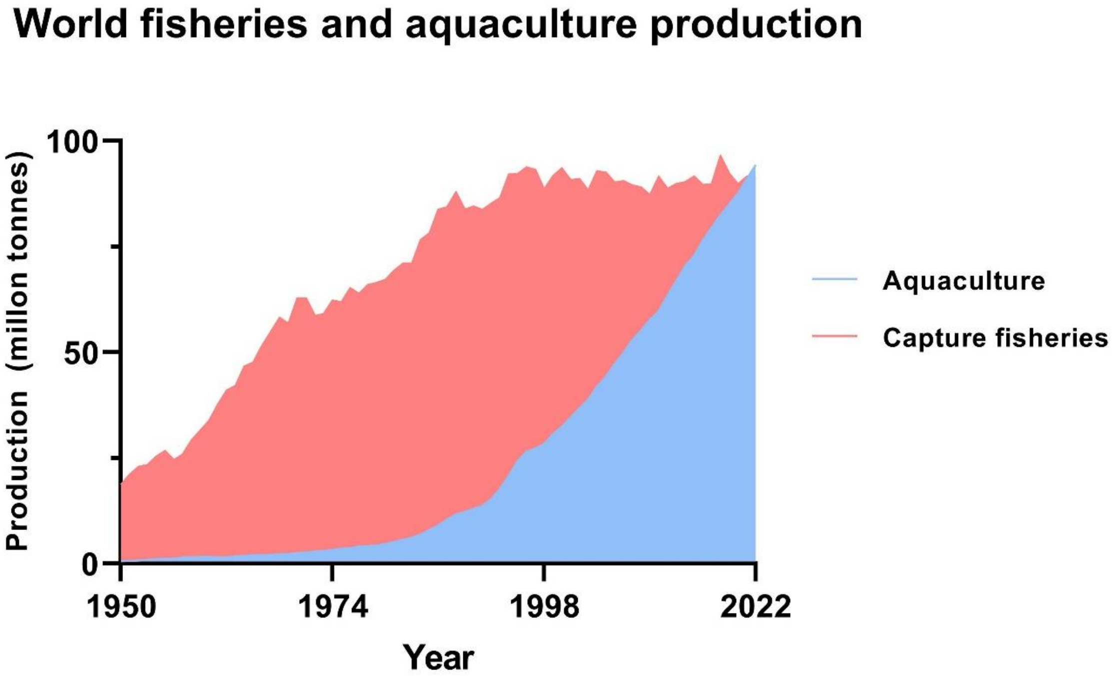

Figure 3: World capture fisheries (red) and aquaculture (blue) production from 1950 to 2022. Aquatic animals such as aquatic mammals, crocodiles, alligators, caimans, aquatic products (corals, pearls, shells and sponges) and algae were excluded from the data analysis. Data was extracted from the FishStatJ® Software for fishery statistical time series (FAO, 2024) and represented as live weight equivalent.

1.1.2. Aquaponics

As aquaculture continues to expand, other innovative food production methods, such as aquaponics, have also gained attention in recent years as sustainable food sources. Aquaponics integrates aquaculture within hydroponics production, creating a mutually beneficial system where fish wastes provide nutrients for plant growth, and plant crops contribute to filtering fish water (Figure 4) (Lennard and Goddek, 2019). This closed-loop system provides an important environmental advantage by optimising the use of water as well as dissolved nutrients that would otherwise be lost in traditional aquaculture effluents. Therefore, by utilising limited space and resources effectively, aquaponic systems could potentially contribute to the sustainable global production of aquatic animals. However, despite its remarkable benefits, this industry also faces challenges and limitations that need to be addressed for its widespread adoption.

One limitation of aquaponics is the initial investment required to set up these systems, which can be prohibitive for small-scale farmers or those with limited financial resources (Bosma *et al.*, 2017; Goddek *et al.*, 2015). Additionally, certifications that add market value to food products (e.g., organic labelling) are not available worldwide, particularly in regions like Europe. This may hinder the marketability of aquaponic products and limit the appeal to investors and producers (Kledal *et al.*, 2019). Furthermore, the restrictive range of species compatible with aquaponic farming also shapes the potential for diversification and scaling up production. Maintaining optimal water conditions for both fish and plant species can also be complex and requires careful monitoring and management to prevent issues such as nutrient deficiencies or toxicity (Estim *et al.*, 2020). Despite advancements in research and the development of decoupled aquaponic systems, where water exchange between fish and plants is not continuous (Goddek *et al.*, 2019), each species presents specific environmental requirements (i.e., temperature, lighting, pH, nitrogen compounds toxicity, among others) that may limit compatibility.

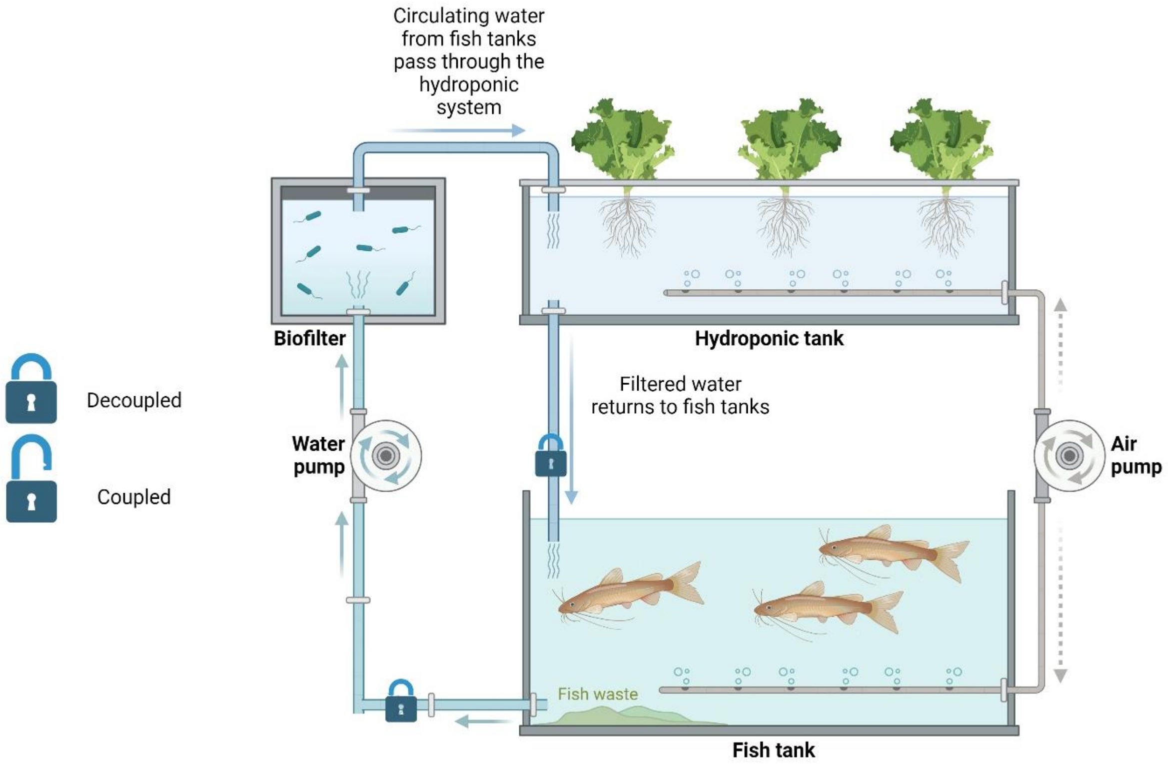

Figure 4: Illustrative scheme of aquaponic systems consisting of an aquaculture and a hydroponic component. Water circulates from the aquaculture to the hydroponic system, transporting aquatic animals' waste and nutrients to plants. Then, filtered water from hydroponic tanks returns to fish tanks. Aquaponics can operate in coupled configurations, where water exchange among components is continuous, or in decoupled, where the water flow between systems can be adjusted as needed.

1.2. Fish welfare

1.2.1. Animal welfare concept

Defining animal welfare has been — and still is — a difficult task, mainly due to the missing consensus on welfare criteria within the scientific community. This ambiguity is not surprising, given that the concept of animal welfare may encompass a broad range of ethological, physiological, and even ecological variables that are difficult to unify under a single definition (Broom, 1991). Consequently, numerous definitions of animal welfare have been proposed over the years, each capturing different aspects of what welfare could imply (Broom, 2011; Huntingford, 2008). Among them, three broad categories have been widely accepted by the scientific community, but each prioritises distinct animal outcomes as welfare indicators (Fraser, 2003; FSBI, 2002).

For instance, the *function-based* definition is set on the ability of animals to cope with the environment. This concept emphasises the importance of maintaining physical health and proper biological functioning, suggesting that welfare is determined by how well it can adapt and maintain homeostasis in the face of environmental challenges (Broom, 1991; Broom, 2000). From this perspective, welfare is closely linked to practical management practices that ensure survival, growth, and reproduction of the animal, often with a focus on minimising diseases, injuries or other physical threats. This approach has been particularly applied in intensive farming systems, where production efficiency is a primary concern, and animal welfare is often assessed in terms of health and productivity (Taylor, 1972; Fraser, 2003). Conversely, the *natural-based* approach considers the inherent biological nature of species and their necessity to display the full repertory of behaviours as they manifest in the wild. These behaviours include exploration, hunting, foraging or mating (Fraser, 2008, Hemsworth and Coleman, 2011; Mellor, 2015). It argues that restricting these behaviours, even if animals are physically healthy, can result in poor welfare outcomes (Hemsworth and Edwards, 2020). Distinctly, the *feeling-based* concept centres on the affective and mental state of individuals, aiming to minimise negative experiences such as pain, fear, and distress while promoting positive experiences (i.e., play, exercise, sleep, thermal comfort) (Duncan and Fraser, 1997; Yeates and Main, 2008). This definition relies on the assumption that animals have conscious feelings (Huntingford, 2008).

Nevertheless, given the lack of consensus about what welfare means for animals reared in captivity, animal welfare guidelines have been committed to identifying different conditions that must be accomplished if welfare is to be considered acceptable.

Therefore, welfare assessments have traditionally relied on widely accepted frameworks such as the Five Freedoms, which basically supports that animals should at least have freedom from (1) hunger and malnutrition, (2) discomfort, (3) injury and disease, (4) fear, pain and distress and (5) to express normal behaviour (FAWC, 2012). As animal cognition and behaviour research have advanced, newer models have emerged, from the *'Four Welfare Principles'* to the '*Five Provisions*' or the '*Five Domains Model*' (Broom, 2011; Mellor, 2016; Mellor, 2020). Thus, these frameworks collectively acknowledge that animal welfare extends beyond practical management to include the fulfilment of animals' biological needs and the consideration of their emotional and psychological well-being.

In aquaculture, where diverse species can be reared in a wide range of environmental conditions and systems, welfare assessment becomes even more complex. Therefore, systematic approaches integrating multiple welfare indicators- even if collected from diverged welfare definitions- are essential for a comprehensive analysis of fish health and welfare status (Broom, 1998). This has become one of the major challenges of the aquaculture industry over the last few years (Bergqvist and Gunnarsson, 2013; Browning, 2023; Martos-Sitcha *et al.*, 2020; Hvas *et al.*, 2021).

1.2.2. Welfare concerns in aquaculture

In recent years, fish welfare awareness has gained increasing attention in public perception and, thus, growing relevance for scientific research and farmers. This concern partly arises from the ongoing estimates suggesting that the number of individuals killed annually in aquaculture and fisheries likely exceeds the approximately 70 billion animals involved in terrestrial agriculture (FAO, 2022; Mood *et al.*, 2012; Mood *et al.*, 2023). Additionally, nowadays, it is widely accepted that fish can experience pain, stress and discomfort, cognitive abilities that were traditionally and exclusively assigned to mammals and birds (Sneddon, 2003a; Braithwaite, 2010). Collectively, these findings have shifted consumers' concerns towards welfare issues within fish products. Furthermore, this awareness has been gradually translated into premium prices for products labelled with animal welfare certification, increased attention to welfare issues in global policy agendas, and research efforts for improving the living conditions of farmed fish species (Brown and Dorey, 2019; Franks *et al.*, 2021; Saraiva *et al.*, 2019; Seibel *et al.*, 2020). Despite some advancements, the welfare of farmed animals is still largely overlooked within the aquaculture industry and is certainly not guaranteed across fish husbandries (Franks *et al.*, 2021; Mustapha, 2014).

Unlike terrestrial animal production, farmed fish species are either wild or recently domesticated animals (Teletchea *et al.*, 2014). This implies that most species farmed in modern aquaculture are not biologically adapted to life in captivity, giving rise to serious welfare risks when held captive (Franks *et al.*, 2021). Furthermore, the diversification of aquaculture production has rapidly succeeded over the last 40 years and, despite undoubtedly strengthening the economy and resilience of the industry, it has also compromised the living conditions of recently introduced species (Chandararathna *et al.*, 2021; FAO, 2022). The number of globally farmed species in aquaculture has increased from 73 in 1950 to over 700 in 2022 (FAO, 2024; Metian *et al.*, 2020; Sicuro, 2021). This diversified production exceeds by far those from terrestrial animals (Carral *et al.*, 2021).

Moreover, the lack of reports on the biology of non-traditional farmed species has directly contributed to inadequate welfare standards in aquaculture settings (Sánchez-Súarez *et al.*, 2020). For instance, there is a lack of research on welfare aspects for at least 56% of farmed aquatic species (Franks *et al.*, 2021; Wuertz *et al.*, 2023). Thereby, it is imperative to describe adequate welfare criteria and farming environments for each cultivated species to uphold optimal husbandry conditions in captivity.ç

In Europe, there has been a progressive development and enhancement of legal frameworks that protect the welfare of fish farmed for human purposes (Toni *et al.*, 2019). Since 2009, these regulations have been based on the principle that vertebrate animals should be treated as sentient beings, and their use should ultimately benefit human, animal, and environmental health (Toni *et al.*, 2019). However, despite the broad range of animal welfare guidelines available for farmed aquatic animals, the legal protections for fish are currently limited to basic and general principles that are often not applicable or even contradictory to their specific welfare needs. This is largely because EU legislation tends to apply the same standards to both terrestrial and aquatic animals (i.e., Council Directive 98/58/EC; Council Regulation EC Nº 1/2005; Council Regulation EC Nº 1099/2009). While additional frameworks such as the COE Recommendations (2005) and the OIE Aquatic Code offer some guidance, they establish only minimal protection standards and fail to address species-specific needs (Giménez-Candela, 2020). This highlights the urgent need for tailored research and welfare assessment that specifically addresses the varied and complex needs of the wide range of farmed species.

1.3. Biology of stress in fish

1.3.1. Fish stress response

Stress is a well-conserved and adaptive response in animals, including fish, that plays a crucial role in survival. Stress occurs when an individual perceives a threat or challenge to its homeostasis (i.e., stressor), exceeding the natural and regulatory capacity of an organism (Koolhaas *et al.*, 2011). This response is essential as it triggers a range of behavioural and physiological adjustments that enable animals to cope with immediate threats. For instance, stress can enhance alertness, improve physical performance, and temporarily suppress non-essential functions, allowing individuals to respond quickly to dangers such as predation, competition for resources, or sudden environmental changes (Boyce and Ellis, 2005; Ellis, 2006; Wingfield, 2013). While this response is adaptive and ultimately enhances the fitness of species, prolonged or repeated exposure to stress can significantly impair the health and welfare of individuals, even compromising their survival (Wiepkema and Koolhaas, 1993). This is a major concern in captive animals, such as those fish reared in aquaculture settings, since stressors are commonly present across daily management procedures (Conte, 2004; Huntingford, 2006; Huntingford, 2008). Consequently, modern farming environments provide evidence of poor welfare conditions throughout the entire life cycle of most farming species (Ashley, 2007; Browning, 2023; Saraiva *et al.*, 2019; Stien *et al.*, 2020b).

When a stressor is perceived by the central nervous system of fish, a cascade of hormones and molecules gives rise to what is known as the primary stress response. This first physiological response to a stressor is modulated by the Brain-Sympathetic-Chromaffin (BSC) axis and the hypothalamic-pituitary-interrenal (HPI) axis (Wendelaar, 1997). The activation of these axes leads to the release of stress hormones such as cortisol and catecholamines into the blood plasma. The secondary stress response occurs when stress hormones bind to receptors in target tissues, activating distinct metabolic pathways that modulate osmoregulation processes and stimulate energy mobilisation. This response includes cardiovascular and respiratory adjustments that allow fish to cope with the stressor (Rodnick and Planas, 2016; Schreck and Tort, 2016). If fish cannot overcome the imbalance caused by the stressor and recover their homeostasis, the stress becomes chronic and leads to the tertiary stress response. This stage is characterised by the increased energy demand associated with stress (i.e., allostatic load), where energy resources are redirected from essential physiological processes such as growth, reproduction and immune function to support fish stress coping mechanisms. Thereby, depending on the intensity and duration of the stressor, chronic stress can induce maladaptive consequences such as impaired development, abnormal behaviour, increased disease susceptibility, reduced growth and reproductive success (Iwama, 2007). Figure 5 summarises the stress response in fish.

In aquaculture, stressed fish not only experience compromised health and welfare but also exhibit reduced growth and increased incidence of diseases, ultimately affecting the overall productivity and profitability of aquaculture operations (Conte, 2004; Huntingford *et al.*, 2006). Indeed, recent studies have documented that stress can negatively impact fish meat quality, including its texture, flavour and nutritional composition (Boaru, 2022; Castro *et al.*, 2016; Daskalova, 2019; Silva Maciel *et al.*, 2014; Sigholt *et al.*, 1997). Therefore, achieving effective stress management in farmed species has become one of the major challenges in aquaculture. This is crucial for meeting ethical standards and ensuring the sustainability and economic success of fish farming practices.

Nevertheless, numerous stressors present in aquaculture are inherent to farming conditions and generally unavoidable throughout daily maintenance procedures. For instance, it is often necessary to transfer live fish between aquaculture facilities. This procedure has been shown to elicit stress-inducing responses as a consequence of loading, transport and discharge of live animals (King, 2009; Southgate, 2008). Furthermore, handling practices are also frequent in routine farming procedures and have directly been associated with increased cortisol levels, weight losses, increased oxygen consumption or physical abrasion (Barcellos *et al.*, 1999; Brydges *et al.*, 2009; Wedemeyer, 1996). Similarly, the isolation of naturally group-living species has been shown to increase cortisol levels, downregulate immune systems and promote abnormal behaviours (Brandão *et al.*, 2015; Waheed, 2023). Crowding, harvesting, noises or vaccination are among other critical and intrinsic practices commonly observed in farming environments that act as stressors for fish, threatening the welfare and survival of animals (Ashley, 2007). However, various strategies can be explored to diminish and/or mitigate the effects of these stressors under farming conditions.

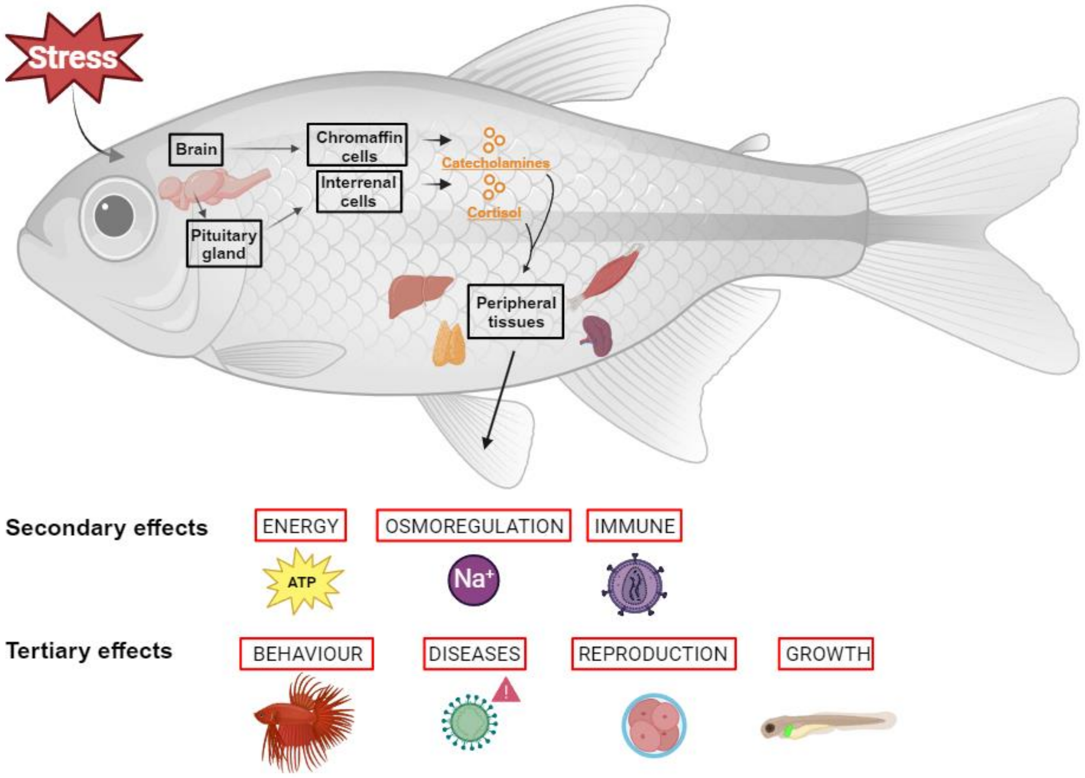

Figure 5: Illustrative scheme of the biology of stress in fish.

1.3.2. Circadian rhythms and stress

Fish, like many other organisms, have evolved complex biological clocks that allow them to adapt their internal processes to the cyclic changes occurring in their surrounding environment. These timekeeping mechanisms are synchronised to external cues, such as temperature or the light-dark cycles determined by the 24-hour Earth rotation on its axis (i.e., circadian clocks). Consequently, fish often exhibit daily variations in a wide range of biological processes that enhance their fitness by improving their ability to cope with environmental challenges (Steindal and Whitmore, 2019; Idda *et al.*, 2012; Zhadanova and Reebs, 2005).

Circadian rhythms are present in many physiological and behavioural processes of fish. For example, melatonin secretion varies according to the time of day, playing a key role in regulating sleep, activity cycles, and overall synchronisation of the internal clock with the external environment (Cowan *et al.*, 2017). Fish behaviour such as feeding and swimming activity also display variations throughout the day, commonly aligned with light cues and environmental factors such as food availability (Hurd *et al.*, 1998; Sánchez-Vázquez and Tabata, 1998; Spieler, 2001). Reproductive processes, including courtship, spawning, and gonadal development also follow circadian patterns (Hansen *et al.*, 1992; Oliveira *et al.*, 2010). Similarly, the stress response has also been found to vary with different likelihoods throughout the day in several fish species. However, this has been mostly documented by daily rhythms in physiological traits (López-Olmeda *et al.*, 2013; Manuel *et al.*, 2014; Vera *et al.*, 2014) and its implication for improving fish welfare has been generally overlooked (Gregory, 2022; Sánchez-Vázquez *et al.*, 2019).

Previous studies have shown that the activity of the HPI axis, which governs the primary stress response in teleost species, exhibits significant variations over the 24 h cycle. Indeed, evidence suggests this variation directly depends on the activity pattern of species. Thus, cortisol responses to stress peaked during the light phase in the nocturnal sole *Solea senegalensis* (López-Olmeda *et al.*, 2013). Conversely, cortisol stress responses were higher during the dark phase in the diurnal gilthead seabream *Sparus aurata* (Vera *et al.*, 2014). Similarly, the zebrafish *Danio rerio,* a diurnal species, has found stronger physiological stress responses during the dark phase than during the light phase (Manuel *et al.*, 2014). Hence, results indicated that fish stress response generally peaks during the resting phase of species and declines during the active phase (Sánchez-

Vázquez *et al.*, 2019). Accordingly, the extensive data indicating that the immune response also differs throughout the day in fish adds weight to the hypothesis that individuals are differently susceptible to stress depending on the time of day (Ellison *et al.*, 2021; Montero *et al.*, 2019). However, further research is needed to support this hypothesis across a broader range of species and welfare indicators.

1.3.3. Stress responsiveness across ontogeny

Other variables are however intrinsic to each species, but also showed a significant impact on fish stress responses. For instance, stress reactivity has been found to differ depending on the age of individuals, showing differences across ontogeny in various teleost species (Barcellos *et al.*, 2012; Cheyadmi *et al.*, 2022; Koakoski *et al.*, 2012). Reports on zebrafish showed that young individuals exhibit a higher degree of resiliency to stressors than adults, according to behavioural and physiological indicators of stress (Aponte and Rutherford, 2019; Tran *et al.*, 2014; Tran and Gerlai, 2015). Similarly, studies on the physiological stress response to heat stress in the starry flounder (*Platichthys stellatus*) suggested that heat tolerance is stronger in juveniles than in adults (Lee *et al.*, 2021). Age-related differences were also found in the time-course response of physiological indicators of the silver catfish (*Rhamdia quelen*), possibly due to a distinct maturation of the HPI axis (Barcellos *et al.*, 2012). Conversely, studies in other fish species such as the African killifish *Nothobranchius furzeri* or the mosquitofish *Gambusia holbrooki* herefore evidenced that younger fish exhibited stronger stress responses than adults (Mariën *et al.*, 2024; Polverino *et al.*, 2016). For this reason, it is essential to design species-specific welfare standards that consider the age of the individuals and/or the specific stages of aquaculture production (i.e., hatchery, nursery, and grow-out phases).

1.3.4. Role of conspecifics in attenuating stress

Moreover, the presence of conspecifics is another variable that can directly influence the welfare of fish, though its impact varies depending on the social nature of the species. For instance, in gregarious species, social interactions can provide benefits such as reduced stress and increased feeding efficiency, as these fish are naturally inclined to live in groups (Culbert *et al.*, 2019; Gilmour and Bard, 2022). Conversely, in solitary or territorial species, the presence of conspecifics can lead to increased stress due to competition, aggression, disruption of social hierarchies or even increased disease susceptibility (Myrberg and Thresher, 1974; Perrone *et al.*, 2019; Silva *et al.*, 2013). Other studies however supported that solitary species can also show welfare benefits since the presence of social companions (Dunlap *et al.*, 2021). Additionally, social hierarchies of individuals can further modulate these effects (Dara *et al.*, 2022; Ejike and Schreck, 1980; Fox *et al.*, 1997), making it crucial to consider the species-specific social structure when designing welfare protocols for aquaculture (Arechavala-López *et al.*, 2022; Zhang *et al.*, 2022).

1.3.5. Species-specificity of stress response

Despite the influence of the various external and internal factors discussed, it is well-documented that stress responses vary significantly across fish species (Balasch *et al.*, 2019; Coulter *et al.*, 2016; Fanouraki *et al.*, 2011). These differences are largely attributable to the distinct evolutionary and ecological backgrounds that shape the physiology and behaviour of each species (Birnie-Gauvin *et al.*, 2017; Eronen *et al.*, 2023; Pankhurst, 2011; Petitjean *et al.*, 2019; Schreck *et al.*, 2016). Thus, different fish species may exhibit varying responses to the same stressor (Fanouraki *et al.*, 2011; Pottinger, 2010). Therefore, understanding these species-specific stress responses is crucial when designing welfare protocols, as what may be stressful for one species could be tolerable or even beneficial for another. This knowledge contributes to effectively minimising stress and optimising the welfare of fish held captive.

1.3.6. Functional nutrition in stress management

Fish nutrition also plays a crucial role in improving fish welfare within the aquaculture industry. Studies have demonstrated that incorporating functional ingredients like probiotics, prebiotics, and immunostimulants in fish diets can improve welfare indicators such as growth rates, feed efficiency or disease resistance (Oliva-Teles, 2012). Accordingly, functional ingredients such as taurine or tryptophan have been widely adopted in aquaculture due to their stress-reducing properties (Azeredo *et al.*, 2019; Peixoto, 2024; Sampath *et al.*, 2020). However, in recent years, there has been a growing emphasis on the sustainability of functional diets. The industry focus has shifted towards incorporating sustainable and alternative ingredients that reduce reliance on traditional fish oil and fishmeal, which are not only limited resources but also have significant environmental impacts (Antonelli *et al.*, 2023; Boyd *et al.*, 2020). Therefore, to achieve optimal growth and health in farmed fish, researchers are exploring the properties of agricultural and animal by-products, plant-based proteins, and ingredients derived from circular economy practices. Notably, many of these alternative sources have also demonstrated promising effects in reducing stress levels and/or improving fish welfare (Mahmoud *et al.*, 2018; Mattos *et al.*, 2019; Sánchez-Velázquez *et al.*, 2024).

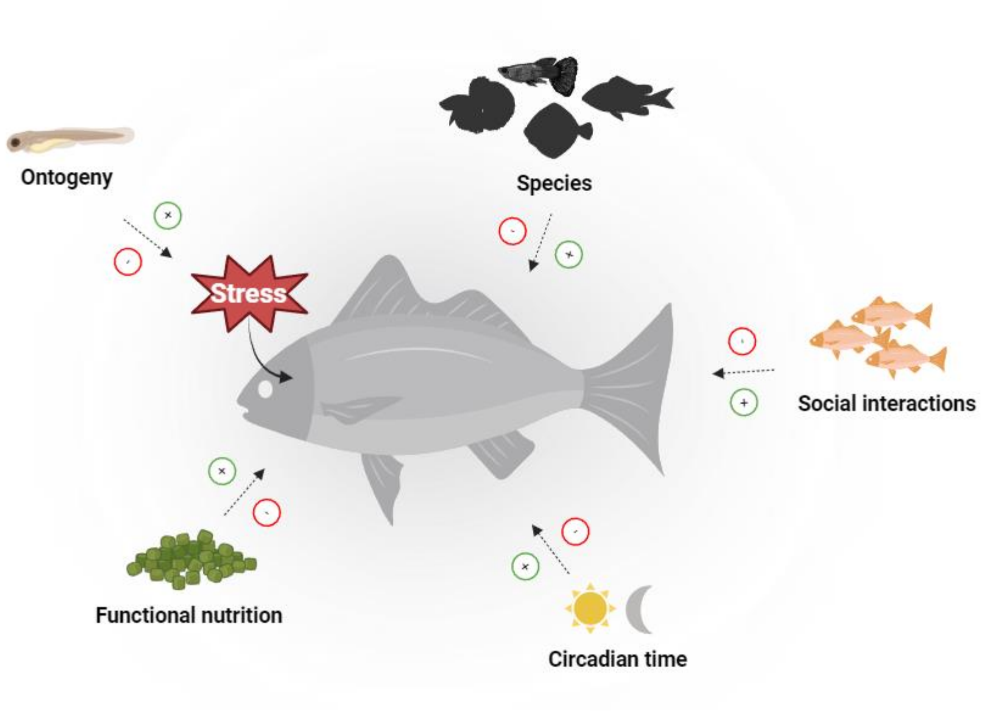

Figure 6: Illustrative scheme of external and internal factors influencing stress response in fish.

1.4. The assessment of fish welfare

1.4.1. Welfare indicators

As in other animals reared captive, biological, ecological, ethical and legal knowledge needs to be considered to assess welfare in fish husbandry. All these variables should lead to standardised and quantifiable parameters to objectively evaluate animal welfare (Barreto *et al.*, 2022; Nilsson *et al.*, 2018). In aquaculture, available reports of scientifically validated protocols and methods to assess welfare are largely missing (Huntingford, 2006; Tschirren *et al.*, 2021). Although in recent years welfare standards have been improved in model species (i.e., zebrafish *Danio rerio*; Lee *et al.*, 2022; Stevens *et al.*, 2021) as well as in traditionally and economically farmed species (i.e., Atlantic salmon *Salmo salar*; Cao *et al.*, 2017; Pettersen *et al.*, 2014), the broad range of current farmed species highlights an urgent need for further research in developing species-specific welfare assessment protocols and standards (Browning, 2023; Franks *et al.*, 2021; Giménez-Candela, 2020).

Many indicators have been proposed to assess fish welfare, from cortisol and glucose plasma levels to cellular and tissular oxidative stress status, the expression of key genes involved in the stress axis (i.e., HPI) and the incidence of morpho-anatomical abnormalities (Barreto *et al.*, 2022; Maria-Poli *et al.*, 2009; Sopinka *et al.*, 2016). While cortisol plasma levels have become one of the primary biomarkers of stress in fish (Ellis *et al.*, 2012; Sadoul and Geffroy, 2019), modern aquaculture is deeply committed to developing non-invasive methods which do not expose fish to unnecessary stress or harm. Invasive techniques typically involve direct physical interaction with the fish, which can induce stress and discomfort to individuals. Although blood sampling methods can provide valuable insights into the welfare state of fish, it is known to be stressful for fish as it involves handling and potential pain, which can lead to physiological changes that may skew welfare assessments (Seibel *et al.*, 2021; Sloman *et al.*, 2019). Fin clipping is another technique commonly used for individual identification or research purposes that has been shown to cause pain and stress in fish (Roques *et al.*, 2010). Given this evidence, reports on non-invasive techniques, such as behavioural monitoring, have gained growing recognition in aquaculture research over the last few years (Barreto *et al.*, 2022; Carbajal *et al.*, 2019a; Carbajal *et al.*, 2019b; Cavallino *et al.*, 2023; Pedrazzani *et al.*, 2023; Wiese *et al.*, 2023). Figure 7 shows the number of articles published by year containing keywords such as “aquaculture”, “non-invasive”, and “fish behaviour”.

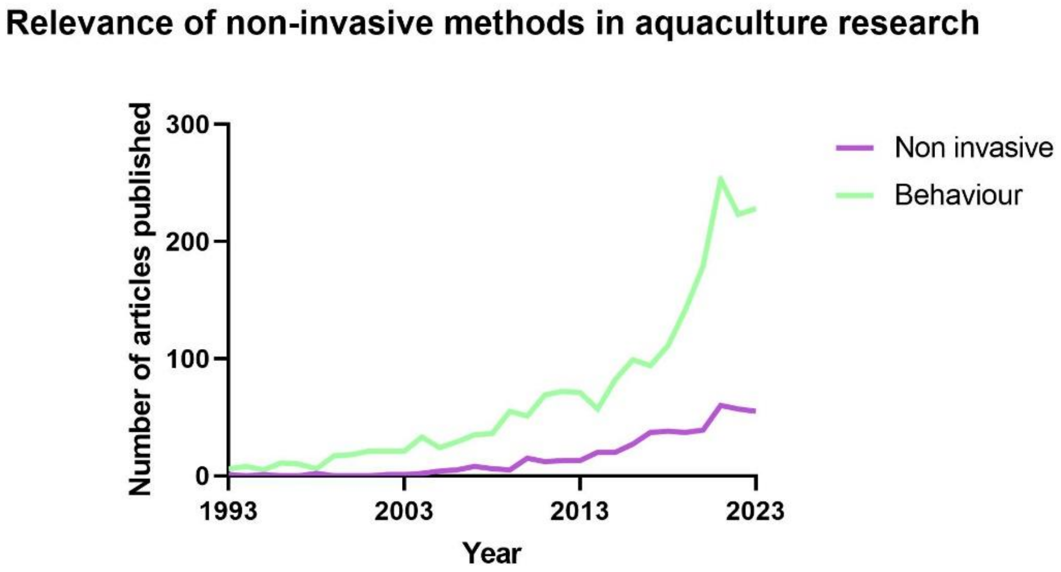

Figure 7: Number of articles published from 1993 to 2023 containing "Aquaculture" and "Fish behaviour" (green) or "Non-invasive" (purple) as keywords. Data was extracted from the Web of Science Core Collection database (Clarivate™).

1.4.2. Fish behaviour as a non-invasive proxy

The development of non-invasive behavioural welfare indicators has become a promising tool for assessing the welfare of fish. Behavioural indicators can be easily implemented, quickly observed, and provide early signs of potential welfare problems such as acute and chronic stress, illnesses or pathologies (Höglund *et al.*, 2022; Martins *et al.*, 2012; Relic *et al.*, 2010; Rousing *et al.*, 2001). Furthermore, video-recording fish behaviour in housing or novel environment conditions does not represent a harmful stimulus for individuals (Best *et al.*, 2008; Blaser and Vira, 2014; Hong and Zha, 2019). Therefore, behavioural proxies can provide reliable information about the welfare state of individuals under various experimental conditions without compromising fish health and welfare (Cavallino *et al.*, 2023; Kane *et al.*, 2004; Miller *et al.*, 2020; Scherer, 1992; Weirup *et al.*, 2022).

A wide range of behavioural parameters have been commonly used as welfare indicators in fish, including feed intake activity, swimming patterns, ventilation rates, food-anticipatory or anxiety-evoked responses (Alfonso *et al.*, 2020; Carbonara *et al.*, 2019; Huntingford *et al.*, 2006). Social behaviours such as shoal cohesion or agonistic encounters have also been incorporated as welfare state estimators in fish (Cavallino *et al.*, 2023; Hamilton *et al.*, 2021; Oldfield, 2011; Rosa *et al.*, 2020; Zhang *et al.*, 2021). Indeed, despite the differences between fish farming environments and natural habitats, natural fish behaviour is often used as a welfare proxy within aquaculture and is considered a positive welfare symptom of individuals (Martins *et al.*, 2012; Saraiva *et al.*, 2019). However, while nowadays it is well-accepted that behavioural outcome offers valuable insights into the welfare of fish, these proxies are not without limitations. For instance, fish behaviour can fluctuate throughout the day due to natural biological rhythms, affecting the interpretation of welfare indicators (Krylov *et al.*, 2021; Thoré *et al.*, 2021). Moreover, individual differences among fish within the same species (Castanheira *et al.*, 2017), as well as interspecific variations (Christensen and Persson, 1993; Katz *et al.*, 1999), can further complicate the assessment of welfare using behavioural proxies. Additionally, environmental factors such as water quality (i.e., temperature, pH, oxygen, nitrates or nitrites concentration) have also evidenced an impact on fish behaviour (Fontana *et al.*, 2021; Gray, 1983; Serra-Toro *et al.*, 2010; Vanderzwalmen *et al.*, 2021). Furthermore, the developmental stage of fish may also play a role in shaping behaviour and stress responses (Brown and Colgan, 1985; Ryer *et al.*, 1991). Therefore, when developing stress indicators and describing welfare standards, it is worth considering these factors comprehensively to ensure accurate and reliable assessment.

1.4.3. **Novel environment paradigms**

As in many other taxa (i.e., birds, rodents, insects, humans), fish display robust stress responses to novelty (Cachat *et al.*, 2010; Øverli *et al.*, 2006; Stewart *et al.*, 2010; Wong *et al.*, 2010). When exposed to a novel environment, fish generally exhibit a natural instinct to seek protection in an unfamiliar and potentially threatening environment (Cachat *et al.*, 2010; Egan *et al.*, 2009). This behavioural repertoire has been exploited by several behavioural paradigms for assessing acute anxiety-evoked responses and stress (Maximino *et al.*, 2010a). For instance, the open field (OF) test studies fish horizontal spatiotemporal exploration across a novel environment (Champagne *et al.*, 2010; Csányi and Gerlai, 1988; Figure 7). As in rodents, during the first 6-30 min of behavioural testing, fish typically avoid the centre of the OF arena and spend most of testing time in the outer part, near the walls (i.e., thigmotaxis behaviour) (Schnör *et al.*, 2012; Sataa *et al.*, 2020; Lucon-Xiccato *et al.*, 2020). Furthermore, they often reduce locomotion, although hyperactivity has also been reported as an anxiety response to the OF test in some fish species (Joseph *et al.*, 2022; López-Patiño *et al.*, 2008). Moreover, immobility states (i.e., freezing) can also be driven by novel environment expositions (Blaser *et al.*, 2010; Maximino *et al.*, 2010a).

Conceptually similar, the diving test (DT) analyses the vertical locomotion of fish in a novel arena (Figure 7). In this paradigm, fish often prefer to stay at the bottom of the tank (i.e., bottom-dwelling) and reduce activity during the first minutes of response (Blaser *et al.*, 2012; Tran and Gerlai, 2016). Erratic movements measured by angular velocity and/or turn angle of fish path have also been described as an anxiety proxy in the DT (Blaser *et al.*, 2010; Cachat *et al.*, 2011). Other behavioural paradigms evaluate stress response by measuring fish avoidance behaviours to light stimulus (i.e., light-dark preference test), novel objects or even novel odours (Blaser *et al.*, 2011; Lucon-Xiccato *et al.*, 2020; Sneddon *et al.*, 2003b).

Therefore, conducting these behavioural analyses can allow researchers to collect welfare information and compare stress responses under various experimental conditions.

This approach also helps to identify which behaviours are particularly sensitive to stress, facilitating the development of behavioural welfare indicators. However, anxiety-like behaviours have been found to differ among species (Maximino *et al.*, 2010b), highlighting the need for careful validation before applying these indicators across different fish species. While extensive research on anxiety-like behaviours has been conducted in teleost model species such as in zebrafish *D. rerio*, guppy *Poecilia reticulata* or medaka *Oryzias latipes*, there remains a notable gap in the behavioural biology of farmed species and thereby in the validation of suitable behavioural stress markers (Boissy *et al.*, 2007; Martins *et al.*, 2012). Despite their well-established and widespread use in various research fields such as biomedicine (Soares *et al.*, 2018; Zhang *et al.*, 2020), pharmacology (Abreu *et al.*, 2021; Huerta *et al.*, 2016) or toxicology (Dutra-Costa *et al.*, 2020; Thoré *et al.*, 2021), these behavioural tests have generally been overlooked in aquaculture studies. However, in recent years, there has been a growing recognition of their value in assessing fish welfare, leading to an increasing number of studies applying these tests to economically important farmed species (Alfonso *et al.*, 2020; Barreto *et al.*, 2011; Ibarra-Zatarain *et al.*, 2016; Vasconcelos *et al.*, 2023; Zhang *et al.*, 2023). Indeed, studying how fish respond and thrive in new environments should be prioritised to ensure the success of new aquaculture production systems and technologies (Lazado *et al.*, 2022) but also for restocking purposes (Gro and Braithwaite, 2006).

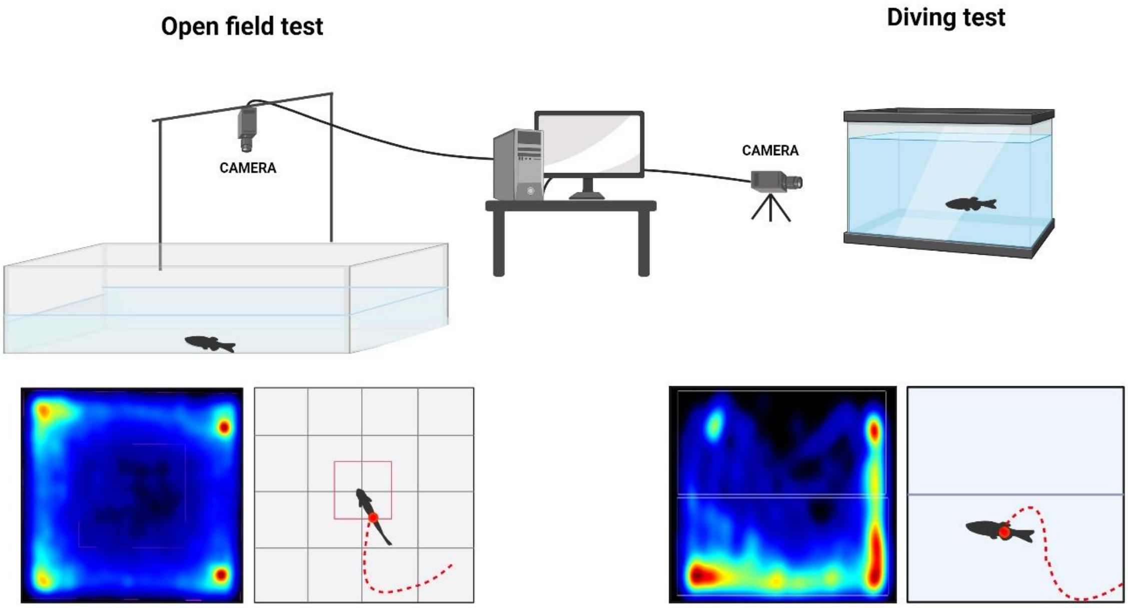

Figure 8: Illustrative scheme of two of the most conducted novel environment paradigms to assess behavioural stress response in fish, such as the Open field and the Diving tests. In both tests, fish behaviour is recorded by video cameras for 6-30 minutes and then analysed by computational tracking software. Anxiety-like behaviours such as the fish position over testing time are quantified to assess acute and/or chronic stress responses.

1.5. Species in focus

1.5.1. Zebrafish (*Danio rerio*)

The zebrafish (*Danio rerio*) is a freshwater species from the Cyprinidae family, native to the Ganges and Brahmaputra River basins in north-eastern India, Bangladesh, and Nepal. This social species typically inhabits shallow, slow-moving waters rich in aquatic vegetation (Spence *et al.*, 2008). Over recent decades, the zebrafish has emerged as a vertebrate model organism in numerous research fields, including genetics, developmental biology, neuroscience and biomedicine (Spence *et al.*, 2008, Vascotto *et al.*, 1997; Grunwald and Eisen, 2002; Cross *et al.*, 2003; Amsterdam and Hopkins, 2006). Its advantages as a model species include small size, high fecundity, translucent embryo, short life-spawn and genetic similarities with humans (Kimmel *et al.*, 1995; Spence *et al.*, 2008). Moreover, this species tolerates a wide range of environmental conditions and water quality that make it relatively easy to maintain in the laboratory (Spence *et al.*, 2008). For these advantages, zebrafish has also been proposed as a model species for aquaculture research (Dahm and Geisler, 2006; Lee-Estevez *et al.*, 2018; Ribas and Piferrer, 2014).

Regarding zebrafish behaviour, this species displays strong group-living behaviours and preferences for social interactions along with marked social hierarchies and agonistic behaviours in the wild as well as in laboratory settings (Filby *et al.*, 2010; Gerlai, 2014; Paull *et al.*, 2010; Schneider, 2011; Scerbina *et al.*, 2012; Spence *et al.*, 2008; Spence and Smith, 2005; Suriyampola *et al.*, 2016). Furthermore, zebrafish individuals typically exhibit diurnal activity patterns that are synchronised with light/dark cycles and regular feeding cycles (López-Olmeda *et al.*, 2010; Zhdanova and Reebs, 2005). Some studies have reported that a first activity peak occurs after the light is on, and two further peaks occur in the early afternoon and the last hour of the light phase (Baganz *et al.*, 2005; Plaut, 2000).

Physiological and molecular responses to stress have been extensively studied in zebrafish, providing valuable insights into the stress physiology of teleost species (Clark *et al.*, 2011; Pavlidis *et al.*, 2013; Pavlidis *et al.*, 2015). Moreover, several behavioural traits have been well-established as stress indicators of environmental stressors in this species (Tudorache *et al.*, 2015; Wong *et al.*, 2012). Thus, the zebrafish has certainly become a model species for behavioural research in recent years (Bonan and Norton, 2015; Steenbergen *et al.*, 2011; Subbiah and Kar, 2013). This is mainly due to its well-described behavioural repertory (i.e., from anxiety-like responses to shoaling behaviour, predator avoidance, learning and memory tasks), highly conserved neural circuitry related to anxiety phenotypes and well-established underlying mechanisms (Hong and Zha, 2019; Lang *et al.*, 2000).

1.5.2. **Nile tilapia (*Oreochromis niloticus*)**

Nile tilapia is one of the most widely farmed fish species globally, particularly in regions with tropical and subtropical climates (FAO, 2024; Nobrega *et al.*, 2017). Originating from the Nile River basin in Africa, Nile tilapia has been extensively domesticated and bred for aquaculture purposes due to its rapid growth rate, adaptability to various environmental conditions, and robustness against diseases (El-Sayed and Fitzsimmons, 2023; Fabrice *et al.*, 2018).

Nile tilapia (*Oreochromis niloticus*) has generally been described as a diurnal species, with activity primarily occurring during daylight hours (Fortes-Silva *et al.*, 2010; Vera *et al.*, 2009). However, studies have also documented nocturnal and crepuscular behaviours, particularly related to foraging and reproductive activities, suggesting that their activity patterns may vary under different environmental conditions (Pratiwy and Kohbara, 2018; Vera *et al.*, 2009). In captivity, as with many other cichlid species, the aggressive nature of Nile tilapia poses significant challenges, including increased stress, injuries, and reduced growth rates, all of which can negatively impact productivity (Gonçalves *et al.*, 2019; Evans *et al.*, 2008). To mitigate these welfare risks, various management practices have been explored, such as optimising stocking densities, providing environmental enrichment to minimise confrontations, implementing specific feeding strategies to reduce competition, and selectively breeding for less aggressive traits (Barreto *et al.*, 2011; Ramadan *et al.*, 2018; Fattah *et al.*, 2020; Fattah *et al.*, 2021; Khaw *et al.*, 2016). However, these efforts often prioritise production efficiency over the welfare of the fish, leading to a relative lack of research into comprehensive welfare indicators for Nile tilapia (Pedrazzani *et al.*, 2020).

1.5.3. Tench (*Tinca tinca*)

Tinca tinca, commonly known as tench, is a nocturnal freshwater fish species widely distributed across Europe and Asia, which was also introduced in America, South Africa and Australia (Gonzalez *et al.*, 2000; Herrero *et al.*, 2003; Rosa *et al.*, 1958). This species is one of the most valued species of the *Cyprinidae* family (Wang *et al.*, 2006) due to its essential role in natural environments as well as in local economy and research. Its bottom-dwelling nature contributes to recirculating nutrients and minerals that are deposited on the bottom and helps in reducing eutrophication (Dulski *et al.*, 2020). Furthermore, in Europe, tench is used as a food source, an ornamental fish species, and for angling purposes (Svobodova and Kolarova, 2004). In turn, tench farming has considerably grown in recent years since consumer demand has increased, mainly due to its meat flavour and relatively low price (Dulski *et al.*, 2020). While projections support that this species will be reared in RAS in the following years (Demska-Zakes *et al.*, 2012), its farming has been primarily done in farm ponds by monoculture production or alongside common carp *Cyprinus carpio* farming.

Despite its ecological significance and commercial importance, tench has received comparatively less attention in scientific research than other freshwater species. Considering the lack of reports on its welfare and stress indicators, further research has to be conducted to ensure optimal husbandry practices and promote the well-being of the species.

1.5.4. Turbot (*Scophthalmus maximus*)

The turbot *Scophthalmus maximus* is a marine species of flatfish belonging to the *Scophthalmidae* family, widely distributed in coastal waters of the North Atlantic, from the North Sea to the Mediterranean Sea (Blanquer *et al.*, 1992; Nielsen, 1986; Aneer and Westin, 1990). In recent years, this flatfish species has become highly valued in aquaculture and commercial fishing due to its delicate flesh and high gastronomic value (Dong *et al.*, 2018; Fernández-González *et al.*, 2021). However, despite its economic importance and potential for aquaculture, there are few reports focusing on turbot welfare (Bonaldo *et al.*, 2015; Liu *et al.*, 2019; Morzel *et al.*, 2003), possibly due to the lack of knowledge about its ecology and behaviour. This is similar to that reported for other flatfish species reared in captivity, commonly cultured in sub-optimal husbandry conditions that lead to poor welfare (Arechavala-López *et al.*, 2022).

1.5.5. Seabream (*Sparus aurata*)

The gilthead seabream *Sparus aurata* is a marine fish species native to the coastal waters of the Mediterranean and the north-eastern Atlantic Ocean (Rossi *et al.*, 2006). This species has become essential for European aquaculture due to its meat flavour and quality, rapid growth, tolerance to a wide range of environmental conditions, and adaptability to captivity. Intensive farming systems such as ponds and sea cages are commonly used to rear gilthead seabream in controlled conditions, enabling efficient production and stocking densities. Nevertheless, there are challenges within its culture such as disease susceptibility, dietary requirements, and welfare considerations (Dara *et al.*, 2023; Mhalhel *et al.*, 2023; Paperna, 1984; Porcino and Genovese, 2022; Roncarati *et al.*, 2006).

1.5.6. Koi carp (*Cyprinus carpio*)

Alongside Nile tilapia, the common carp is one of the most farmed species worldwide (FAO, 2022). This cyprinid species native to Europe and Asia has been praised for its resilience, adaptability, and fast growth. As one of the earliest domesticated fish species, carp have been selectively bred over centuries to enhance desirable traits such as growth rate, disease resistance and flesh quality (Li and Moyle, 1993; Lever, 1996; Koehn, 2004; Mahboob *et al.*, 2015). Furthermore, the ornamental variant of common carp, known as 'koi,' holds significant economic importance in the ornamental fish trade, contributing to the popularity of carp cultivation in aquaculture worldwide (Xu *et al.*, 2014).

C. carpio can generally be raised in distinct aquaculture systems, including ponds, raceways or recirculating aquaculture systems. Their ability to thrive in a wide range of environmental conditions makes them suitable for aquaculture operations in diverse geographical regions (Soltani *et al.*, 2010; Manjappa *et al.*, 2011; Rahman, 2015). However, this feature has also contributed to becoming an invasive species worldwide (Badiou *et al.*, 2011). Similarly to Nile tilapia, despite being one of the most produced fish in captivity, its welfare standards are poorly studied.

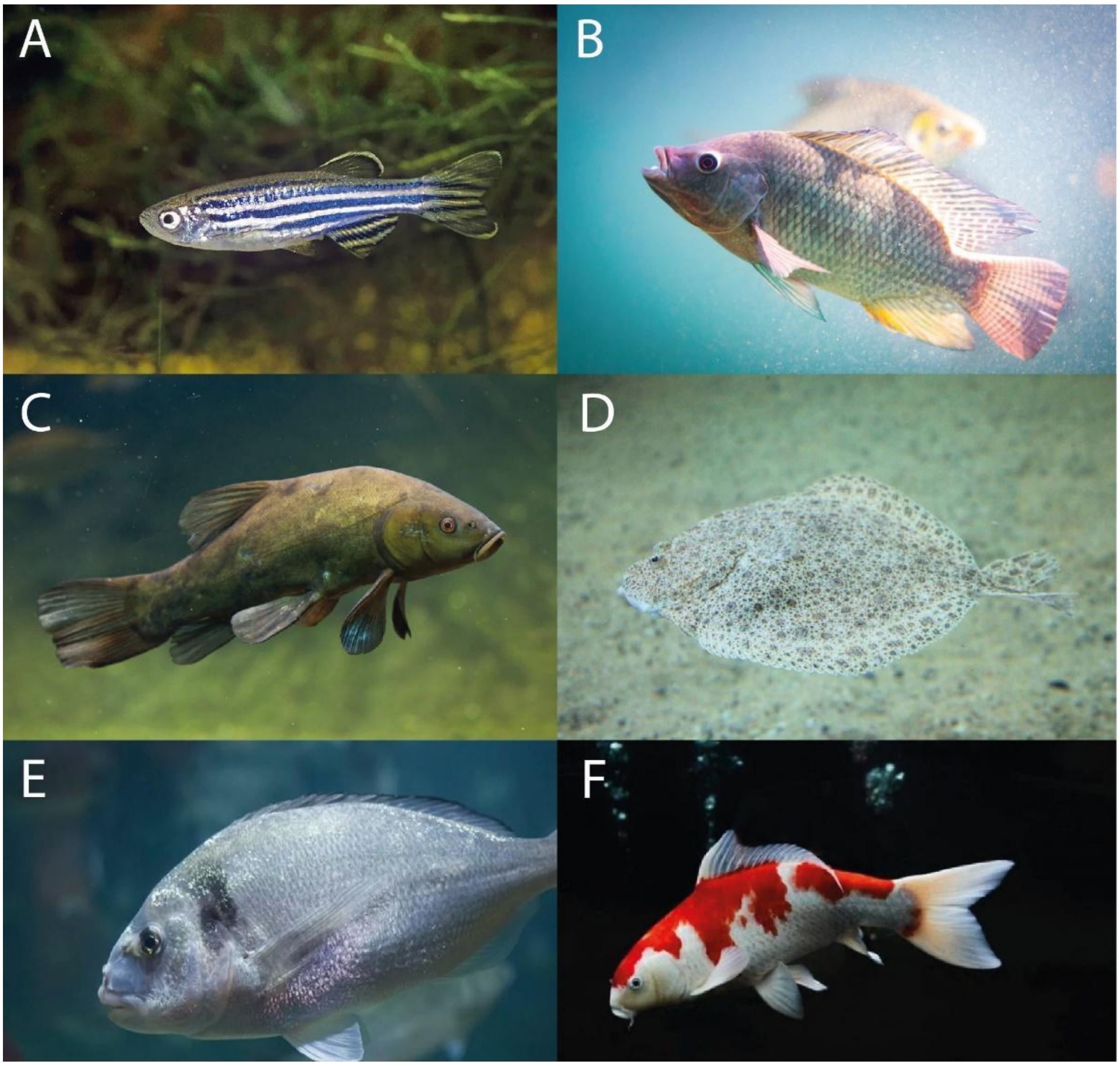

Figure 9: Studied species in this doctoral thesis: (A) Zebrafish *Danio rerio*. (B) Nile tilapia *Oreochromis niloticus*. (C) Tench *Tinca tinca*. (D) Turbot *Scophthalmus maximus*. (E) Glithead seabream *Sparus aurata* (F) Koi carp *Cyprinus carpio*.

Objectives

This doctoral thesis aimed to improve the welfare assessment of fish, with a particular focus on species farmed within aquaculture and aquaponic systems. To achieve this objective, this thesis investigated the potential of behavioural stress responses and behavioural traits as non-invasive indicators to assess animal welfare. Furthermore, this study intended to improve the living conditions of fish held captive by optimising management practices and procedures. To fulfil this general objective, the following specific objectives were proposed:

1. To develop behavioural stress indicators in farmed fish species (Nile tilapia, seabream, turbot, seabream, tench) with limited knowledge of welfare indicators.
2. To explore the impact of social companions on behavioural stress indicators in farmed species (Nile tilapia, seabream, koi carp, and tench).
3. To examine the impact of the time of day on the stress response of model (zebrafish) and farmed fish species (Nile tilapia and tench) with contrasting activity patterns (diurnal *versus* nocturnal).
4. To assess the impact of age on behavioural indicators of stress in zebrafish, throughout the 24-hour daily cycle.
5. To evaluate the impact of sustainable and alternative aquafeed diets on welfare indicators in Nile tilapia.
6. To describe daily variations in water quality parameters in large-scale aquaponic systems.

Chapter 1.

Developing behavioural stress indicators in farmed fish species

1. Behavioural assessment of turbot *Scophthalmus maximus* juveniles in novel environment paradigms: non-invasive welfare indicators

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Abstract

As aquaculture production grows to meet global nutrition needs, understanding the welfare of farmed fish has become increasingly important. The turbot (*Scophthalmus maximus*) is a flatfish teleost species with significant economic importance in European aquaculture. However, this species lacks comprehensive research to assess its welfare, especially in terms of behavioural indicators. Hence, this study investigated the stress response of turbot using three well-established behavioural assays to evaluate fish stress responses: the open field test, the diving test and the light-dark preference test. To this end, juvenile fish were individually subjected to behavioural testing according to two experimental groups: the stress group, where fish were acutely stressed (i.e., 2-minute confinement event) prior to behavioural testing; and the control group, where fish were directly subjected to the behavioural test. Contrary to expectations, turbot displayed anxiety-related behaviours opposite to those reported in model species. This was true for both diving and light-dark preference tests. Conversely, turbot did not display stereotyped stress behaviours in response to the open field test. Overall, these findings highlight the need for species-specific research to interpret welfare indicators in farmed species accurately. Furthermore, this underscores the potential of using novel environment paradigms as non-invasive tools for evaluating stress and welfare in aquaculture species, although further research is required to correlate behavioural traits with physiological stress markers.

Introduction

Aquaculture has become one of the most important activities for meeting global nutrition demands and food security. However, as this fast-growing industry expands, there is a growing recognition of the need to better address the living conditions of animals reared in captivity. Several fish studies have shown that poor living conditions promote chronic stress, reduce immune responses and even increase disease susceptibility (Daskalova, 2019; Refaey *et al.*, 2017; Tort, 2011). Furthermore, it is well-known that flesh quality and welfare issues are intrinsically linked in fish. For instance, inappropriate husbandry conditions can lead to lower meat quality, as reported in the flatfish sole *Solea senegalensis* and the rainbow trout *Oncorhynchus mykiss* (Ribas *et al.*, 2007; Robb *et al.*, 2000). Accordingly, harvesting stress or slaughter methods directly affect the organoleptic characteristics of fish (Matos *et al.*, 2010; Lefèvre *et al.*, 2008; Periago *et al.*, 2005), which is one of the major concerns of aquaculture product consumers. Therefore, collected evidence suggests that ensuring the welfare of farmed fish is crucial for the future sustainability and success of aquaculture practices.

In recent years, remarkable research efforts have been made to develop welfare indicators that do not compromise animal health and can be continuously and easily monitored (Carbajal *et al.*, 2019; Cavallino *et al.*, 2023; Pedrazzani *et al.*, 2023). These non-invasive indicators include physiological (e.g., skin mucus and waterborne cortisol), environmental (e.g., water quality), and behavioural (e.g., stress behaviour) parameters (Barreto *et al.*, 2022; Fernández-Alacid *et al.*, 2019; Palme *et al.*, 2012). In particular, behavioural traits have been gaining relevance in fish since they are easily implementable and generally effective in detecting early stress symptoms (Martins *et al.*, 2012). Nevertheless, developing suitable behavioural indicators of stress requires a thorough understanding of the species' behavioural biology and how they react to distinct stressors.

This knowledge has been shown to be crucial for accurately interpreting behavioural changes and for applying these indicators effectively (Huntingford and Adams, 2005; Maia *et al.*, 2024; Mesquita, 2011).

In the last few years, novel environment paradigms have become useful behavioural assays to evaluate stress response in fish across different research fields, including biomedicine, toxicology, neuroscience and even aquaculture (Alfonso *et al.*, 2019; Fulcher *et al.*, 2017; Hong and Zha, 2019; Tran and Gerlai, 2016). These tests exploit the behavioural response of fish in novel and potentially threatening environments to assess their stress levels. This is accomplished by measuring different anxiety-like indicators such as activity, freezing, erratic movements or space-use proxies such as thigmotaxis or bottom-dwelling behaviours (Collier *et al.*, 2017; Maximino *et al.*, 2010). However, although interspecific variations of stress response have been widely documented in fish (Maximino *et al.*, 2007; Lucon-Xiccato *et al.*, 2022; Pintos *et al.*, 2024), most of these studies have been carried out on model species such as the zebrafish *Danio rerio* (Collier *et al.*, 2017; Egan *et al.*, 2009; Maximino *et al.*, 2010).

The turbot (*Scophthalmus maximus*) is a teleost flatfish species of significant economic importance in aquaculture, especially in Europe. Turbot is valued for its rapid growth, high market demand, and adaptability to farming conditions (Fernández-González *et al.*, 2023). However, despite considerable recent advancements in turbot farming technologies and practices (Aramburu González, 2024; Fiorin *et al.*, 2024; Hoerterer *et al.*, 2022), there is a notable lack of studies on its behavioural biology and welfare indicators (EFSA, 2009; Liu *et al.*, 2019; Saraiva and Arechavala-López, 2019).

In this study, we evaluated the behavioural response of juvenile turbot to distinct well-established behavioural tests to assess stress responses in fish (Godwin *et al.*, 2012; Maximino *et al.*, 2010). To determine which behavioural traits were sensitive to stress, turbot were assigned to a stress (i.e., 2 minutes of confinement stress prior to the behavioural assay) or a control (i.e., directly exposed to the behavioural assay) group. Then, fish were individually subjected to the open field, diving or light-dark preference tests while different behavioural proxies were monitored. We hypothesised that 1) the confinement stress event elicits behavioural changes in turbot exposed to novel environment paradigms and 2) turbot anxiety-like behaviours differ from those reported in model species.

Material and methods

Experimental subjects

Turbot were obtained from a commercial hatchery at the end of the larval stage and then reared at SPAROS facilities (Olhão, Portugal) in recirculating aquaculture systems for one month at 18 ± 1 ºC in 8 L community tanks (10-12 fish/tank). Tanks were exposed to a 12:12 h light-dark photoperiod (6500 K LED; Superlight Technology Co. Ltd., Shenzhen, China) and were equipped with constant aeration and supplied with filtered saltwater from mechanical and biological filters. All tanks were kept barren to simulate the typical conditions of commercial facilities (Figure 1). Turbot were fed ad libitum 5 times per day and were approximately 30-45 days post fertilisation at the time of the experiment, reaching an average length of 2.86 ± 0.45 cm (mean ± standard deviation). The administered diet was manufactured by extrusion at SPAROS, mimicking a typical current commercial formulation used for turbot.

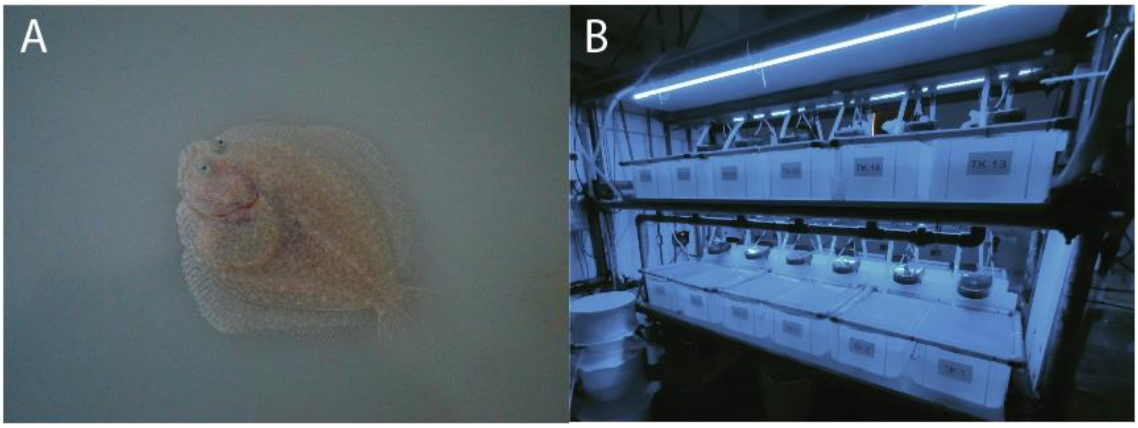

Figure 1: A. Picture of juvenile turbot used in this study. **B.** Recirculating aquaculture system containing turbot housing tanks.

Testing procedure

A total of ninety-six juvenile turbot were randomly assigned to the experimental groups (i.e., control or stress; n= 48 fish/group) and subsequently subjected to one of the following assays: 1) open field test 2) diving test or 3) light-dark preference test. Whereas turbot from the control group were directly exposed to the behavioural assay, turbot from the stress group were acutely stressed prior to the behavioural testing. The acute stressor consisted of a 2-minute confinement in a 250 mL chamber. Restraining and confinement events procedures have been shown to trigger physiological and behavioural stress responses in fish (Assad *et al.*, 2020; Höglund *et al.*, 2020; Huising *et al.*, 2004). This finally resulted in sixteen turbot analysed in each behavioural assay for each experimental group (n=16 fish/group/test).

All experimental subjects were fed 30 minutes prior to behavioural testing. Subjects were randomly selected from housing tanks and individually transported to the corresponding experimental arena using an opaque jar to minimise stress. After the release in the centre of the arena, turbot behaviour was recorded for 10 minutes for all behavioural trials. The water was changed between each trial to prevent exposure to the chemical cues produced by the previous experimental subject.

Experimental apparatus and behavioural parameters

All experimental arenas were illuminated from 1 m above with a white LED strip (6500 K; Superlight Technology Co. Ltd., Shenzhen, China). A full-HD camera (ELP USBFHD08S-MFV, Shenzhen Ailipu Technology Co. Ltd, Shenzhen, China) was used to record behavioural trials for 10 minutes. The camera was placed in different positions to record fish behaviour according to the corresponding behavioural assay. Videos were captured in 1920 × 1080 pixels of resolution and 30 frames per second.

After recording, all videos were analysed with computer software for automatic tracking (Ethovision XT®, Noldus Information Technology, Wageningen, The Netherlands). The software was used to collect behavioural traits commonly used to assess fish stress responses.

Open field test

The open field arena consisted of a white plastic and rectangular arena (25 × 25 × 15 cm) filled with 10 cm of filtered seawater. The experimental arena was empty, and the camera was placed 50 cm above the arena (Figure 2A).

Behavioural traits collected in open field trials included: thigmotaxis as the time spent in the edge of the arena (centre: 12.5 × 12.5 cm), which generally increases when fish are stressed (Champagne *et al.*, 2010); activity as the distance travelled across the arena, commonly used as a proxy of fish locomotion and anxiety (Levin *et al.*, 2007); and freezing as the time spent motionless with a speed lower than 0.5 cm/s, which is often linked to anxiety states (Egan *et al.*, 2009).

Diving test

The diving test arena consisted of a rectangular arena (15 × 6 × 20 cm) filled with 18 cm of filtered seawater. The experimental arena was empty, and its walls were opaque from behind and transparent from the front and sides. The camera was located 40 cm from the front of the tank (Figure 2B).

As in the open field test, freezing and activity behaviours were collected in the diving test. Additionally, the time spent in the lower half of the apparatus (i.e., bottom-dwelling) was measured in diving test trials. This behaviour is the main stress indicator in this behavioural assay and is expected to increase when fish are stressed (Blaser and Rosemberg, 2012; Cachat *et al.*, 2010; Maximino *et al.*, 2010).

Light-dark preference test

The light-dark preference arena consisted of a white plastic (40 × 20 × 15 cm) filled with 10 cm of filter seawater. This arena was half covered by a dark plastic lid from the sides above and the sides. This design divided the experimental arena in two differently illuminated zones (i.e., light and dark) (Figure 2C).

Behavioural traits collected in this assay also included activity and freezing. Furthermore, behavioural parameters such as the time spent and the activity in the light zone or the number of entries in each zone. These parameters are commonly measured to assess phototaxis behaviour and the preference/aversion of fish to distinct light stimulus (Maximino *et al.*, 2007; Maximino *et al.*, 2010).

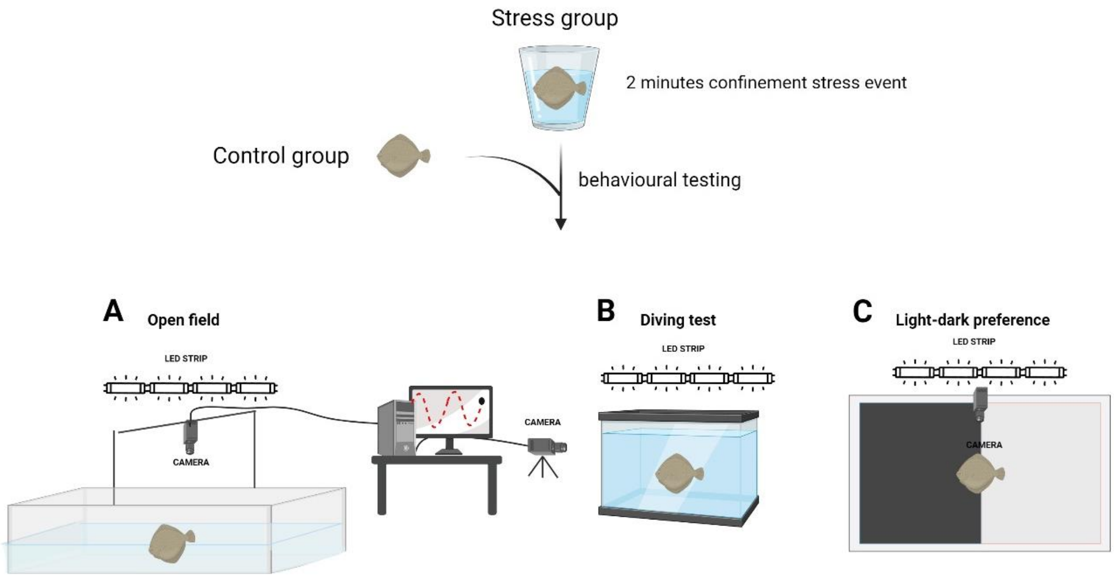

Figure 2: Illustrative scheme of the experimental design. Juvenile turbot from the control and stress group were subjected to different behavioural assays well-established to assess fish stress responses (n=16 fish/group/test): **A.** Open field test (arena: 25 × 25 × 15 cm). **B.** Diving test (arena: 15 × 6 × 20 cm). **C.** Light-dark preference test (arena: 40 × 20 × 15 cm). All experimental arenas were illuminated with a white LED strip, and video recordings were captured by a full-HD ELP camera. Fish behaviour was recorded for 10 minutes from the beginning of each trial and consecutively tracked by an automated fish tracking software (Ethovision XT®).

Statistical analysis

Statistical analyses were performed using R Statistical software version 4.0.1 (The R foundation for Statistical Computing Vienna Austria http://www.r-project.org) and all analyses were conducted by functions from the R base package. To analyse the effect of the experimental group (two levels: stress and control) on fish behaviour, two tailed *t*-tests were performed. The interquartile range (IQR) technique was used to detect outliers from each dataset. Moreover, one sample *t*-tests were performed to study if thigmotaxis, bottom dwelling and the time spent time in the light zone are driven by stress or associated with random movements across the experimental arena.

Results

Open field test

On average, turbot from the control group spent 65.61 ± 8.66 % (mean ± standard error) of testing time in the outer part of the open field arena. Similarly, turbot from the stress group spent an average of 67.09 ± 6.97 % in the outer part of the arena, indicating no significant differences in thigmotaxis between experimental groups (*t-test:* $t_{30} = 1.84$, $p = 0.07$). Furthermore, turbot from both experimental groups did not spend more time in the outer part of the open field arena than expected by random movements. This indicated that turbot did not display the fish's expected thigmotaxis response to the open field arena (*t-test*: control: $t_{15} = 1.08$, $p = 0.29$; stress: $t_{15} = 1.13$, $p = 0.27$; Figure 3A).

Moreover, turbot from the control group spent an average of 22.93 ± 8.52 % of testing time motionless. Similarly, turbot from the stress group spent 19.93 ± 9.71 % of testing time motionless. These values indicated no significant differences in freezing behaviour between experimental groups (*t-test:* $t_{30} = 0.23$, $p = 0.81$; Figure 3B).

Regarding activity behaviour, whereas turbot from the control group travelled an average distance of 403.5 ± 41.65 cm across the open field arena, turbot from the stress group travelled 494.6 ± 26.54 cm. Similarly to thigmotaxis and freezing, this behaviour did not evidence significant differences between experimental groups (*t-test:* $t_{30} = 0.71$, $p = 0.47$; Figure 3C).

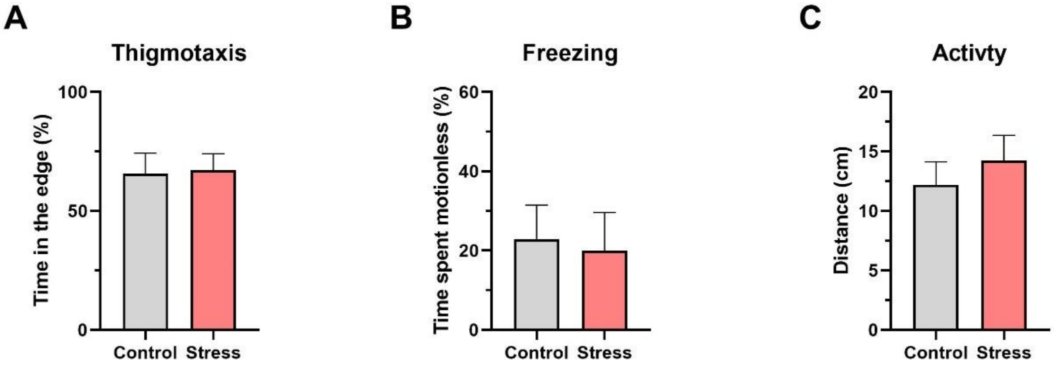

Figure 3: Turbot behaviour in the open field test according to the experimental group (control = grey; red = stress; n=16/group). **A.** Thigmotaxis. **B.** Freezing. **C.** Activity. Data are presented as mean ± standard error and asterisks indicate statistical differences between experimental groups.

Diving test

In the diving test, turbot from the control group spent an average of 32.78 ± 11.54 % of testing time in the lower half of the arena. This value did not differ from those expected for random movements across the apparatus (*t-test*: $t_{15} = 1.49$, $p = 0.15$). Conversely, turbot from the stress group spent 0.40 ± 0.25 % of testing time at the bottom of the diving test arena. This indicated that stressed turbot spent less time in the bottom than expected for random movements (*t-test*: $t_1 = 198.10$, $p = 0.15$). These differences in bottom-dwelling between experimental groups were significant, indicating increased time spent in the bottom of the arena in the control group than in the stress group (*t-test*: $t_{30} = 2.8$, $p < 0.01$; Figure 4A).

For activity behaviour, control turbot travelled an average distance of 6.80 ± 0.59 cm across the arena, and the stressed turbot was 5.48 ± 0.68 cm. No significant differences were registered between experimental groups for this behaviour (*t-test*: $t_{29} = 1.45$, $p = 0.15$; Figure 4B). Similarly, control and stressed turbot displayed freezing values (control:

17.54 ± 7.86 %; stress: 16.82 ± 6.68 %) that did not statistically differ between them (*t-test*: $t_{30} = 0.06$, $p = 0.94$; Figure 4C).

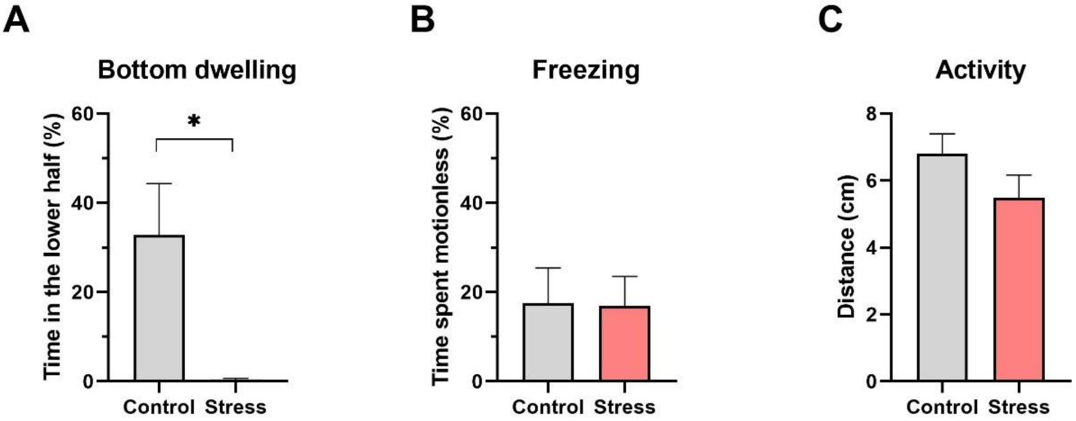

Figure 4: Turbot behaviour in the diving test according to the experimental group (control = grey; red = stress; n=16/group). **A.** Bottom dwelling. **B.** Freezing. **C.** Activity. Data are presented as mean ± standard error and asterisks indicate statistical differences between experimental groups.

Light-dark preference test

Both control and stressed turbot exhibited dark avoidance behaviour by spending 93.74 ± 2.09 % and 82.33 ± 8.91 % of the testing time in the light zone, correspondingly (*t-test:* control: $t_{14} = 20.84$, $p < 0.01$; stress: $t_{14} = 3.62$, $p < 0.01$). However, no significant differences were found between experimental groups for this behaviour (*t-test*: $t_{28} = 1.24$, $p = 0.22$; Figure 5A). Conversely, the number of entries to the dark and covered zone showed increased values in the control group when compared to the STR group (control: 4.92 ± 1.45 entries; 1.30 ± 0.32 entries; *t-test*: $t_{27} = 3.26$, $p < 0.01$; Figure 5B).

Furthermore, the average activity measured in the light zone was 10.29 ± 2.09 cm in the control group and 2.44 ± 1.05 cm in the stress group. This higher activity level in the control group was supported by significant differences between experimental groups (*t-test*: $t_{25} = 2.34$, $p = 0.02$; Figure 5C).

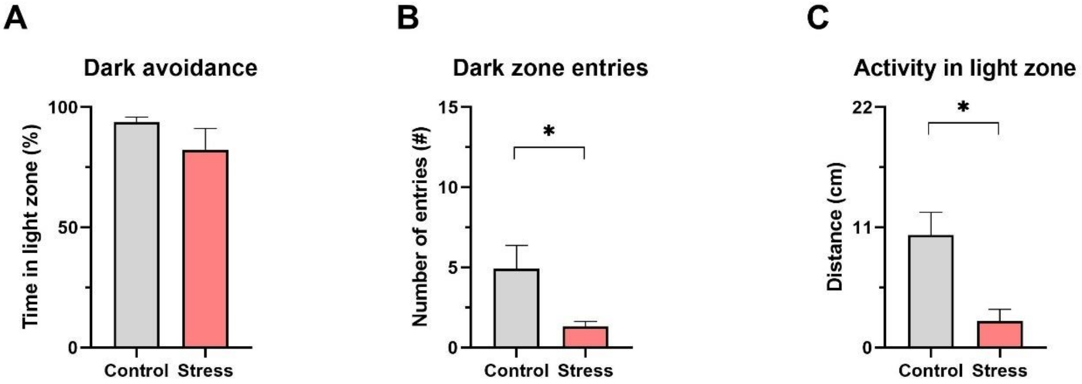

Figure 5: Turbot behaviour in the light-dark preference test according to the experimental group (control = grey; red = stress; n=16/group). **A.** Dark avoidance. **B.** Cover zone entries. **C.** Activity in the light zone. Data are presented as mean ± standard error and asterisks indicate statistical differences between experimental groups.

Discussion

This study examined for the first time the behavioural performance of turbot in a set of behavioural assays commonly used to assess stress responses in fish. Our findings revealed that juvenile turbot exhibited behavioural responses contrary to those previously reported in model teleost species. Critically, stressed turbot avoided the bottom of the tank and preferred dark zones over illuminated ones. Furthermore, our results generally support two out of three analysed behavioural paradigms to assess stress responses in this species. Moreover, we found that the confinement stress event used in our experiments significantly elicited behavioural changes in turbot, suggesting it can be an adequate stress challenge for the species.

In the open field test, turbot did not display the expected thigmotaxis response of fish to open areas (Norton, 2012; Richendrfer *et al.*, 2012; Watanabe *et al.*, 2021). This was true for both control and stressed groups, further suggesting that turbot do not exhibit this well-described and stereotyped fish behavioural response to novelty (Axling *et al.*, 2022; Schnörr *et al.*, 2012; Watanabe *et al.*, 2021). Furthermore, none of the variables analysed showed significant differences between stressed and control fish. This might suggest that this behavioural assay is not suitable for assessing stress in turbot. Alternatively, it is also possible that the experimental design used in this study was not adequate to trigger behavioural stress responses in this behavioural assay. Various studies have shown that fish anxiety-like responses can be influenced by numerous factors, including the type of stressor (i.e., biological, chemical, mechanical), the size of the experimental arena or other environmental conditions such as water temperature (Angiulli *et al.*, 2020; Blaser *et al.*, 2010; Shishis *et al.*, 2022; Wong *et al.*, 2012). Therefore, further research exploring these variables in turbot is necessary to determine whether the open field test is appropriate for assessing stress in this species.

On the contrary, both diving and light-dark preference tests showed clear avoidance behaviour in turbot. This was supported by significant spatial preferences across the arena between stressed and control fish. In the diving test, the only variable that revealed this difference was bottom-dwelling behaviour. Several studies have shown that more time spent at the bottom of the arena is associated with anxiety in a wide range of teleost species (Almeida Silva *et al.*, 2021; Huerta *et al.*, 2016; Thompson *et al.*, 2016). Surprisingly, we found that stressed turbot remarkably avoided the lower half of the arena and spent almost all testing time in the upper half. Accordingly, studies conducted on the Atlantic halibut (*Hippoglossus hippoglossus* L.), another farmed flatfish species with demersal habits, showed that individuals tended to swim close to the surface of the tank when they were under stressful and poor welfare conditions such as high stocking densities (Björnsson, 1994; Holm *et al.*, 1986; Kristiansen *et al.*, 2004). Therefore, this result indicated that behavioural stress indicators in flatfish species such as turbot may differ from those reported in most teleost species, highlighting the importance of considering inter-specific variation in welfare proxies.

Notably, the behavioural response of turbot to the light-dark preference test also revealed avoidance behaviours that were opposite to that expected from several teleost species. Behavioural studies have documented that species such as zebrafish, guppy, the cardinal tetra *Paracheirodon axelrodi* or the Nile tilapia *Oreochromis niloticus* exhibited a preference for dark environments when placed in a novel arena (Maximino *et al.*, 2007; Maximino *et al.*, 2010). In our study, stressed fish showed fewer entries in the dark zone than those observed in control fish, suggesting dark avoidance behaviour in turbot. Additionally, fish from both experimental groups displayed a significant preference for the light zone of the arena, further supporting the avoidance of dark environments in turbot. These findings were in agreement with previous studies supporting that juvenile turbot displayed a preference for white backgrounds and a dislike for black, red or brown backgrounds. Indeed, this preference was associated with improved welfare through higher growth rate, feed intake and metabolic rates (Li *et al.*, 2006). Collectively, these findings support the potential of this avoidance behaviour to measure stress in turbot. Furthermore, in our study, reduced activity in the light zone was observed in stressed turbot when compared to the control. This result also indicated that activity may function as an anxiety-like behaviour in turbot, as reported in other teleost species (López-Patiño *et al.*, 2008; Pintos *et al.*, 2024).

The causes underlying behavioural differences between teleost species are likely driven by distinct ecological and evolutionary backgrounds. In the case of turbot, this flatfish species inhabits shallow and sandy bottom habitats at a depth of about 100m (Hammen *et al.*, 2013; Knijn *et al.*, 1993; Nita, 2011). Many authors supported that scototactic patterns (i.e., light/darkness-seeking behaviours) could be explained by adaptive features of species in terms of crypsis-based strategies against predators (Fuiman *et al.*, 1994; Maximino *et al.*, 2007; Shaklee, 1963). This has been observed in Atlantic halibut juveniles which, in nature, lay camouflaged on the bottom of the water column, covered by sand (Kristensen *et al.*, 2004). Indeed, burying behaviour in sandy substrates has been shown to reduce predation risk in turbot as well as other flatfish species such as the sole *Solea solea* or the flounder *Paralichthys flesus* (Ellis *et al.*, 1997; Kristensen *et al.*, 2014). Therefore, it is plausible that the observed preference of turbot for light zones was influenced by the natural and cryptic behaviour of the species. Indeed, the experimental subjects used in our study were translucid without appreciable pigmentation, further supporting this hypothesis. Similarly, the bottom avoidance observed in the diving test might be interpreted as an escape response, a common anti-predatory behaviour in fish (Domenici *et al.*, 2010). This behaviour has been previously described in flatfish species as surface swimming, possibly serving as a strategy to carry out individuals from threatened areas by using water currents (Gibson *et al.*, 1998).

Overall, this study described for the first time the behavioural performance of turbot in a set of well-established behavioural assays to evaluate fish stress responses. The implications of our findings are mostly two. First, accurately describing and understanding the basic behavioural biology of each species is essential for interpreting their stress and welfare indicators in captivity. Otherwise, referring to literature from model species could lead to significant biases in understanding the stress conditions of farmed species, such as flatfish. Second, novel environment paradigms may serve as useful non-invasive tools for assessing stress and welfare in aquaculture species. However, it is necessary to conduct further research on this topic, including correlational studies among behavioural, physiological and molecular stress markers.

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RESEARCH ARTICLE

Social buffering of behavioural stress response in two fish species, Nile tilapia (*Oreochromis niloticus*) and koi carp (*Cyprinus carpio*)

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Abstract

The presence of conspecifics plays a crucial role in mitigating stress responses in social teleost species and holds potential for enhancing welfare in captive fish. While studies on social buffering effects have typically focussed on single species, marked interspecific differences can exist. Here, we conducted an analysis of social buffering of stress response in two of the most extensively farmed fish, the Nile tilapia and the koi carp. Subjects were exposed to a behavioural stress response assay (open-field test) in three conditions simulating increasing levels of social enrichment: isolation, pairs, or shoals of five fish. We obtained five stress indicators from the assay: thigmotaxis, freezing, activity, erratic movements and interindividual distance in conditions with more than one fish. In both species, erratic movements significantly decreased with increasing levels of social enrichment, suggesting a similar social buffering effect. However, other indicators revealed species differences. Koi carp, but not Nile tilapia, showed a socially-mediated reduction in thigmotaxis, whereas Nile tilapia, but not Koi carp, showed a socially-mediated reduction in freezing behaviour. Furthermore, social enrichment determined opposite effects on the activity of the two species: Nile tilapia were more active as group size increased, whereas the opposite trend was found in koi carp. Finally, Nile tilapia showed increased interindividual distance with increasing social group size, whereas no changes were observed for koi carp. Our study indicates that the buffering effects of social enrichment on the behavioural stress response do not completely overlap between different fish species, highlighting the importance of developing finely-tuned species-specific enrichments and welfare indicators.

KEYWORDS
anxiety-like behaviour, *Cyprinus carpio* koi, fish behaviour, open field test, *Oreochromis niloticus*, social buffering

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3. Developing behavioural indicators to assess acute stress responses in gilthead seabream *Sparus aurata*

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Abstract

Intensive fish production may result in poor farming conditions that compromise the health and overall well-being of animals held captive. Consequently, ensuring fish welfare has become an urgent priority for the long-term sustainability of the aquaculture industry, which necessarily relies on a thorough understanding of what welfare implies for each farmed species. The objective of our study was to identify behavioural traits sensitive to stress in the seabream *Sparus aurata* following exposure to the diving test, a widely reported behavioural assay to study stress in teleost species. To this end, we subjected juvenile seabream individuals to the diving test under two relevant stressors in aquaculture settings: salinity changes (i.e., low, control or high salinity) and lack of social enrichment (i.e., isolation or pair of fish). Our findings on the salinity challenge showed that a 5-ppt salinity variation had no effect on seabream behaviour. However, our results indicated that isolation significantly increased two over four studied behaviours (i.e., freezing and erratic movement), suggesting potential anxiety-like behaviours in the species and reduced stress when fish are exposed to the test in pairs. Overall, this study explored for the first time the behavioural performance of seabream individuals in the diving test. While our findings highlighted the existence of social buffering effects on the behavioural stress response of seabream, they also indicated that slight variations in salinity do not significantly affect its behaviour. These insights can contribute to identifying species-specific stress indicators and enhancing welfare practices in seabream farming.

Introduction

Aquaculture is one of the fastest-growing sectors in global food production, providing a significant source of protein and contributing to food security worldwide (FAO, 2024). However, current farming practices may result in poor farming conditions that fail to uphold the welfare of most farmed species (Conte, 2004; Huntingford *et al.*, 2006; Saraiva *et al.*, 2019). As projections indicate a significant increase in aquaculture production in the coming years (Edwards *et al.*, 2019; FAO, 2024), ensuring the welfare of farmed fish has become a critical priority, aiming to guarantee the long-term sustainability of fish farming (Berrill *et al.*, 2012; Stien *et al.*, 2020; Toni *et al.*, 2019).

In aquaculture settings, fish are exposed daily to a wide variety of stressors such as handling procedures, crowding, social isolation or even poor water quality (Ashley, 2007; Dara *et al.*, 2023; Iwama *et al.*, 1998; Martos-Sitcha *et al.*, 2020). These stressors generally compromise the health and overall welfare of animals held captive, suggesting that their impact should be minimised within fish farming. Although several studies have explored multiple strategies to reduce stress in aquaculture, most of them rely on traditionally farmed species such as the Atlantic salmon *Salmo salar* (Hvas *et al.*, 2024; Iversen *et al.*, 1998; Iversen *et al.*, 2005; Liu *et al.*, 2017) or even on model teleost such as the zebrafish *Danio rerio* (Dahm and Geisler, 2006; Gronquist and Berges, 2013; Pavlidis *et al.*, 2013; Piferrer and Ribas, 2020). Besides, they are mostly based on physiological or molecular indicators of stress (Barton and Iwama, 1991; Eissa and Wang, 2016, Davis, 2006).

Behavioural indicators of stress have recently gained attention for assessing fish welfare, as they can offer a non-invasive and real-time assessment of welfare conditions (Barreto *et al.*, 2022; Cavallino *et al.*, 2023; Laursen *et al.*, 2011; Martins *et al.*, 2012; Miller *et al.*, 2020; Wiese *et al.*, 2023). However, despite vast evidence collected in teleost model species (Collier *et al.*, 2017; Kysil *et al.*, 2017; Lucon-Xiccato *et al.*, 2022; Parker *et al.*, 2012), behavioural stress indicators are still unexplored across farmed species.

In this study, we examined the behavioural stress response of the gilthead sea bream (*Sparus aurata*), arguably the most important Mediterranean-farmed species (FAO, 2024; Haberle *et al.*, 2024), using the diving test. This behavioural paradigm has been widely exploited to assess stress responses in fish. Our objective was to identify behavioural traits sensitive to stress within the species by analysing stress responses under different stressors commonly encountered in aquaculture, such as salinity changes and social isolation (Herrera *et al.*, 2012; Ojelade *et al.*, 2022; Martos-Sitcha *et al.*, 2014) since seabream has been characterised as a social species (Dara *et al.*, 2022; Montero *et al.*, 2009). Hence, we hypothesise that 1) stress response increases under acute salinity changes and 2) the presence of a conspecific attenuates individuals' stress responses.

Material and methods

Experimental subjects

Gilthead seabream fish were obtained from a local farm at the fry stage and then reared in the University of Murcia fish facility (Murcia, Spain) for one month at 18 ± 1 ºC. Fish were housed in 250 L community tanks equipped with skimmers, constant aeration and supplied with filtered artificial saltwater (salinity: 25 ppt) from mechanical and biological filters. This salinity has shown optimal growth performance and survival in gilthead seabream (Appelbaum *et al.*, 2009; Tandler *et al.*, 1995). All tanks were kept barren to simulate the typical conditions of commercial facilities, and the housing room was under a 12:12 h light-dark artificial photoperiod. Seabream fish were fed once a day at 2% of total biomass with a commercial pelleted diet (Skretting, Spain) and were approximately 2-3 months old at the time of the experiment. They exhibited an average length of 7.12 ± 1.02 cm (mean ± standard deviation) and an average weight of 7.75 ± 2.65 g.

Social companion – testing procedure

Thirty-two experimental subjects randomly assigned to the isolation or the pair condition were evaluated in this experiment. For the isolation condition, twelve fish were randomly selected from housing tanks and individually exposed to the diving test (n=12 fish). In the pair condition, ten pairs of fish were exposed to the diving test (n= 10 pairs). The same experimental arena was used for both experimental groups, consisting of a glass rectangular tank (50 × 35 × 20 cm) covered with white plastics from the sides and behind. A white LED strip illuminated (6500 K; Superlight Technology Co. Ltd., Shenzhen, China the arena from above while a full-HD camera (ELP USBFHD08S-MFV, Shenzhen Ailipu Technology Co. Ltd, Shenzhen, China) was located 40 cm from the front of the arena to record the experiments in 1920 × 1080 pixels of resolution and 30 frames per second (Figure 1). Once experimental subject/s were released in the centre of the arena, fish behaviour was recorded for 10 minutes and tracked by a computer running the Ethovision XT® software (Noldus Information Technology, Wageningen, Netherlands). Different well-reported fish anxiety-like behaviours were measured, including: time spent in the lower half of the apparatus (hereafter 'bottom-dwelling'), which often increases in stressed fish (Blaser and Rosemberg, 2012; Cachat *et al.*, 2010; Maximino *et al.*, 2010); locomotor activity, measured as the distance travelled across the arena (Levin *et al.*, 2007); freezing, as the time spent motionless with a speed lower than 0.5 cm/s, a behavioural trait often linked to anxiety states (Egan *et al.*, 2009); and erratic movement, as the angular velocity of the path, which usually increases in stressed fish (Blaser *et al.*, 2010). All experiments were conducted between 09.00 a.m. and 13.00 p.m to minimise the effect of time-of-day on stress responses (Pintos *et al.*, 2023).

Salinity challenge – testing procedure

Thirty-six fish were evaluated in this experiment and randomly assigned to the control, low or high salinity group (n=12 fish/group). Experimental subjects were randomly selected from housing tanks and individually exposed to the diving test (Figure 1). The diving test arena was filled with artificial seawater prepared at different salinities depending on the experimental group (control: 25 ppt; low: 20 ppt; high: 30 ppt; Figure 1B). All details of the analysed behaviours and testing conditions were kept as described in the previous experiment, following the same procedure.

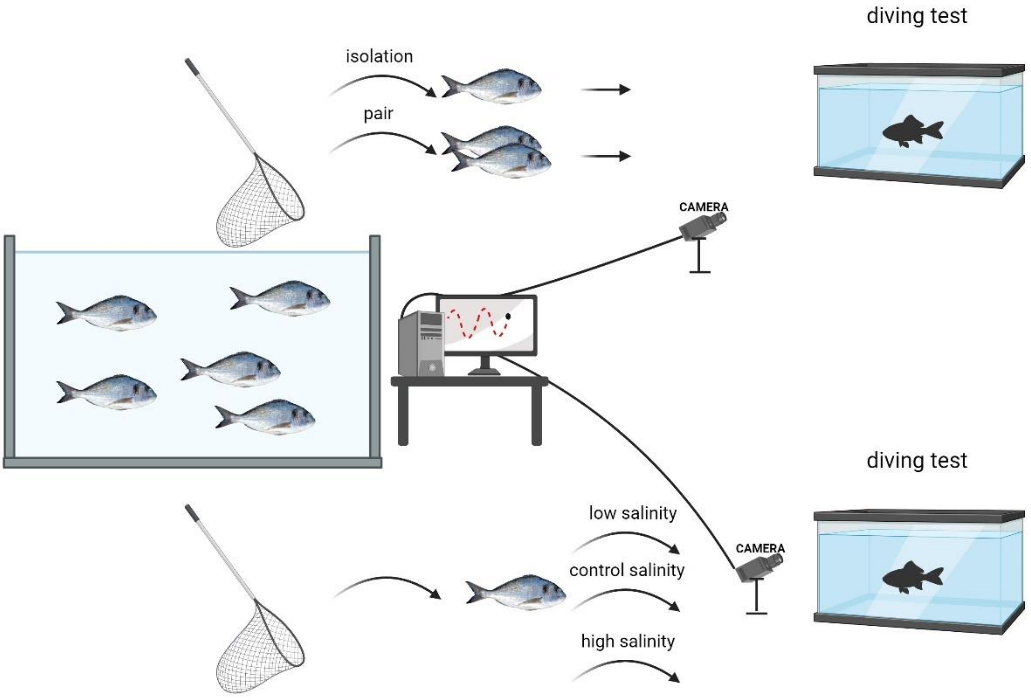

Figure 1: Illustrative scheme of the experimental design. Juvenile seabream were behaviourally assessed according to social condition (i.e., isolation or pair) and salinity changes (i.e., control, low and high salinity) in the diving test. The diving test arena consisted in a 50 × 35 × 20 cm glass tank. Fish behaviour was recorded for 10 minutes from the beginning of each trial and consecutively tracked by an automated fish tracking software (Ethovision XT®).

Statistical analysis

Statistical analyses were performed using R Statistical software version 4.0.1 (The R foundation for Statistical Computing Vienna Austria http://www.r-project.org) and all analyses were conducted by functions from the R base package. To analyse the effect of social companions on seabream behaviour, multiple two-sampled *t-test*s were performed. The effect of water salinity on seabream behaviour was studied by one-way analysis of variance (ANOVA). Model assumptions were verified by the Shapiro-Wilk (normality) and Levene's tests (homoscedasticity). Behavioural data that did not meet the assumptions for parametric analysis were transformed to improve model fitting by square root (activity and erratic movement) and rankit (bottom dwelling) transformations. Moreover, one sample *t*-tests were performed to study if bottom-dwelling behaviour was driven by stress or associated with random movements across the experimental arena.

Results

Social companions

On average, fish spent 77.50 ± 3.23 % of testing time in the lower half of the arena in isolation and 71.35 ± 6.55 % in pairs, denoting the expected bottom-dwelling response to the diving test in both experimental groups (*t-test:* isolation: $t_{11} = 8.42$, $p < 0.01$; pair: $t_{11} = 3.25$, $p < 0.01$). However, no significant differences were observed between isolated and paired fish in bottom dwelling behaviour (t-test: $t_{20} = 0.88$, $p = 0.38$; Figure 2A).

Fish travelled an average of 1886.16 ± 372.16 cm across the diving test arena when isolated and 984.69 ± 112.62 cm when paired. The *t-test* showed no significant differences between isolated and paired fish in activity behaviour (t-test: $t_{20} = 0.67$, $p = 0.50$; Figure 2B).

Fish displayed freezing behaviour during 16.92 ± 4.58 % of the testing time when isolated and 4.90 ± 0.75 % when paired. These differences were significant, indicating higher freezing behaviour in those fish exposed to the diving test in isolation (t-test: $t_{20} = 2.36$, $p < 0.01$; Figure 2C).

Regarding erratic movement behaviour, significant differences were found between isolated and paired fish (t-test: $t_{20} = 3.5$, $p < 0.01$). Thus, fish exhibited an average angular velocity of 488.25 ± 77.78 deg/s when isolated and 185.92 ± 12.33 deg/s when paired (Figure 2D).

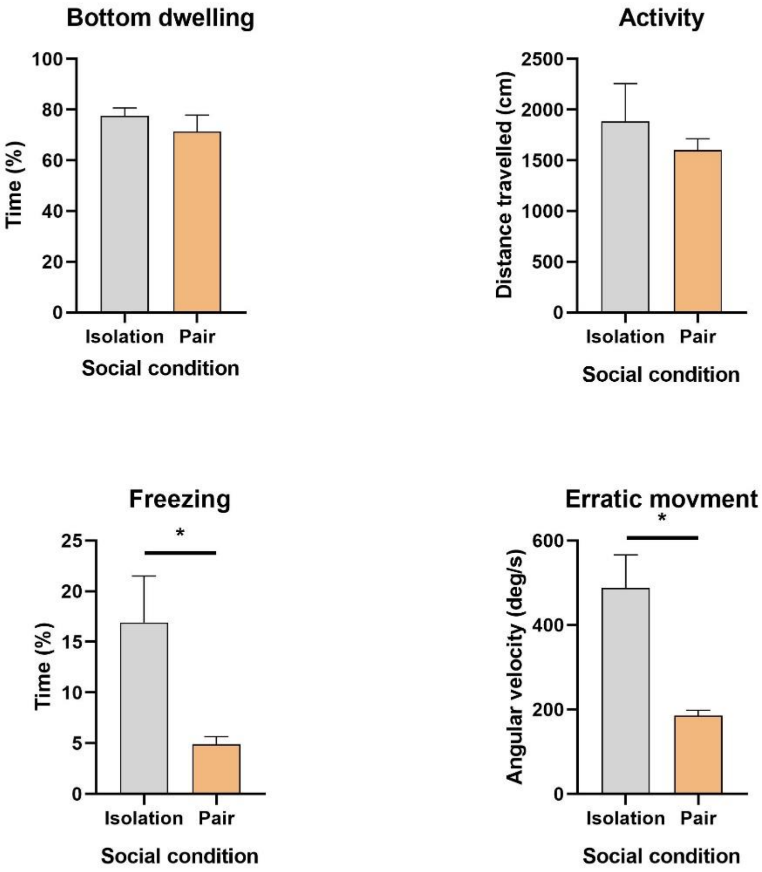

Figure 2: Seabream behaviour in the diving test according to social condition (isolation = grey; pair = orange). **A.** Bottom dwelling. **B.** Activity. **C.** Freezing. **D.** Erratic movement (n= 12 fish/social condition). Data are presented as mean ± standard error and asterisks indicate statistical differences between experimental groups.

Salinity challenge

Control fish spent on average 89.63 ± 4.20 % of testing time in the lower half of the diving test arena, whereas fish exposed to high or low salinity spent 93.97 ± 2.41 %, and 84.34 ± 6.17 %, respectively. All groups showed the expected bottom-dwelling response to the diving test (*t-test:* control: $t_{11} = 9.42$, $p < 0.01$; low: $t_{11} = 18.17$, $p < 0.01$; high: t_{11}

= 5.56, $p < 0.01$) but no significant differences were observed between experimental groups (ANOVA: $F_{2,33} = 0.53$, $p = 0.59$; Figure 3A).

On average, fish travelled 3996.81 ± 1137.86 cm across the diving test arena when exposed to the control condition. Conversely, fish exposed to high or low salinity travelled an average of 1403.78 ± 255.42 cm and 2713.31 ± 573.80 cm, respectively. No significant differences were found in activity behaviour between experimental groups (ANOVA: $F_{2,33} = 2.90$, $p = 0.06$; Figure 3B).

Moreover, fish displayed freezing behaviour at an average of 26.49 ± 6.49% of testing time in the control condition, 36.39 ± 7.10 % in high salinity and 32.34 ± 7.51 % in the low salinity. This behaviour did not show significant differences between salinity groups (ANOVA: $F_{2,33} = 0.49$, $p = 0.61$; Figure 3C).

Regarding erratic behaviour, fish exhibited an average angular velocity of 59.22 ± 8.24 deg/s in the control condition, 49.22 ± 5.29 deg/s in the high salinity condition and 48.54 ± 7.53 deg/ in the low salinity condition, although differences were not significant between experimental groups (ANOVA: $F_{2,33} = 0.68$, $p = 0.51$; Figure 3D).

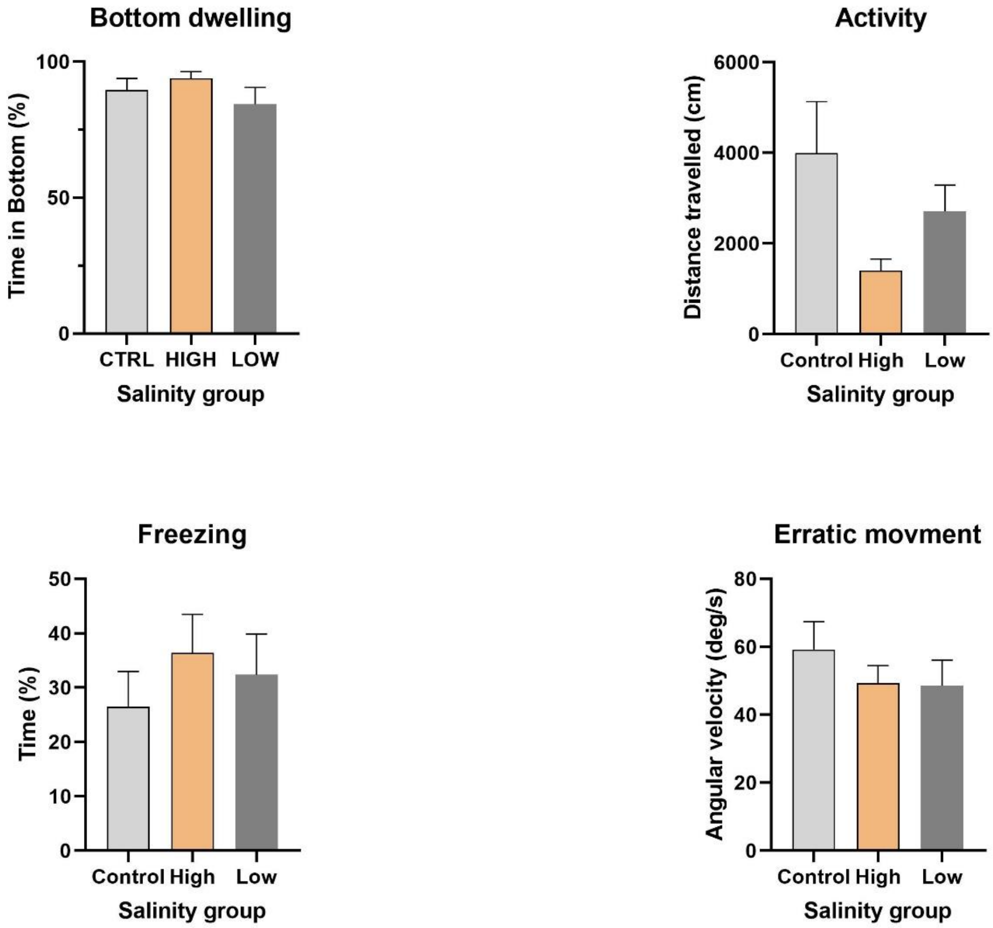

Figure 3: Seabream behaviour in the diving test at different water salinity levels (control = light grey; high = orange; low = dark grey). **A.** Bottom dwelling. **B.** Activity. **C.** Freezing. **D.** Erratic movement (n= 12 fish/salinity group). Data are presented as mean ± standard error and asterisks indicate statistical differences between experimental groups.

Discussion

This study documented how behavioural stress indicators in gilthead seabream vary in response to two stressors of relevance in aquaculture: social isolation and acute salinity changes. Our findings suggested social buffering effects within the species, showing reduced individuals' anxiety-like behaviours in the presence of social companions. Furthermore, results highlighted potential behavioural traits as stress indicators in seabream. Conversely, our findings also showed that none of the analysed behaviours were influenced by a deviation of 5-ppt in water salinity, whether above or below the control levels.

Critically, the presence of a social companion showed a reduction in two out of four analysed behaviours (i.e., freezing and erratic movement). Previous studies have supported both freezing and erratic movement as anxiety indicators in fish exposed to the diving test (Blaser and Rosemberg, 2012; Cachat *et al.*, 2010; Tran and Gerlai, 2016). Indeed, recent studies in Nile tilapia agree with our findings by showing that at least one social companion could reduce these anxiety-like behaviours in another novel environment paradigm, such as the open field test (Pintos *et al.*, 2024). Collectively, this evidence would indicate the existence of social buffering effects in seabream behaviour. Surprisingly, although seabream exposed to the diving test denoted the expected bottom dwelling response in isolation and in pairs, the presence of a conspecific did not modify this stereotyped anxiety-evoked response (Maximino *et al.*, 2012). This indicated that bottom dwelling may act as an anxiety indicator in seabream, but the social buffering effect was not enough to mitigate this response. Similarly, activity behaviour neither showed differences between social conditions. However, it is worth noting that this proxy can solely estimate locomotion rather than stress (Sharma *et al.*, 2019; Wiles *et al.*, 2020). More research should gather data on seabream behavioural stress responses to further explore species-specific stress indicators as well as the importance of social behaviour for attenuating stress within the species.

Seabream behaviour did not change in response to a 5-ppt salinity change above and below the control values. Although bottom-dwelling behaviour suggested that all experimental groups exhibited the expected stress response of fish to the diving test, the slight change in salinity used in this study did not elicit differences in any of the analysed behaviours. Previous studies have shown that this species can adjust their homeostasis within a salinity range of 5 to 60 ppt (Laiz-Carrion *et al.*, 2005; Sangiao-Alvarellos *et al.*, 2005a; Sangiao-Alvarellos *et al.*, 2005b). While physiological stress responses to short-term salinity changes have been observed in seabream (i.e., increased hypothalamic-pituitary-interrenal axis activity), these typically occur in response to more abrupt shifts, such as from 38 ppt to 5 or 55 ppt, representing hypo- or hyperosmotic conditions (Martos-Sitcha *et al.*, 2013; Martos-Sitcha *et al.*, 2014). Therefore, it is possible that seabream can indeed tolerate a 5-ppt salinity change, as used in this study, without exhibiting increased anxiety-like responses. To verify this hypothesis, future research should investigate whether such salinity changes trigger physiological stress responses in seabream, providing a more comprehensive understanding of their tolerance to salinity fluctuations.

Overall, this study documented for the first time the behavioural performance of seabream in the diving test. Although numerous behavioural studies have examined the effects of chronic stress and stocking densities on gilthead seabream, most of them were focused on housing behaviour rather than acute stress responses to novel environments (Alfonso *et al.*, 2020; Arechavala-Lopez *et al.*, 2020; Carbonara *et al.*, 2019; Herrera *et al.*, 2014), a method commonly used to assess stress in other teleost species. Our findings support the application of novel environment paradigms, such as the diving test, to evaluate seabream welfare through behavioural assessments. Furthermore, our results highlighted distinct behavioural traits as potential candidates for anxiety-like behaviours within the species.

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Chapter 2.

The time-of-day matters: daily rhythms in the behavioural stress response of model and farmed fish species

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Daily rhythms in the behavioural stress response of the zebrafish *Danio rerio*

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ABSTRACT

In nature, animals are exposed to stressors that occur with different likelihood throughout the day, such as risk of predation and human disturbance. Hence, the stress response is expected to vary plastically to adaptively match these challenges. Several studies have supported this hypothesis in a wide range of vertebrate species, including some teleost fish, mostly through evidence of circadian variation in physiology. However, in teleost fish, circadian variation in behavioural stress responses is less understood. Here, we investigated the daily rhythm of stress response at the behavioural level in the zebrafish *Danio rerio*. We exposed individuals and shoals to an open field test every 4 h over a 24 h cycle, recording three behavioural indicators of stress and anxiety levels in novel environments (thigmotaxis, activity and freezing). Thigmotaxis and activity significantly varied throughout the day with a similar pattern, in line with a stronger stress response in the night phase. The same was suggested by analysis of freezing in shoals, but not in individual fish, in which variation appeared mostly driven by a single peak in the light phase. In a control experiment, we observed a set of subjects after familiarisation with the open-field apparatus. This experiment indicated that activity and freezing might present a daily rhythmicity that is unrelated to environmental novelty, and thus to stress responses. However, the thigmotaxis was constant through the day in the control condition, suggesting that the daily variation of this indicator is mostly attributable to the stress response. Overall, this research indicates that behavioural stress response of zebrafish does follow a daily rhythm, although this may be masked using behavioural indicators other than thigmotaxis. This rhythmicity can be relevant to improve welfare in aquaculture and reliability of behavioural research in fish models.

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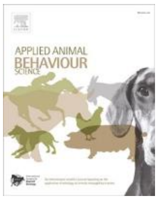

Circadian modulation of behavioural stress indicators varies between diurnal and nocturnal fish species

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ABSTRACT

In the wild, most animals experience daily fluctuations in threats and resources that are synchronised with environmental time cues such as the light-dark cycle. Consequently, animals have evolved daily behavioural patterns (i.e., diurnal or nocturnal) that enhance their fitness by, for example, reducing the temporal overlapping with predators. In fish, previous studies revealed stronger physiological stress responses during the resting period of the species. However, little is known about the circadian modulation of stress indicators and how they are influenced by daily behavioural patterns. In this research, we investigated the behavioural stress responses of two farmed fish species with different activity patterns: the diurnal Nile tilapia (*Oreochromis niloticus)* and the nocturnal tench (*Tinca tinca)*. To this end, we examined the behavioural response of individuals exposed to the diving test every 4 h over a 24 h cycle (n = 12 fish/species/time point). Results indicated that most behavioural indicators varied according to the time of day, aligning with the daily rhythmic pattern of the two species. Tilapia exhibited stronger stress responses to novelty during the dark phase, while tench displayed higher stress during the light phase. This was supported by stress-related behaviours such as freezing and erratic movements (in both tilapia and tench) and bottom-dwelling (in tench only). These results indicated that stress responses peaked during the resting phase of each species, although behavioural indicators exhibiting this daily variation did not completely coincide between the studied species. Overall, these findings suggest interspecific differences in the daily modulation of behavioural stress indicators in farmed fish, an effect with potential relevance for welfare. Understanding the activity rhythmic patterns, resting periods, and associated daily variation in stress for each fish species of interest can precisely help tailor farming procedures to minimise suffering.

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3. Is social buffering consistent across the time of the day? A study in a diurnal (Nile tilapia *Oreochromis niloticus*) and a nocturnal fish species (tench *Tinca tinca*).

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Abstract

In social animals, interactions with conspecifics provide several benefits that ultimately enhance welfare and fitness, including an attenuation of individuals' stress responses to environmental challenges often referred to as social buffering. This effect has been reported across several fish taxa. However, evidence suggests that social buffering is remarkably variable in fish, depending on intrinsic factors of species and environmental conditions. In this research, we explored whether social buffering effects vary throughout the time of the day in two social fish species with opposed activity patterns: the diurnal Nile tilapia (*Oreochromis niloticus)* and the nocturnal tench (*Tinca tinca)*. To this goal, we collected behavioural indicators of stress in fish subjected to two different social conditions (i.e., isolation or groups of five fish) for 24 h every 4 h. Our results revealed social buffering effects in both species in almost all analysed indicators. Critically, while the social buffering effect was constant throughout the day in tench, it varied between day and night in tilapia. Furthermore, isolation disrupted daily rhythmicity only in tilapia, highlighting interspecific variation in the influence of conspecifics on behavioural rhythms. Overall, our findings supported the presence of social companions to improve fish welfare in captivity and underscored the importance of considering species-specific behavioural rhythms in the management of social enrichment strategies.

Keywords: behavioural plasticity; biological clock; circadian rhythms; social behaviour.

Introduction

In social animals, the opportunity to interact with conspecifics plays a key role in the well-being of the individuals. For example, the presence of social companions can increase foraging efficiency (Costa *et al.*, 2015; Velázquez-Martínez *et al.*, 2010), reduce the risk of predation (Crane *et al.*, 2013; Lung *et al.*, 2006), and attenuate stress responses to environmental challenges (Eisenberger *et al.*, 2007; Hennessy *et al.*, 2009). These benefits enhance the overall welfare of the individuals and ultimately improve their fitness (Snyder-Mackler *et al.*, 2020). In mammals, several studies have documented that individuals housed in isolation displayed greater physiological responses to stress than those housed in pairs or in groups. For instance, squirrel monkeys *Saimiri sciureus* separated from conspecifics exhibited abnormal, prolonged peaks of circulating cortisol (Mendoza *et al.*, 1992; Lyons *et al.*, 1994) and stronger physiological responses to threats (Stanton *et al.*, 1985). Guinea pigs *Cavia porcellus* showed reduced cortisol responses to novelty in the presence of conspecifics than isolated (Hennessy *et al.*, 2008). This phenomenon has been often referred to as social buffering and it has been recently described in a wide range of taxa, including insects (Oliveira and Faustino, 2017; Tian *et al.*, 2017), reptiles (Martin *et al.*, 2023) and birds (Edgar *et al.*, 2015).

In fish, the existence of social buffering has been documented across various species, in particular in those commonly reared in captivity (Culbert *et al.*, 2019; Gilmour and Bard, 2022; Pavlidis *et al.*, 2013). Consequently, many authors have proposed social buffering as a strategy to refine the impact of husbandry procedures and improve the welfare conditions of fish held captive (Arechavala-Lopez *et al.*, 2022; Jones *et al.*, 2023; Yusishen *et al.*, 2020). Nevertheless, social enrichment effects showed considerable inter- (Pintos *et al.*, 2024) and intra-specific variation in relation to sex (Akinrinade *et al.*, 2023), developmental stage (Hesse *et al.*, 2015), and stocking density (Fattah *et al.*, 2020;

Turnbull *et al.*, 2005). This evidence suggests that social interactions and their benefit are variable, and as such, they should be carefully evaluated in each species to be interpreted in terms of welfare (Barreto *et al.*, 2011; Brown *et al.*, 1992).

One factor known to significantly affect fish welfare is the time of day. Its effect is mainly due to the daily modulation of the stress response, which is well-reported in various species. Fish stress responses are frequently stronger when the stressor occurs during the resting period of a given species, suggesting adequate periods to manipulate individuals and thereby minimise stress (Figueiredo *et al.*, 2020; López-Olmeda *et al.*, 2013; Vera *et al.*, 2014). Reports on circadian regulation of stress responses in fish are mostly based on physiological traits (Sánchez-Vázquez *et al.*, 2019; Hernández-Pérez, 2019) and generally conducted in isolated fish (Cousineau *et al.*, 2014; Oliveira *et al.*, 2012; Vera *et al.*, 2014). Therefore, in this study, we assessed whether the social buffering effect on stress responses varies according to the time of the day in fish. This possibility has been suggested by a few studies in primates (Mendoza *et al.*, 1991; Lyons *et al.*, 1995) and birds (Chaturvedi *et al.*, 2024).

In our experiment, we analysed the variation of the social buffering effect on fish welfare across the day in two social fish species with opposed activity patterns (diurnal: Nile tilapia *Oreochromis niloticus*; nocturnal: tench *Tinca tinca*). To this end, we collected behavioural indicators of stress in fish subjected to two social conditions (i.e., isolation or groups of five fish) for 24 h every 4 h. The indicators collected included freezing, activity, erratic movement and bottom-dwelling behaviour. We hypothesised that 1) the presence of social companions decreases behavioural indicators of stress, thereby increasing welfare, in both species, 2) the social buffering effect exhibits daily variations and 3) the effect of social companions on daily behaviour is different (i.e., opposed) across the day between diurnal and nocturnal species.

Material and methods

Experimental subjects

Juvenile tilapia (length: 4.67 ± 1.39 cm; n = 52) and tench (length: 5.48 ± 1.04 cm; n = 52) were reared separately in the fish facility of the University of Murcia. The tilapia subjects were obtained through the spawning of adult individuals obtained from an aquaculture company (Fishgen, Swansea, Wales, UK). Tench were purchased at the fry stage from a commercial supplier (Tencas-Atanasio S. L., Badajoz, Spain). The housing tanks (28 × 25 × 16 cm; 11 L) contained 10-12 individuals. The water in the housing tanks was kept at a constant temperature: 26 ± 1 °C for tilapia and 21 ± 1 °C for tench. Housing tanks were exposed to a 12:12 h light-dark (LD) artificial photoperiod with lights on at 09.00 am ("zeitgeber" time 0; ZT0). All tanks were provided with constant aeration and mechanical and biological filters and were kept barren to simulate the typical conditions of commercial facilities.

All fish were fed to satiety once per day for 20 days prior to behavioural testing. The feed was randomly administered during the active phase of species to avoid influencing fish behaviour by determining feeding rhythms (López-Olmeda and Sánchez-Vázquez, 2010). Whereas tilapia has been classified as a diurnal species (Fortes-Silva *et al.*, 2010), tench has been described as strictly nocturnal (Herrero *et al.*, 2003). Therefore, the feed was administered during the light phase for tilapia (ZT0 to ZT12) and during the dark phase for tench (ZT12 to ZT0).

Testing conditions

Experimental settings

The behavioural test was performed following a well-established paradigm (Blaser and Rosemberg, 2012; Cachat *et al.*, 2010; Tran and Gerlai, 2016). The paradigm exploited a common antipredator response in fish as a stress indicator. When small fish are threatened, they often reduce their activity and descend close to the bottom of their habitat, where they are likely less visible to eventual predators (Brown *et al.*, 2004; Templeton and Shriner, 2004; Wisenden and Sargent, 1997). For the testing, we used a plastic arena filled with dechlorinated tap water aged 24 h. This arena was equipped with constant aeration and mechanical and biological filtration to ensure constant water conditions. The water was changed between each trial to prevent exposure to the chemical cues from the previous experimental subject. The size of the arena varied between the two testing conditions as follows: isolation condition, 25 × 15 × 15 cm with 13 cm of water; group condition, 30 × 20 × 18 cm filled with 15 cm of water. The walls of the arena were opaque from behind and transparent from the front and sides. A digital full-HD camera (WebcamIP-002WA) was located 40 cm from the front of the experimental tank. This camera was sensitive to infrared light. An infrared backlight panel irradiated the arena from behind (Figure 1), providing the necessary light for the camera to work during the dark phase. A white LED strip (AquaRay, 6500 K) illuminated the arena from above during light-hour trials (ZT0-12).

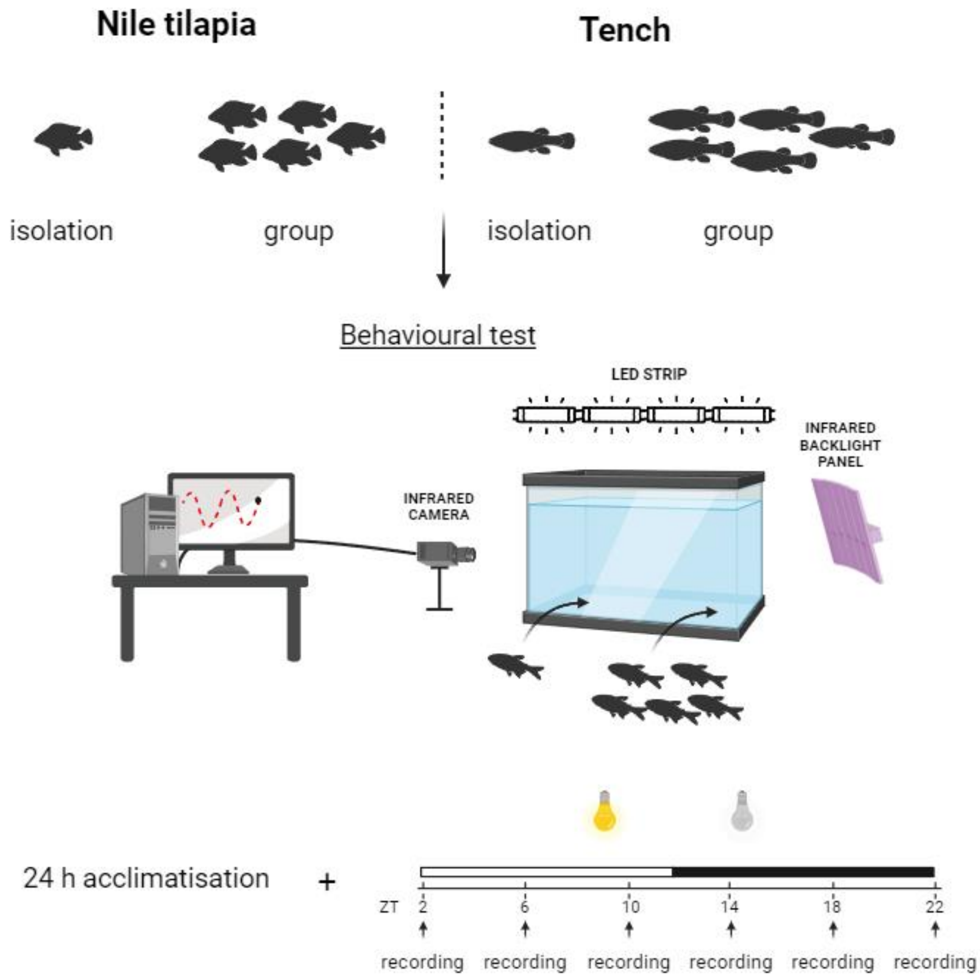

Figure 1. Illustrative scheme of the experimental apparatus and design. Fish from both species (Nile tilapia and tench) were continuously recorded for 24 h (every 4 h) in a novel environment according to different social conditions (isolation: n=12 fish/species/ZT; group: n=8 shoals/species/ZT). Video recordings were consecutively tracked with Ethovision XT® software and different behavioural stress indicators were measured. Experimental arenas (isolation: 25 × 15 × 15 cm; group: 30 × 20 × 18 cm) were illuminated with an LED strip from above during daylight hours (ZT0-12) and by an infrared light panel from behind (ZT0-23).

Isolation condition

Twelve fish from each species (n = 12/species) were randomly selected from the housing tank and individually tested in the arena. These subjects were gently transported by means of an opaque jar to minimise stress. The fish were acclimated to the arena for 24 h. During this period, food was randomly administered in one meal during the light phase for tilapia and during the dark phase for tench. After the acclimatisation, the behaviour of the subjects was recorded for 1 h at different times of the day, every 4h: at ZT 2, 6, 10, 14, 18 and 22. The video recordings were then analysed offline using the software Ethovision XT®. This software allowed us to track the position of the subject in the arena during each minute of the recording. Using the tracking data, the software provided us with the following variables to be used as stress indicators: 1) the time spent in the lower half of the arena (bottom-dwelling), which usually increases when fish are stressed (Blaser and Rosemberg, 2012; Cachat *et al.*, 2010); 2) the distance moved (activity), a common proxy of stress in fish (Levin *et al.*, 2007); 3) the time spent not moving (freezing) with a speed of 0.7 cm/s as threshold for tench and 0.5 cm/s for tilapia, as immobility is a typical antipredator behaviour displayed by several teleost species (Barreto *et al.*, 2013; Egan *et al.*, 2009); and 4) the angular velocity of the path (erratic movement), which is often assumed to increase in response to stressors (Blaser *et al.*, 2010).

Group condition

Eight groups of 5 fish from each species (n = 8 group/species) were used in the experiment. These fish were randomly selected from different housing tanks. After being moved into the arena and left undisturbed for 24 h, the recording began with the same conditions described in the previous section (i.e., 6 recordings, 1 each 2 hours at ZT2, 6, 10, 14, 18 and 22). The same variables analysed in the isolation condition were obtained from the tracking analyses. The Ethovision XT® provided as the output the average values of all the fish in the group for each analysed behaviour.

Statistical analysis

Statistical analysis was performed in R Statistical software version 4.0.1 (The R foundation for Statistical Computing Vienna Austria http://www.r-project.org). All behaviours analysed were subjected to the Cosinor analysis to evaluate the existence of daily rhythmicity. This was done by the *cosinor2* R package (Mutak, 2018). Cosinor analysis employs least-squares regressions to model cosine curves, which are useful to describe circadian variations (Nelson *et al.*, 1979) and estimates a circadian rhythm through a zero-amplitude test, in which $p < 0.05$ constitutes evidence for a statistically significant rhythm of the given period under consideration (i.e., 24 h). Rhythm parameters such as mesor and acrophase were calculated for each behavioural rhythm (Table 1) and compared between social conditions by the *circacompare* R package (Parsons *et al.*, 2020). All acrophases were corrected to locate them in the correct quadrant (Cornélissen *et al.*, 2014) and subsequently transformed from radians to time values (i.e., ZT).

The *nlme* R package was used to study differences in behaviour between ZTs (6 levels: 2,6,10,14,18 and 22) and social condition (2 levels: isolation condition; group condition). For each indicator, we fitted a linear mixed-effects model (LMM; Pinheiro *et al.*, 2017) with ZT and social condition as fixed effects. The models also included as the random effect the experimental subjects' or experimental shoals' ID to account for repeated measurements over time. The interaction between the two fixed effects was evaluated as it would indicate social buffering effects that vary through the day. If necessary, pairwise comparisons between each ZT were performed with the Tukey HSD test. Model assumptions were verified by the Shapiro-Wilk test and Q-Q plot (normality) and by Levene's test (homoscedasticity). Behavioural data that did not meet the assumptions for parametric analysis were transformed through square (bottom-dwelling in tench), square root (angular velocity in tench) and logarithmic (freezing in tench and activity in tilapia) functions.

SPECIES	BEHAVIOUR	SOCIAL CONDITION	ACROPHASE	AMPLITUDE	MESOR
Tench	Bottom	ISOLATION	5.43 ± 0.74	11.21 ± 4.27	89.10 ± 2.41
Tench	Bottom	GROUP	5.08 ± 2.45	17.26 ± 10.41	67.81 ± 5.86
Tench	Activity	ISOLATION	17.77 ± 1.18	4761.77 ± 1461.89	5659.48 ± 824.28
Tench	Activity	GROUP	18.30 ± 1.67	4392.82 ± 1856.19	8871.90 ± 1044.29
Tench	Freezing	ISOLATION	5.73 ± 1.07	38.70 ± 10.75	52.50 ± 6.06
Tench	Freezing	GROUP	6.42 ± 1.22	22.77 ± 7.20	30.96 ± 4.04
Tench	Erratic	ISOLATION	6.15 ± 2.52	48.92 ± 29.97	251.38 ± 16.90
Tench	Erratic	GROUP	5.43 ± 2.78	78.50 ± 52.40	306.59 ± 29.48
Tilapia	Bottom	ISOLATION	4.97 ± 3.50	10.81 ± 8.58	54.22 ± 4.83
Tilapia	Bottom	GROUP	6.22 ± 2.22	10.86 ± 5.97	50.01 ± 3.36
Tilapia	Activity	ISOLATION	N/A	N/A	N/A
Tilapia	Activity	GROUP	3.97 ± 3.28	1515.03 ± 1150.16	6730.36 ± 647.07
Tilapia	Freezing	ISOLATION	N/A	N/A	N/A
Tilapia	Freezing	GROUP	17.35 ± 3.55	8.82 ± 7.04	30.66 ± 3.96
Tilapia	Erratic	ISOLATION	18.60 ± 1.58	43.56 ± 17.43	203.86 ± 9.83
Tilapia	Erratic	GROUP	18.10 ± 1.32	97.29 ± 32.67	315.46 ± 18.38

Table 1. Cosinor parameters of behavioural indicators collected from tench and tilapia individuals over the 24 h cycle and according to social condition. Data are presented as mean ± C.I. 95% and acrophases in time values (ZT).

Results

Tench

Bottom-dwelling

On average, tench from the isolation condition spent 89.10 ± 1.51 % of the testing time in the lower half of the arena, whereas tench from the group condition showed this behaviour in 67.81 ± 3.35 % of the testing time. Both experimental groups denoted more time spent in the lower half of the arena than expected by random movements (one sample t-test: isolation condition: $t_{71} = 25.89$, $p < 0.01$; group: $t_{48} = 5.31$, $p < 0.01$). Cosinor analysis showed that bottom-dwelling behaviour exhibited significant daily variations for both social conditions (isolation condition: $p < 0.01$; group condition: $p < 0.01$), indicating similar diurnal acrophases (isolation condition = 5.43 ZT; group condition= 5.08 ZT; $p_{\text{acrophase}} = 0.74$; Figure 2A) but lower mesor in the group condition as compared to the isolation condition ($p_{\text{mesor}} < 0.01$).

In line with the Cosinor analyses, the LMM on bottom-dwelling behaviour showed significant main effects of ZT ($F_{5,97} = 26.23$, $p < 0.01$). Bottom-dwelling was higher during the light phase when compared to the dark phase (Tukey post-hoc test: Figure 3A). Moreover, the LMM found a significant effect of social condition ($F_{1,97} = 87.48$, $p < 0.01$), indicating a social buffering effect: tench of the group condition spent less time in the lower half of the arena when compared to tench of the isolation condition (Figure 3A). The interaction between the two fixed factors was not significant ($F_{5,97} = 1.53$, $p = 0.18$), suggesting no daily variation in the social buffering effect.

Activity

On average, tench moved 5659.48 ± 570.60 cm across the experimental arena in the isolation condition and 8871.90 ± 680.20 cm in the group condition. Cosinor analysis showed significant daily variations in activity for both social conditions (isolation condition: $p < 0.01$; group condition: $p < 0.01$), with similar acrophases close to the middle of the dark phase (isolation condition = 17.76 ZT; group condition = 18.30 ZT; $p_{\text{acrophase}} = 0.49$; Figure 2B) but increased mesor in tench of the group condition compared to tench of the isolation condition ($p_{\text{mesor}} < 0.01$).

The LMM on activity registered a significant main effect of ZT ($F_{5,97} = 37.49$, $p < 0.01$), due to higher activity during the dark phase as compared to the light phase (Tukey post-hoc test: Figure 3B). Moreover, the effect of social condition was also significant ($F_{1,97} = 43.10$, $p < 0.01$) because of higher activity in the group condition as compared to the isolation condition (Figure 2B). The interaction between the two factors was not significant ($F_{5,97} = 0.45$, $p = 0.81$), suggesting no daily variation in the buffering effect.

Freezing

On average, tench from the isolation condition spent 52.50 ± 4.41 % of testing time displaying freezing behaviour, whereas tench from the group condition displayed freezing for 30.95 ± 3.06 % of testing time. Cosinor analysis showed that freezing behaviour exhibited significant daily variations for both social conditions (isolation condition: $p < 0.01$; group condition: $p < 0.01$), indicating similar acrophases close to the middle of the light phase (isolation condition = 5.73 ZT; group condition = 6.42 ZT; $p_{\text{acrophase}} = 0.41$; Figure 2C) but increased mesor in the isolation condition as compared to the group condition ($p_{\text{mesor}} < 0.01$).

The LMM test on freezing revealed a significant main effect of both ZT ($F_{5,97} = 46.11$, $p < 0.01$) and social condition ($F_{1,97} = 13.98$, $p < 0.01$), and no significant interaction between fixed factors ($F_{5,97} = 1.83$, $p = 0.11$). Post-hoc analyses revealed increased freezing in the light phase compared to the dark phase (Tukey post-hoc test: Figure 3C). Moreover, consistently with the presence of social buffering, we found reduced freezing in the group condition as compared to the isolation condition (Figure 3C).

Erratic movement

Tench from the isolation condition showed an average angular velocity of 251.38 ± 9.30 deg/s, while tench in the group condition displayed an average angular velocity of

306.6 ± 16.45 deg/s. Cosinor analysis showed that this behaviour exhibited significant daily variations for both social conditions (isolation condition: $p < 0.01$; group condition: $p < 0.01$), indicating similar acrophases close to the middle of the light phase (isolation condition = 6.15 ZT; group condition= 5.44 ZT; $p_{\mathrm{acrophase}} = 0.60$; ; Figure 2D) but increased mesor in the group condition as compared to the isolation condition ($p_{\mathrm{mesor}} < 0.01$).

The LMM on angular velocity revealed a main effect of both ZT ($F_{5,97} = 10.98$, $p < 0.01$) and social condition ($F_{1,97} = 8.86$, $p < 0.01$). Post-hoc analyses revealed increased erratic movement during the light phase compared to the dark phase (Tukey post-hoc test: Figure 3D) and graphical inspection suggested increased erratic movement in the group condition (Figure 3D). The interaction between these factors was not significant ($F_{5,97} = 0.51$, $p = 0.76$), indicating no circadian variation of the social buffering effect.

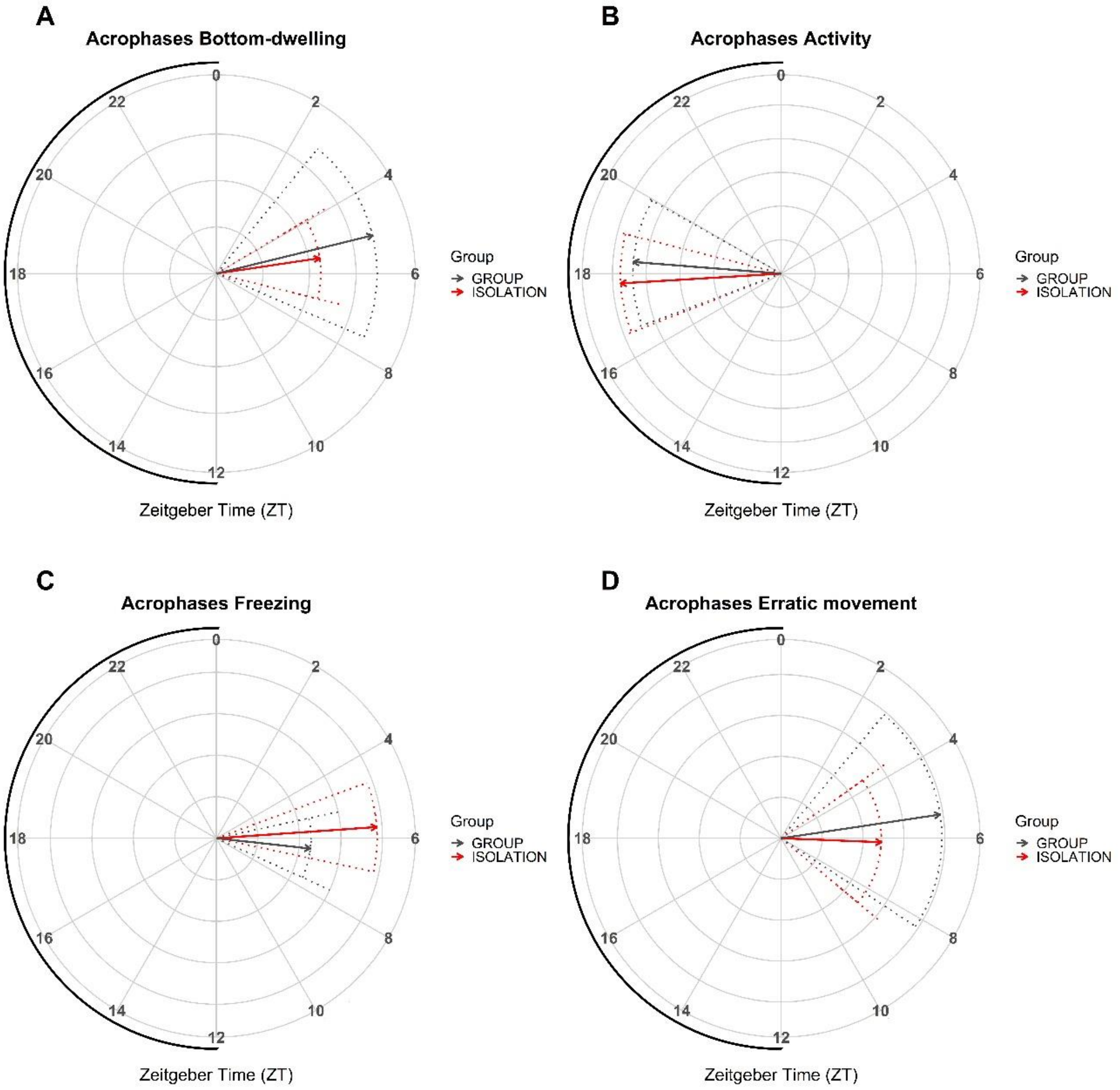

Figure 2. Polarograms of estimated acrophases for significant daily behaviours in tench (Cosinor, $p < 0.05$). **A.** Bottom-dwelling. **B.** Activity. **C.** Freezing and **D.** Erratic movement. Solid arrows indicate the mean acrophase and dotted lines indicate the confidence interval (set at 95%). The radial axis represents the time of the day (ZT) and the vector length represents the amplitude of the rhythm. The black line above radial axis represents the dark phase.

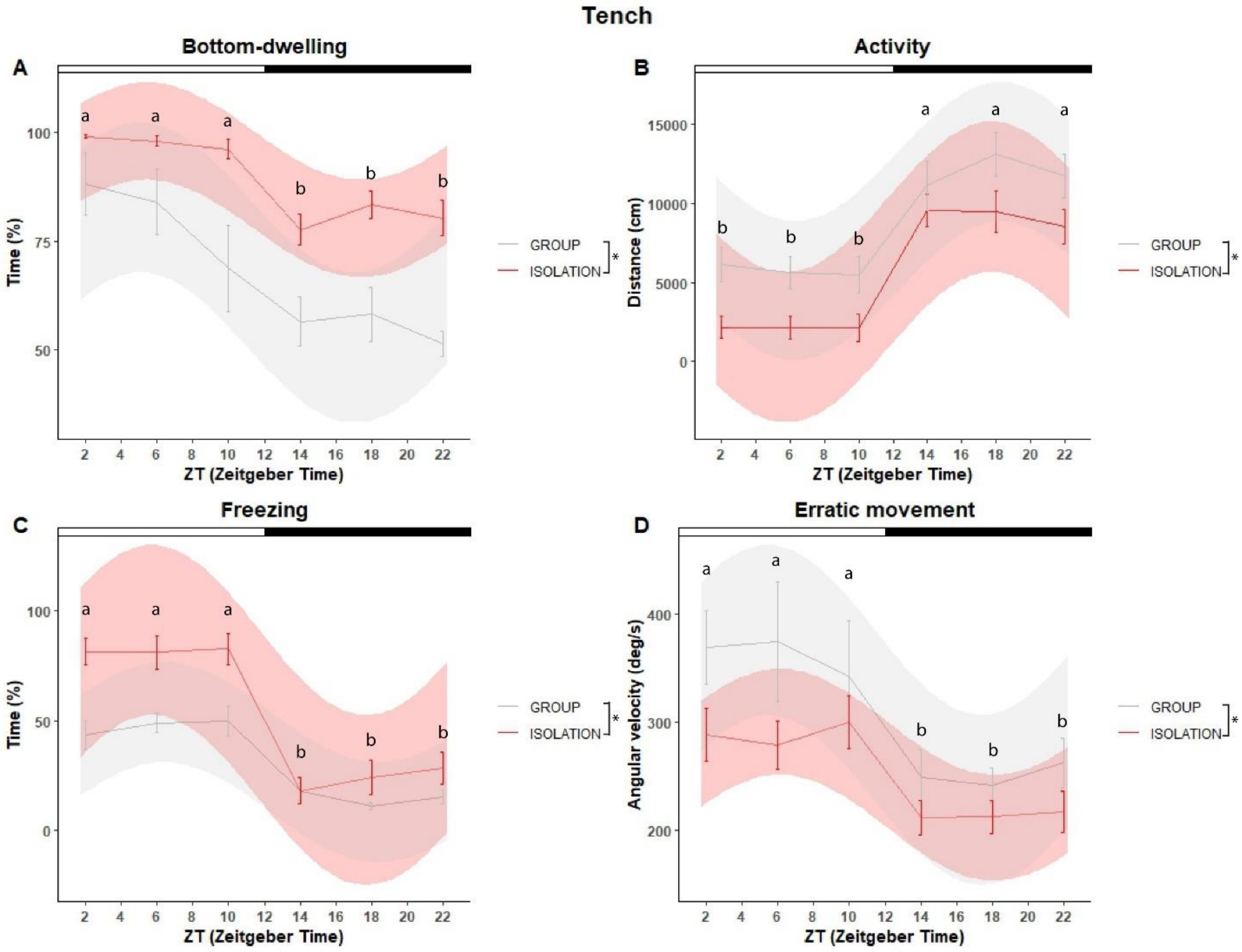

Figure 3. Daily variation of tench behaviour according to social condition (i.e., isolation or group). **A.** Bottom-dwelling. **B.** Activity. **C.** Freezing. **D.** Erratic movement. Data points represent mean ± standard error. Different letters (a,b) indicate statistical differences between ZTs by Tukey HSD test performed on linear mixed models. Asterisks (*) indicate a significant main effect of social condition. Wave area plots represented predicted data based on significant Cosinor models. White and black bars above each graph represent light and dark phases, respectively.

Tilapia

Bottom dwelling

On average, tilapia from the isolation condition spent 54.22 ± 2.55 % of the testing time in the lower half of the arena, whereas tilapia from the group condition spent 50.00 ± 1.97 % of the testing time. Therefore, the fish from the two experimental social conditions did not display a significant preference for the lower part of the arena (one sample t-test: isolation condition: $t_{71} = 1.65$, $p = 0.10$; group condition: $t_{48} < 0.01$, $p = 0.99$). Cosinor analysis showed bottom-dwelling behaviour exhibited significant daily variations for both social conditions (isolation condition: $p < 0.01$; group: $p < 0.01$), with similar acrophases close to the end of the light phase (isolation condition = 4.96 ZT; group condition= 6.23 ZT; $p_{\text{acrophase}} = 0.44$; Figure 4A) and no significant differences in the mesor ($p_{\text{mesor}} = 0.20$).

The LMM on bottom-dwelling behaviour showed a significant main effect of ZT ($F_{5,97} = 6.18$, $p < 0.01$). This indicated that bottom-dwelling varied across the time of the day. In particular, tilapia spent more time in the lower half of the arena at the end of the light phase as compared to the beginning of the dark phase (Tukey post-hoc test: Figure 5A). There was no significant effect of social condition ($F_{1,97} = 1.55$, $p = 0.21$) and no significant interaction between social condition and time of the day ($F_{5,97} = 0.65$, $p = 0.65$).

Activity

Tilapia moved an average of 4792.90 ± 223.63 cm across the diving test arena in the isolation condition and 6730.36 ± 351.05 cm in the group condition. Cosinor analysis revealed daily rhythms in activity only for tilapia of the group condition (isolation condition: $p = 0.20$; group condition: $p < 0.01$). The acrophase of activity behaviour for tilapia in the group condition was located close to the middle of the light phase (3.96 ZT; Figure 4B).

The LMM on activity registered significant main effects of ZT ($F_{5,97} = 3.20$, $p = 0.01$) and social condition ($F_{1,97} = 51.43$, $p < 0.01$). This indicated two independent effects of ZT time and social condition on activity behaviour. Post hoc analysis showed that tilapia exhibited higher activity during the beginning of the light phase as compared to the middle and the end of the dark phase (Tukey post-hoc test: Figure 5B). Moreover, higher activity was found in the group condition when compared to the isolation condition

(Figure 5B). The interaction between the two factors was not significant ($F_{5,97} = 1.85$, $p = 0.10$).

Freezing

On average, tilapia from the isolation condition displayed freezing behaviour at 39.09 ± 1.87 % of the testing time. Similarly, tilapia from the group condition spent 30.66 ± 2.12 % of testing time displaying freezing behaviour. Cosinor analysis showed significant daily rhythms in freezing only for tilapia of the group condition (isolation condition: $p = 0.61$; group condition: $p = 0.01$;). In the group condition, freezing acrophase was located close to the middle of the dark phase (17.35 ZT; Figure 4C).

The LMM test on freezing behaviour found a significant main effect of ZT ($F_{5,97} = 2.41$, $p = 0.04$) and social condition ($F_{1,97} = 36.06$, $p < 0.01$). Furthermore, the interaction between these factors was significant ($F_{1,97} = 3.97$, $p < 0.01$). This indicated diverging effects of ZT on the social buffering of freezing. Post-hoc analysis showed that, during daylight trials, tilapia of the group condition exhibited lower freezing than tilapia of the isolation condition (Tukey post-hoc test: Figure 5C).

Erratic movement

On average, tilapia from the isolation condition showed an angular velocity of 203.85 ± 6.07 deg/s, whereas tilapia from the group condition exhibited an angular velocity of 315.45 ± 13.43 deg/s. Cosinor analysis found significant daily rhythms in erratic movement behaviour for both social conditions (isolation condition: $p < 0.01$; group condition: $p = 0.03$). The acrophases were similarly located between the two social conditions, with maximum values close to the middle of the dark phase (isolation condition = 18.61 ZT; group condition = 18.10 ZT; $p_{\text{acrophase}} = 0.54$; Figure 4D).

Furthermore, increased mesor was observed in the group condition compared to the isolation condition ($p_{\text{mesor}} < 0.01$).

The LMM on angular velocity revealed a main effect of ZT ($F_{5,97} = 26.65$, $p < 0.01$) and social condition ($F_{1,97} = 143.21$, $p < 0.01$). Furthermore, the interaction between these factors was significant ($F_{1,97} = 4.54$, $p < 0.01$), suggesting daily variation in the social buffering effect. However, post-hoc analysis showed higher angular velocity values in tilapia of the group condition compared to tilapia of the isolation condition for all sampled time points (Tukey post-hoc test: Figure 5D).

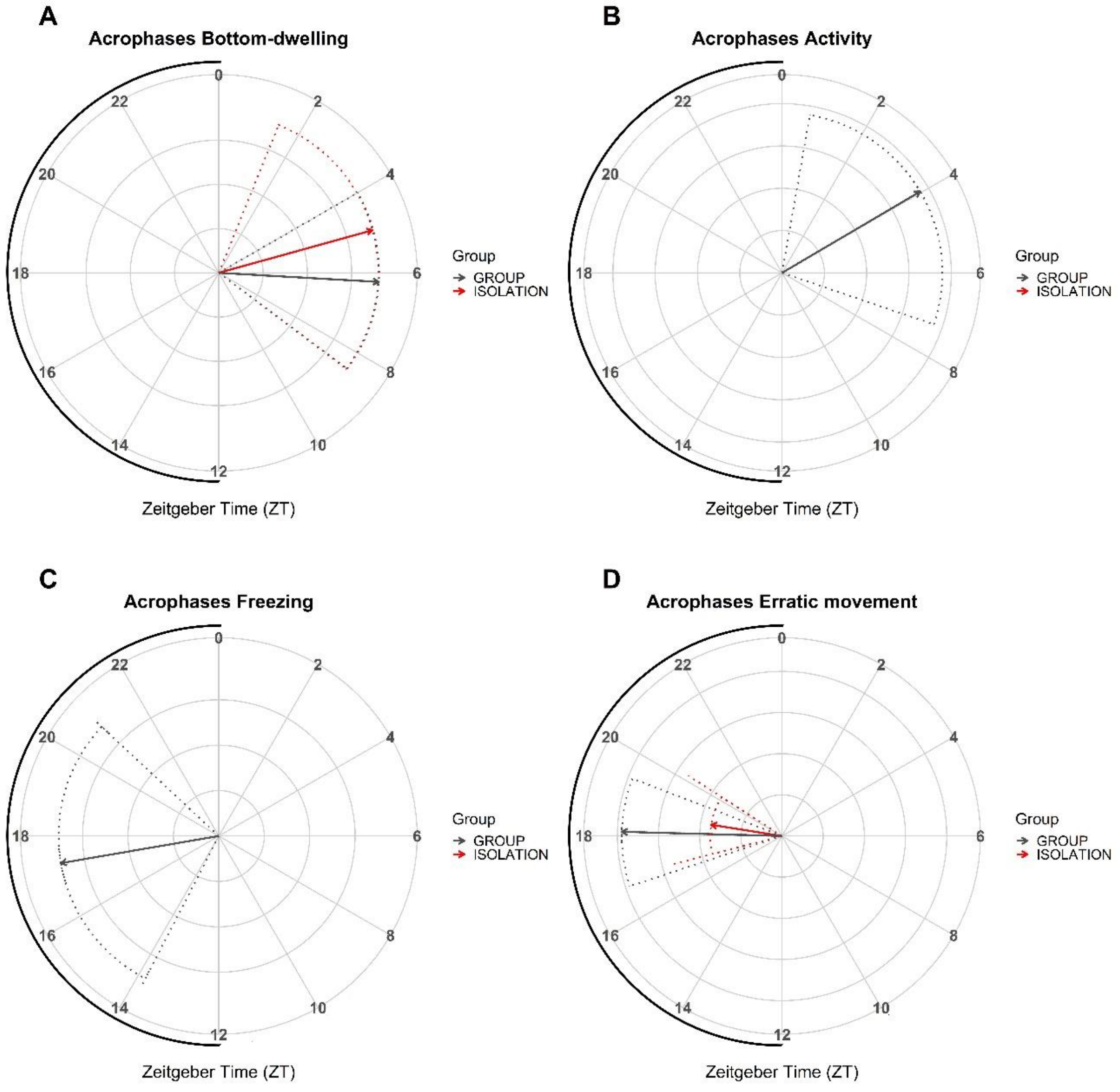

Figure 4. Polarograms of estimated acrophases for significant daily behaviours in tilapia (Cosinor, $p < 0.05$). **A.** Bottom-dwelling. **B.** Activity. **C.** Freezing and **D.** Erratic movement. Solid arrows indicate the mean acrophase and dotted lines indicate the confidence interval (set at 95%). The radial axis represents the time of the day (ZT) and the vector length represents the amplitude of the rhythm. The black line above radial axis represents the dark phase.

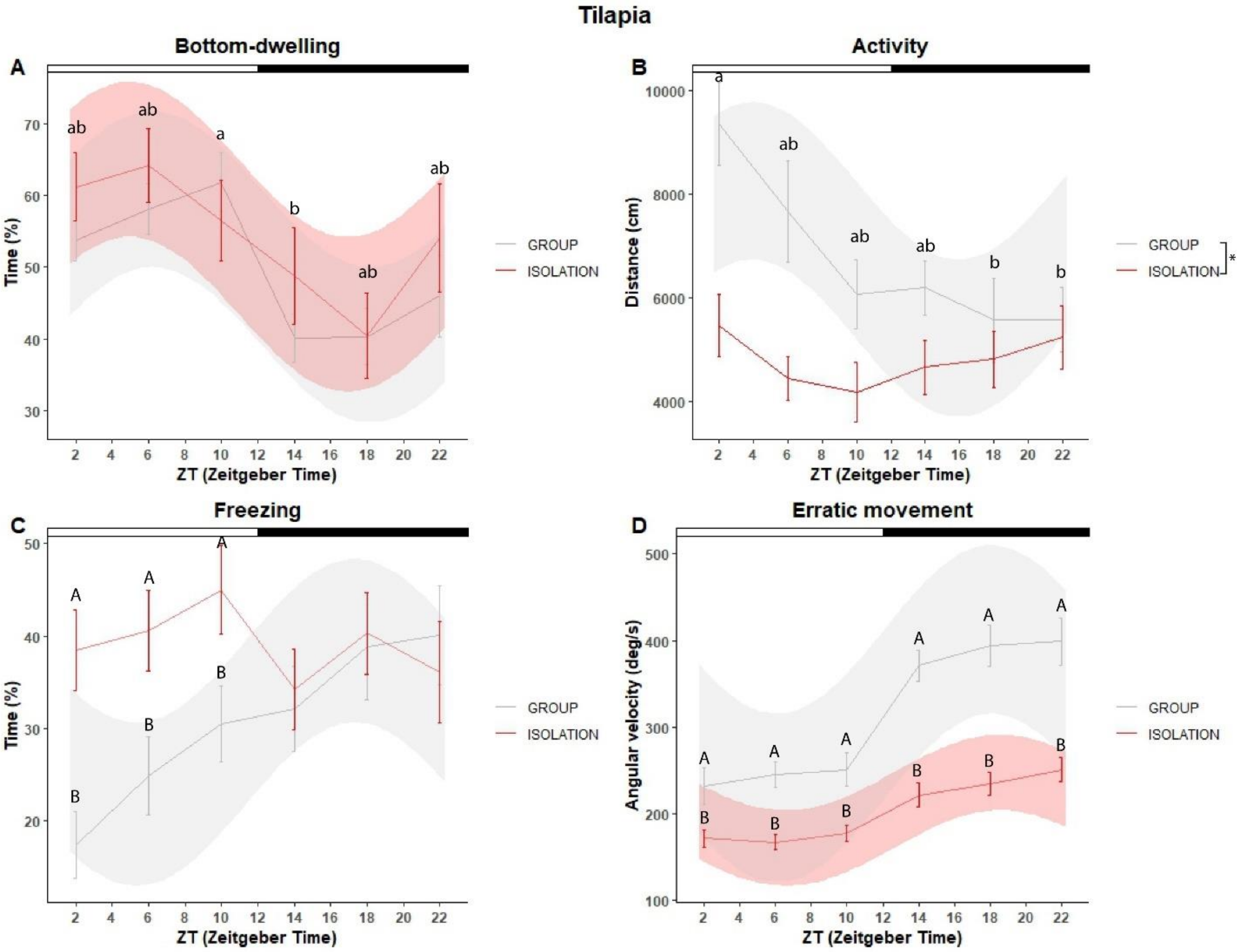

Figure 5. Daily variation of tilapia behaviour according to social condition (i.e., isolation or group). **A.** Bottom-dwelling. **B.** Activity. **C.** Freezing. **D.** Erratic movement. Data points represent mean ± standard error. Asterisks (*) indicate significant main effects of social condition. Lowercase letters (a, b) indicate significant differences between ZTs. Uppercase letters (A, B) indicate significant differences between social conditions at the same ZT. Post-hoc differences were obtained by the Tukey HSD test performed on linear mixed models. Wave area plots represented predicted data based on significant Cosinor models. White and black bars above each graph represent light and dark phases, respectively.

Discussion

To provide indications on welfare requirements and strategies, this study investigated the buffering effect provided by social companions on the stress behaviour of two farmed species with opposed activity patterns (Nile tilapia and tench) throughout the circadian cycle. We found general support for the presence of social buffering: group testing affected indicators of stress in both tench and tilapia in 7 out of 8 cases. Moreover, isolation conditions disrupted daily rhythmicity in two indicators in tilapia. Critically, while in tench the social buffering was constant throughout the day, in tilapia social buffering varied between day and night in two indicators. We conclude that social buffering might vary throughout the 24 h cycle, but only in some species and considering certain welfare indicators.

In tench, cosinor analyses found daily rhythms for all analysed behavioural indicators, both in group and isolation conditions. The acrophases were similar between the two social conditions, suggesting that the presence of social companions did not influence the rhythmic pattern of the behavioural indicators analysed. In line with the presence of social buffering effects, the social condition affected the overall levels of the behavioural indicators over the 24 h cycle, as evidenced by mesor comparisons and LMMs. In general, the effect indicated that social companions decreased stress and increased welfare. Bottom-dwelling and freezing, two behaviours indicative of stress in teleost species (Chin *et al.*, 2018; Levin *et al.*, 2007; Maximino *et al.*, 2010; Qiu *et al.*, 2017; Thompson *et al.*, 2016), decreased in the group condition. Moreover, the activity, which under some circumstances has been considered evidence of positive welfare in teleost species such as zebrafish *Danio rerio*, the mackerel *Scomber japonicus*, the bloodfin tetra *Aphyocharax anisitsi*, and the Nile tilapia (Golla *et al.*, 2020; Nakamura *et al.*, 2022; Pintos *et al.*, 2021; Pintos *et al.*, 2024), was increased in the social condition.

Therefore, these three indicators suggested that the group condition reduced stress and increased welfare behaviours in tench. Interestingly, erratic movement apparently deviated from the aforementioned pattern: whereas some studies supported it as an anxiety indicator in fish (Sireeni *et al.*, 2020; Speedie and Gerlai, 2008), our findings detected increased values in grouped fish. However, this variable evidenced controversial results and a weaker relation to anxiety (Blaser *et al.*, 2010; Blaser and Gerlai, 2006; Gerlai *et al.*, 2006). Therefore, it is possible this proxy was mainly influenced by other factors such as activity levels (Tran and Gerlai, 2016) or even the shoaling behaviour of the group rather than their stress level. Critically, all the effects of social condition observed in the tench were visible across the entire day (i.e., we did not detect significant interactions between social condition and time of the day). The general conclusion from the tench data is the presence of a social buffering effect that is consistent across the entire day, including the resting phase of the species.

In the case of tilapia, all behavioural indicators exhibited circadian rhythmicity in the group condition. However, two indicators did not show rhythmicity in the isolation condition (i.e., activity and freezing). For instance, in the presence of social companions, tilapia followed the circadian activity pattern expected in a diurnal species (Iigo and Tabata, 1996; Pozo *et al.,* 2011; Ueda and Oishi, 1982). This rhythmicity was however absent in the isolation condition. In agreement, previous studies showed that stressors (i.e., isolation) can disrupt circadian processes across taxa, including locomotor activity patterns (mammals: Albrecht, 2010; birds: Chaturvedi *et al.*, 2023; fish: Leliavski *et al.*, 2014; López-Patiño *et al.*, 2014; Ota *et al.*, 2018). This supported that social buffering effects might be important for the development of natural behavioural rhythmicity in tilapia. Furthermore, we found evidence of social buffering in 3 of the behavioural indicators analysed in tilapia (activity, freezing, and erratic movements). However, the effect was consistent throughout the day, as observed in the tench, only in one indicator. In particular, activity was increased by group testing during the entire day. Conversely, freezing was reduced by the social condition only during the light phase and erratic movement was increased by the group condition only at night. We believe that the effect of the erratic movement is difficult to interpret because of the arguments reported for the results on tench. However, the effect of freezing clearly demonstrates that the social buffering effect might vary across the time of the day. Certainly, our work suggests that this daily modulation of social buffering occurs only in limited circumstances.

Ecological and evolutionary factors may underpin the interspecific differences observed in the circadian modulation of social buffering. Some fish studies revealed that nocturnal benthic species often exhibit strict daily behavioural patterns, with no plasticity in response to different environmental conditions. For instance, two bottom dwellers and nocturnal teleost species, the burbot (*Lota lota*) and the stone loach (*Barbatula barbatula*), did not display changes in their nocturnal behaviour (i.e., foraging and activity) in presence of predators and under starvation (Fischer, 2004). Tench results thereby aligned with this trend characteristic of nocturnal and bottom-dwelling species (Alaş *et al.*, 2010; Erguden *et al.*, 2010). In contrast, diurnal species often show a strong plasticity in their behavioural patterns (Reebs *et al.*, 2002). For instance, the sea bass *Dicentrarchus labrax* individuals that showed diurnal feeding could be turned into nocturnal by restricting food availability to the night-time (Sánchez-Vázquez *et al.*, 1995). Similarly, the Atlantic salmon *Salmo salar* switch between diurnal and nocturnal foraging solely in response to environmental temperature (Fraser *et al.*, 1993). Indeed, previous studies in Nile tilapia reported intraspecific variation and even nocturnal activity rhythms (Vera *et al.*, 2009). Accordingly, our results also documented behavioural plasticity over the day in a diurnal species, underscoring for the first time its dependence on social conditions. Further studies should gather data on this species-specific behavioural plasticity in other fish species with different temporal habits (i.e., crepuscular) and social structures, including solitary species.

Our study bears three implications relevant to the welfare of captive fish. First, both species evidenced a social buffering effect through the reduction of behavioural stress indicators in the presence of conspecifics. As supported by previous studies, social rearing is an important enrichment strategy that can increase welfare in captive conditions and decrease the negative effects of manipulation and other farm procedures in fish (Arechavala-Lopez *et al.*, 2022; Cavallino *et al.*, 2023; Saraiva *et al.*, 2016; Silva *et al.*, 2020). Second, our work supports the hypothesis that the social buffering effect might vary across the day, although not in all species and not for all indicators. This effect requires specific investigations for each species of interest. Third, the behavioural indicators typically adopted to assess welfare in fish vary through the time of the day, and this variation depends on the diurnal versus nocturnal habit of the species. Therefore, caution should be adopted when using behavioural indicators of welfare in applied conditions.

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RESEARCH ARTICLE OPEN ACCESS

Circadian Modulation of Behavioral Stress Responses in Zebrafish Is Age-Dependent

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ABSTRACT

In the wild, stressors occur with varying likelihood throughout the day, leading animals to evolve plastic stress responses that exhibit circadian rhythmicity. In mammals, studies have revealed that the circadian plasticity of stress response may differ with age. However, such developmental effects have been largely overlooked in other vertebrate groups. In our research, we explored the presence of developmental variation in the daily pattern of behavioral stress response in a teleost fish model: the zebrafish (*Danio rerio*). We compared juvenile and adult individuals in two behavioral paradigms commonly used to analyze fish stress response, such as the open-field test and the diving test. Our comparisons were conducted every 4 h during a 24-h cycle to analyze daily variations. Significant daily rhythms were detected for almost all analyzed behaviors in both tests. In general, the analyses suggested a greater stress response in adults during the daytime and in juveniles during the night-time, although not all indicators aligned in this direction. Moreover, we found average differences in zebrafish behavior, suggesting that juveniles were more sensitive to stress. Overall, these findings highlight the importance of considering developmental variation in the circadian pattern of stress response in non-mammalian species like zebrafish.

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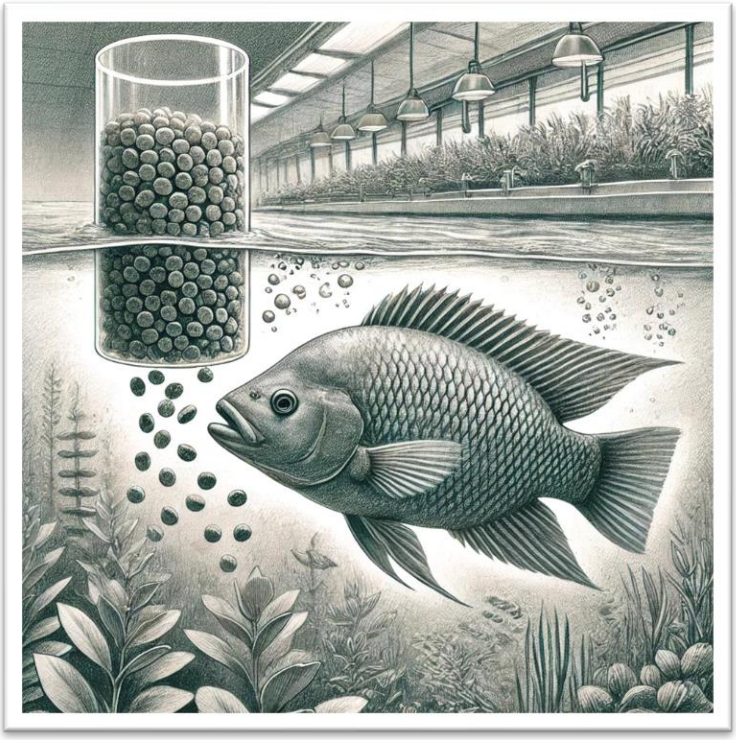
Chapter 3.

Impact of alternative and sustainable feed sources on the welfare of farmed fish



1. Effect of spirulina and quinoa as alternative in-feed ingredients on the welfare of Nile tilapia (*Oreochromis niloticus*)

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Abstract

Nutrition is a critical aspect of aquaculture, representing over half of the total costs in fish farming and being essential for ensuring the growth, health, and flesh quality of farmed fish. To reduce pressure on wild fish stocks and enhance sustainability, the industry has recently shifted towards alternative protein sources such as insect meal, microalgae, crop-based proteins, and terrestrial animal by-products. However, these in-feed ingredients present challenges, particularly in meeting the specific nutritional requirements of fish and ensuring palatability. This study evaluated the effects of novel and sustainable diets, incorporating quinoa (*Chenopodium quinoa*) and spirulina (*Arthorspira platensis*) as main ingredients, on the welfare of Nile tilapia (*Oreochromis niloticus*). To this end, three experimental diets (control, eco-efficient and organic) were administered to juvenile fish over 36 days while welfare indicators were assessed. Results indicated that tilapia fed both novel diets exhibited reduced weight gain compared to the control group, likely due to low palatability and acceptance, as observed in voluntary feed intake data. However, no differences in basal cortisol levels, weight loss, and mortality were observed between diet groups. Notably, the eco-efficient diet showed a weaker acute stress response in the open field test. Overall, while sustainable diets formulated with quinoa and spirulina showed promise in terms of enhancing the welfare and stress resilience of Nile tilapia, challenges related to palatability and growth performance remain significant. Further research is needed to determine whether this low palatability persists when diets are introduced from the earliest stages of development or under longer periods of feed habituation.

Introduction

Nutrition stands out as one of the most important fields within aquaculture research, primarily because aquafeeds account for more than half of the total cost of aquaculture farming operations (Gong *et al.*, 2019; Llagostera *et al.*, 2019). Additionally, proper fish nutrition is essential for ensuring optimal growth, health and even flesh quality in farmed species (Oliva-Teles, 2022). However, to reduce the pressure on wild fish stocks and promote sustainability, the industry has recently shifted to include alternative sources in aquafeeds as substitutes for fishmeal and fish oil ingredients. These alternative and sustainable sources include insect meal, crop-based proteins and microalgae (Fry *et al.*, 2016; Fry *et al.*, 2018). Furthermore, the environmental impact of aquaculture is closely tied to its carbon footprint and contributions to global warming (Crippa *et al.*, 2021; Hilborn *et al.*, 2018; Macleod *et al.*, 2020), making the inclusion of low-carbon ingredients (i.e., recycled and by-products) a key objective in the quest for more sustainable practices. Nevertheless, this transition still presents several challenges.

One major challenge is ensuring that these alternative ingredients meet the specific nutritional requirements of species, as each has unique dietary requirements in terms of protein, lipid, and amino acid composition (Gatlin *et al.*, 2007). Additionally, the palatability of novel ingredients is a critical factor, as poor acceptability can lead to reduced feed intake and, consequently, impaired growth and health (Hua and Bureau, 2012). Furthermore, nowadays, it is also essential to understand how these alternative ingredients influence other indicators of animal welfare such as stress markers (Ashley, 2007; Dara *et al.*, 2023; Sneddon *et al.*, 2016). Most evidence of alternative aquafeed ingredients explored their effects on growth and digestibility (Naylor *et al.*, 2009), but very few have investigated their impact on broader welfare traits such as behavioural or physiological indicators of stress (Ciji and Akhtar, 2021; Mendes *et al.*, 2024a).

In recent years, the spirulina (*Arthorspira platensis*) has emerged as a promising alternative protein source for aquafeeds. This microalga contains high-quality protein levels (Mulokozi *et al.*, 2019), vitamins (Madeira *et al.*, 2017), minerals (Christaki *et al.*, 2013) and fatty acid profile (Bernaerts *et al.*, 2019; Hamed *et al.*, 2015) as well as antioxidant pigments (Hussein *et al.*, 2021). Furthermore, its high tryptophan content (Demelash, 2018) may contribute to reducing aggression behaviours among conspecifics, as evidenced in teleost species such as rainbow trout *Oncorhynchus mykiss* (Winberg *et al.*, 2001), yamú *Brycon amazonicus* (Wolkers *et al.*, 2012) or Nile tilapia *Oreocrhomis niloticus* (Vieira *et al.*, 2021). Similarly, the pseudocereal quinoa (*Chenopodium quinoa*) has gained attention as a potential feed ingredient in aquaculture since its content of essential amino acids, which are scarce in other cereals (FAO, 2011; Molina-Poveda, 2017), and a wide range of vitamins and minerals (Repo-Carrasco *et al.*, 2011). Moreover, its inclusion in fish aquafeeds has been associated with improved digestibility (Gutiérrez-Espinosa *et al.*, 2011) and enhanced gene regulation and immune responses under stress (Ahmed *et al.*, 2020; Kumar *et al.*, 2024).

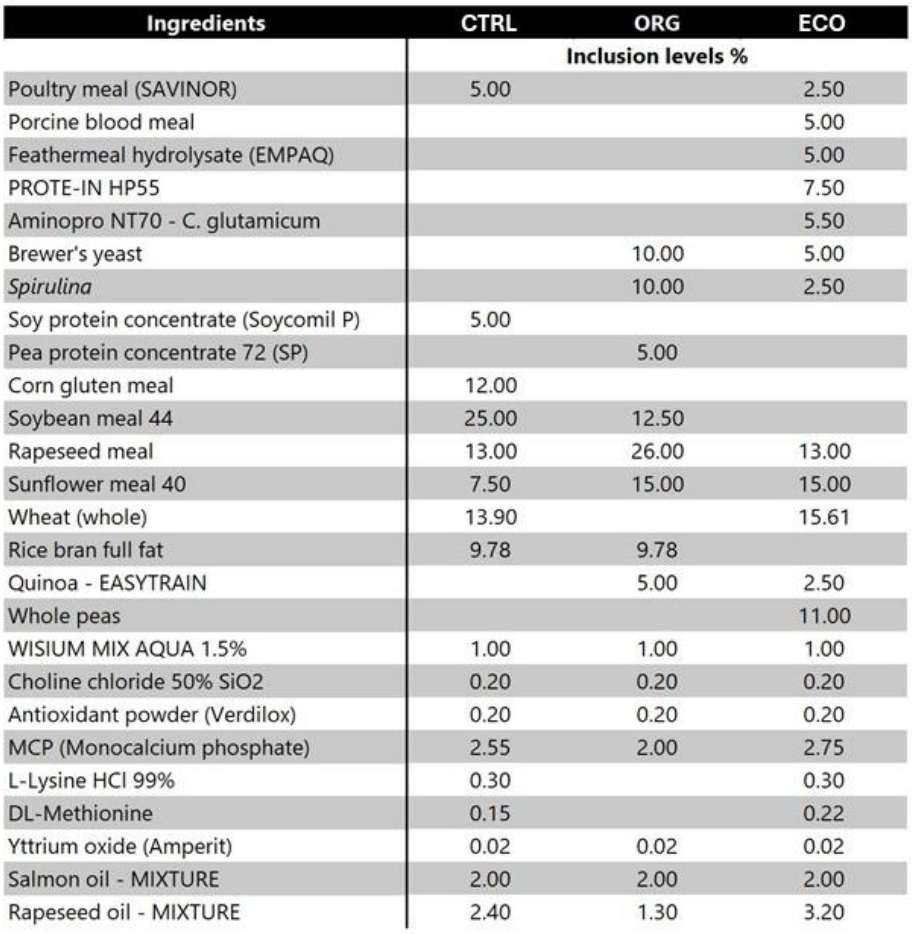
In this study, we aimed to assess the impact of novel sustainable diets on the welfare of Nile tilapia (*Oreochromis niloticus*). To this end, juvenile fish were fed two experimental diets formulated with emergent and circular economy-driven ingredients (i.e., spirulina, quinoa, insect meal, corn gluten meal and rice bran). The experimental diets were administered for 36 days, and the control diet was formulated to mimic current commercial feeds for the species. Then, the welfare of tilapia fish was assessed using a comprehensive set of welfare indicators, including mortality, growth performance, feed intake, and behavioural and physiological stress markers.

Material and methods

Experimental diets

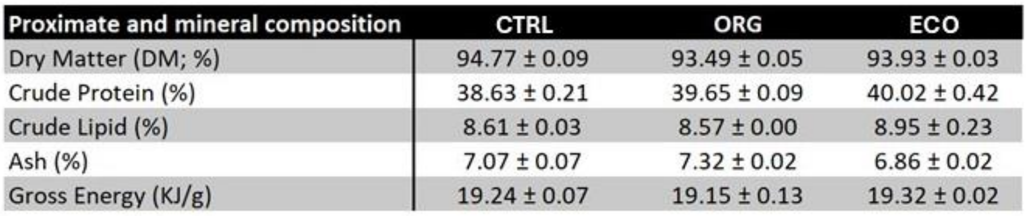
Three experimental diets were tested in this study (CTRL: control; ORG: organic; ECO: eco-efficient), designed and formulated by SPAROS (Olhão, Portugal). The formulation and manufacturing processes followed the methods outlined by Mendes *et al.* (2024b), ensuring consistency in ingredient composition and production.

The ingredient selection (Table 1) was chosen based on an eco-efficient and organic framework, market availability and nutritional composition. The control diet (CTRL) was formulated without fish meal but included corn gluten meal, elevated percentages of soybean meal, and other ingredients such as poultry meal and soy protein concentrate to mimic commercial formulations. The remaining two diets (ORG and ECO) were designed to include functional ingredients (spirulina and quinoa) and replace ingredients that may raise environmental concerns and/or ethical issues with more organic, emergent, and low carbon footprint alternatives. The ORG diet was formulated with ingredients compatible with organic certification and practices. In ORG, poultry and corn meal were removed and replaced by higher percentages of vegetable ingredients, together with spirulina and quinoa with 10.00 % and 5.00 % inclusion levels, respectively. The ECO diet was based on circular-driven subproducts and was highly diverse, having inclusion levels of 2.50 % for both spirulina and quinoa, while being also constituted by, for example, Porcine blood meal, PROTE-IN HP55 and Aminopro NT70 - *C. glutamicum*, which were absent in the other diets. In all diets, when fish oil was used it was in the form of a salmon oil (aquaculture by-product) and was also partially replaced by rapeseed oil. Diets had similar protein, lipid and energetic contents, with an average of 39.43 % crude protein, 8.71 % crude fat, 7.08 % ash, 1.11 % phosphorus and 19.24 KJ g^{-1} gross energy (Table 2). The inclusion levels had to be adjusted for each species, according to existing knowledge on tolerance to different ingredients as well as their nutritional and especially amino acid requirements, without compromising fish growth, development and welfare.



Ingredients	CTRL	ORG	ECO
	Inclusion levels %		
Poultry meal (SAVINOR)	5.00		2.50
Porcine blood meal			5.00
Feathermeal hydrolysate (EMPAQ)			5.00
PROTE-IN HP55			7.50
Aminopro NT70 - C. glutamicum			5.50
Brewer's yeast		10.00	5.00
Spirulina		10.00	2.50
Soy protein concentrate (Soycomil P)	5.00		
Pea protein concentrate 72 (SP)		5.00	
Corn gluten meal	12.00		
Soybean meal 44	25.00	12.50	
Rapeseed meal	13.00	26.00	13.00
Sunflower meal 40	7.50	15.00	15.00
Wheat (whole)	13.90		15.61
Rice bran full fat	9.78	9.78	
Quinoa - EASYTRAIN		5.00	2.50
Whole peas			11.00
WISIUM MIX AQUA 1.5%	1.00	1.00	1.00
Choline chloride 50% SiO2	0.20	0.20	0.20
Antioxidant powder (Verdilox)	0.20	0.20	0.20
MCP (Monocalcium phosphate)	2.55	2.00	2.75
L-Lysine HCl 99%	0.30		0.30
DL-Methionine	0.15		0.22
Yttrium oxide (Amperit)	0.02	0.02	0.02
Salmon oil - MIXTURE	2.00	2.00	2.00
Rapeseed oil - MIXTURE	2.40	1.30	3.20

Table 1 – Nutritional composition of the experimental diets (CTRL, ORG and ECO).



Proximate and mineral composition	CTRL	ORG	ECO
Dry Matter (DM; %)	94.77 ± 0.09	93.49 ± 0.05	93.93 ± 0.03
Crude Protein (%)	38.63 ± 0.21	39.65 ± 0.09	40.02 ± 0.42
Crude Lipid (%)	8.61 ± 0.03	8.57 ± 0.00	8.95 ± 0.23
Ash (%)	7.07 ± 0.07	7.32 ± 0.02	6.86 ± 0.02
Gross Energy (KJ/g)	19.24 ± 0.07	19.15 ± 0.13	19.32 ± 0.02

Table 2 – List of used ingredients in the experimental diets (CTRL, ORG and ECO) with inclusion levels (%).

Experimental subjects and design

Juvenile Nile tilapia (weight: 31.00 ± 0.50 g; n=396) were reared in the facility of the University of Trásos-Montes and Alto Douro (UTAD). Tilapia specimens used in this study were obtained from Tilaqua (Someren, the Netherlands) at the fry stage (~3 g) and then fed (Standard 4 Orange, Sorgal, Portugal; 3.5 mm 43% Crude Protein) and reared in UTAD fish facilities. Fish were approximately 3–4 months old at the time of the experiment. At the start of the trial, tilapia were randomly housed in tanks (70 × 60 × 60 cm; 250 L) containing 33 individuals and were kept at constant temperature of 25 ± 0.5°C and exposed to a 12:12 h light–dark (LD) artificial photoperiod (AquaRay, 6500 K) with lights on at 09.00 am. The initial biomass of experimental tanks was homogeneous (Coefficient of variation: CV < 2 %). All the tanks were equipped with constant aeration and supplied with filtered freshwater water from a mechanical and a biological filter. Tanks were barren to simulate the typical conditions of commercial facilities. The dietary groups (CTRL, ORG and ECO) were randomly assigned to replicate tanks (n= 4 tanks/diet group) and fish were fed with each diet for 36 days. Fish were habituated to the experimental diets for 2 days before starting the trial. Experimental diets were administered by hand until apparent visual satiation twice daily (10.00 a.m. and 15.00 p.m.). The administered feed was quantified throughout the trial for each experimental tank.

Welfare indicators

The total biomass from each experimental tank was weighed at the start (day 0) and at the end of the trial (day 36). Mortality records and feed intake were registered daily in all housing tanks. Once the diet trial was finished, weight gain (i.e., WG %; n= 4 tanks/diet) and voluntary feed intake (i.e., VFI; n=4 tanks/diet) were calculated as follows:

$$\text{WG (\%)} = \frac{Final\ weight\ (g) - Initial\ weight\ (g)}{Initial\ weight\ (g)} \times 100$$

$$\text{VFI} = \frac{Feed\ intake\ (g)}{\left(\frac{Initial\ weight\ (g) + Final\ Weight(g)}{2} \times 100\right)\ /\ days}$$

Moreover, on day 30 and after being fasted for 24 h, blood was collected from the caudal vein of 3 PIT-tagged fish (14.92 ± 1.88 g) per tank (n= 12 fish/diet) and consequently centrifuged (Qlabo, Portugal) at 2500 g for 10 minutes at 4ºC. Thus, plasma was collected for basal cortisol assessment. For every measurement, all fish were previously anaesthetised (2-phenoxyethanol), while obtained samples were stored at -20°C. Plasma cortisol concentrations were determined with a direct competitive ELISA kit (TECAN, Hamburg, Germany). Samples were assayed in triplicate following the recommendations of the manufacturer. The absorbance was assessed in a microplate spectrophotometer (Multiskan™ GO; Thermo Scientific), and a calibration curve was generated based on known standards.

Furthermore, on day 31-33, additional fish from each experimental tank (64.93 ± 27.81 g) were exposed to the open field test (OF) to assess the behavioural stress response to a novel environment. OF tests were conducted during the morning (09.00 a.m. – 11.00 a.m.) or in the evening (16.00 p.m. – 18.00 p.m.) to further explore potential chronobiological effects of the experimental diets on stress responses (Pintos *et al.*, 2023; Sánchez-Vázquez *et al.*, 2011; Sánchez-Vázquez *et al.*, 2019). This resulted in 12 fish from each diet group being exposed to the open field at each time of the day (n=12 fish/diet/time). All fish were fed 30 min before the experiment to avoid conditioning fish behaviour with hunger states. At the beginning of each OF trial, the experimenter collected the subject/s from the maintenance tanks and immediately released it/them into the middle of the OF arena using a net. Once in the OF arena, the behaviour of the subjects was recorded for 10 min using a full-HD camera (ELP USBFHD08S-MFV, Shenzhen Ailipu Technology Co. Ltd, Shenzhen, China) placed 2 m above the arena to record the experiments in 1920 × 1080 pixels of resolution and 30 frames per second. The arena consisted of a white plastic arena (120 × 100 × 40 cm) filled with 15 cm of water. The water was changed between each trial to prevent exposure to the chemical cues from the previous experimental subject. For each video, different well-reported fish anxiety-like behaviours were analysed with Ethovision XT® tracking software (Noldus Information Technology, Wageningen, The Netherlands). These behaviours included time spent in the outer part of the OF arena (thigmotaxis), time spent motionless with a speed lower than 1cm/s (freezing) and distance travelled (activity). The experimental design is summarised in Figure 1.

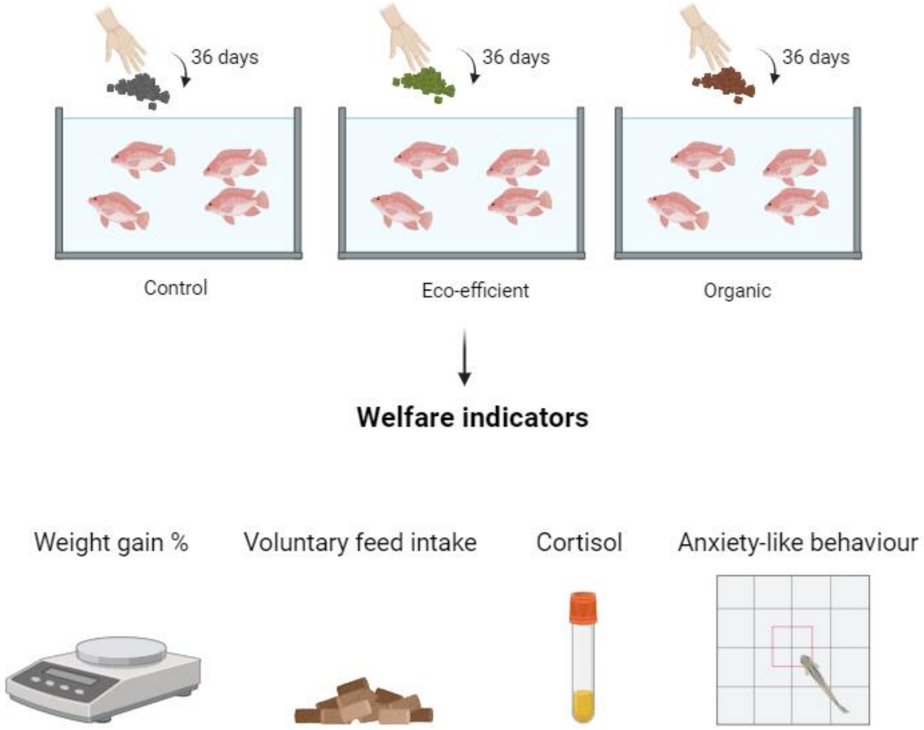


Figure 1: Illustrative scheme of the experimental design. Juvenile tilapia were fed for 36 days with three different experimental diets: control, eco-efficient and organic diet (n=120 fish/diet group). Thereafter, the effect of the experimental diets was assessed through different welfare indicators such as weight gain after 36 days (n= 24 fish/diet group), daily voluntary feed intake in the experimental (n= 4 tanks/diet group), cortisol levels (n=24 fish/diet group) and anxiety-like behaviours in the open field test (n=24 fish/diet group).

Statistical analysis

Statistical analyses were performed using R Statistical software version 4.0.1 (The R foundation for Statistical Computing Vienna Austria http://www.r-project.org) and all analyses were conducted by functions from the R base package. To analyse the effect of diets on weight gain, voluntary feed intake and cortisol, one-way analyses of variance (ANOVA I) were performed considering the diet group as a fixed factor (3 levels: CTRL, ECO and ORG). To analyse the effect of diets on the behavioural stress response to the open field test, ANOVAs II were performed considering the diet group (3 levels: CTRL, ECO and ORG) and the time of the day (2 levels: morning and evening) as fixed factors and the interaction between them. If necessary, post-hoc Tukey HSD test was conducted to study differences between experimental groups. Normality and suitability for the tests were verified by the Shapiro-Wilks test and QQ plot. Data that did not meet normality were transformed through square root (cortisol), logarithmic (freezing behaviour), rankit (thigmotaxis behaviour) transformations.

Results

The ANOVA test on growth performance showed that, on day 36, there were significant differences in weight gain between diet groups (ANOVA: $F_{2,9} = 545.7$, $p < 0.01$). Post-hoc analysis revealed that the weight gain of CTRL tilapia was higher than those registered in the ECO and the ORG groups (Tukey: Figure 2A). Furthermore, the weight gain of the ECO group was higher than those registered in the ORG group (Tukey: Figure 2A). The same pattern was observed for the voluntary feed intake parameter (ANOVA: $F_{2,9} = 91.39$, $p < 0.01$), where the CTRL group ingested a significantly higher amount of feed per day than the ECO and ORG groups: CTRL>ECO>ORG (Tukey: Figure 2B). No mortality was detected in any of the experimental groups.

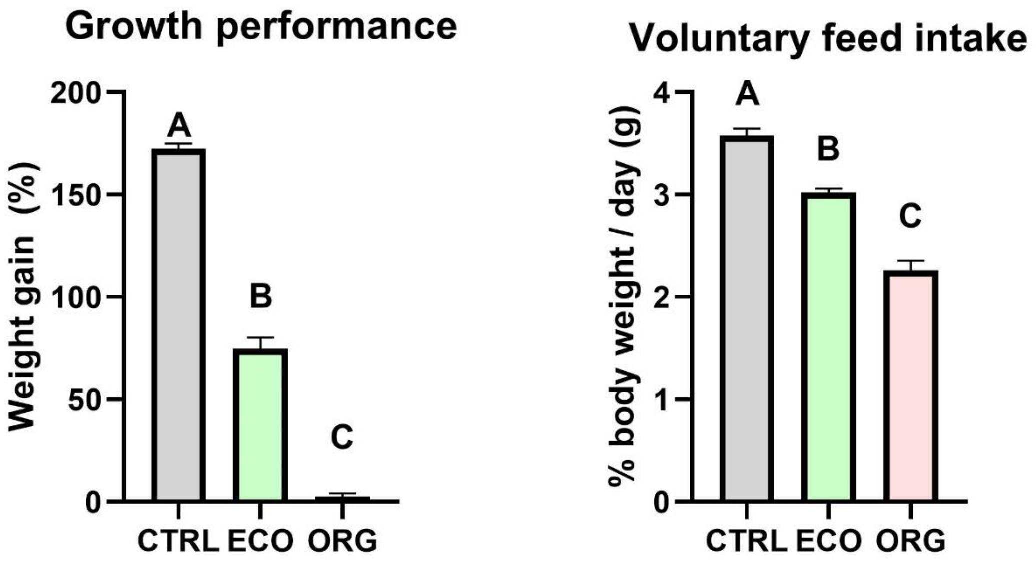


Figure 2: A. Growth performance and **B.** Voluntary feed intake of juvenile Nile tilapia fed with different experimental diets ((CTRL = control diet (grey); ECO = eco-efficient diet (green); ORG = organic (pink)) for 36 days (n=24 fish/diet group). Data are presented as mean ± standard error and different letters indicate statistical differences between diet groups.

Cortisol analysis did not show significant differences among experimental groups at day 30 (ANOVA: $F_{2,32} = 0.03$, $p = 0.96$; Figure 3).

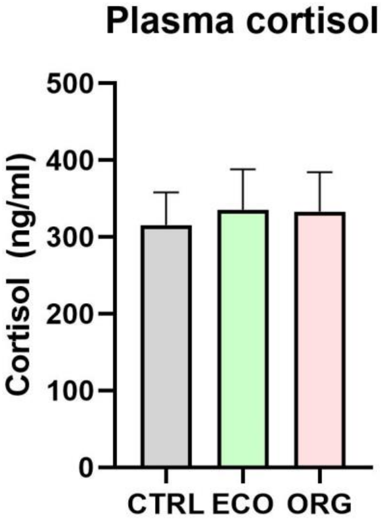


Figure 3: Basal plasma cortisol levels of juvenile Nile tilapia fed with different experimental diets ((CTRL = control diet (grey); ECO = eco-efficient diet (green); ORG = organic (pink)) at day 30 (n= 24 fish/diet group). Data are presented as mean ± standard error and different letters indicate statistical differences between diet groups.

The open field test revealed that thigmotaxis behaviour did not significantly vary according to the time of the day and the diet group (ANOVA: *diet*: $F_{2,66} = 1.07$, $p = 0.34$; *time*: $F_{1,66} = 0.65$, $p = 0.42$; *diet* × *time*: $F_{2,66} = 2.09$, $p = 0.31$; Figure 4A). However, on average, tilapia from all diet groups exhibited more time in the outer part of the experimental tank than expected by random movements across the arena (t-test: CTRL: $t_{23} = 15.70$, $p < 0.01$; ECO: $t_{23} = 2.30$, $p = 0.03$; ORG: $t_{23} = 14.73$, $p < 0.01$). This indicated the expected thigmotaxis behaviour of fish in the open field test.

Similarly, activity behaviour was not affected by the diet group and the time of the day (ANOVA: *diet*: $F_{2,66} = 0.73$, $p = 0.48$; *time*: $F_{1,66} = 3.67$, $p = 0.059$; *diet* × *time*: $F_{2,66} = 0.03$, $p = 0.96$; Figure 4B). Conversely, significant differences between experimental groups were found in freezing behaviour, indicating the diet group influenced freezing according to the time of the day (ANOVA: *diet* × *time*: $F_{2,66} = 9.78$, $p < 0.01$). Post-hoc analysis revealed that tilapia from the ECO group exhibited reduced freezing in the evening when compared to CTRL and ORG group at that time of the day (Tukey: CTRL evening *v.* ECO evening, $t_{12} = 4.28$; $p = 0.03$; CTRL evening *v.* ORG evening, $t_{12} = 5.18$; $p < 0.01$; Figure 4C).

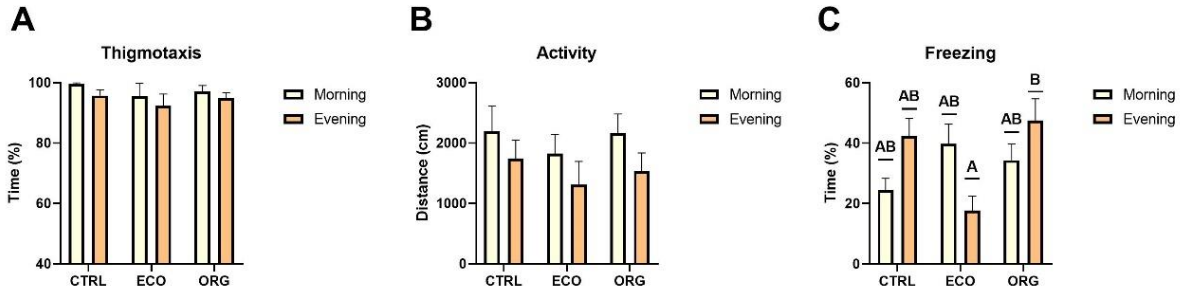


Figure 4: Anxiety-like behaviours of juvenile Nile tilapia exposed to the open field test at different times of the day (morning = yellow; evening = orange) and according to the diet group (CTRL = control; ECO = eco-efficient; ORG = organic; n= 12 fish/diet/time). **A.** Thigmotaxis **B.** Activity and **C.** Freezing behaviours. Data are presented as mean ± standard error and different letters indicate statistical differences between experimental groups

Discussion

This study documented the effect of two diets based on emergent and sustainable ingredients (i.e., spirulina, quinoa, insect meal, animal by-products) on the welfare of juvenile Nile tilapia. Surprisingly, tilapia fed with both eco-efficient (ECO) and organic (ORG) diets did not exhibit the expected growth performance, registering reduced weight gain in comparison to the control. This was, at least, partially due to the low acceptance of these diets by tilapia juveniles. However, our findings indicated no differences in basal physiological stress levels (i.e., cortisol), no weight loss, and no mortality in these groups compared to the control. Indeed, a slight reduction in the acute stress response of tilapia fed with the ECO diet was observed in the open field test. Therefore, it is possible that the low palatability of these diets may have masked the potential benefits of these alternative ingredients on the welfare of Nile tilapia.

Cortisol analysis showed no significant differences in basal cortisol levels among diet groups. This physiological trait is a well-established indicator of chronic and acute stress in fish (Ashley, 2007; Schreck and Tort, 2016; Sneddon *et al.*, 2016). Hence, our results suggested that none of the sustainable diets tested in this study induced significant chronic stress. This finding is particularly noteworthy for the ECO diet, which even exhibited reduced freezing behaviour during the evening in response to the open field test. This behaviour has been reported as a stress indicator in Nile tilapia exposed to the open field test (Pintos *et al.*, 2024). Therefore, this result indicated a slight reduction in the acute stress response of those tilapia fed with the ECO diet. Accordingly, this observed variation in the behavioural stress response throughout the day has been previously described in teleost species (Pintos *et al.*, 2023; Thoré *et al.*, 2021).

Critically, the correlation between the low voluntary feed intake and the reduced growth performance of ECO and ORG groups strongly suggests that the palatability of these novel diets was suboptimal. This is consistent with findings by Mendes *et al.* (2024b), who also reported lower acceptability of these novel diets in an 8-week trial with juvenile tilapia. Therefore, alternative strategies should be considered in future studies to enhance the palatability of alternative aquafeeds. For instance, longer habituation periods could be evaluated to determine if extended exposure (i.e., beyond 2 days of habituation) allows tilapia to adapt to these alternative diets, potentially leading to improved feed acceptability and, consequently, proper growth performances. Previous studies have shown that the acceptance of alternative fish diets without fish meal and oil can be particularly challenging in farmed fish, leading to decreased feed intake and subsequent performance impairment (Watanabe *et al.*, 1998; Slawski *et al.*, 2011). Indeed, studies in tilapia have shown an adequate acceptability of spirulina from the onset of exogenous feeding in larval tilapia (Lu *et al.*, 2002; Olvera-Novoa *et al.*, 1998). Therefore, introducing these diets at earlier developmental stages, when fish are more adaptable to changes in diet, could enhance palatability and acceptance (Clarkson *et al.*, 2017;

Geurden *et al.*, 2013). Additionally, incorporating feed attractants like amino acids, nucleotides, or feeding stimulants such as betaine could significantly improve feed intake and acceptance (Kasumyan and Døving, 2003; Li and Gatlin, 2006). Similarly, reformulating diets with more easily digestible and appealing protein sources, such as protein hydrolysates or enzymatically treated proteins, could also play a crucial role in improving overall diet palatability as well as nutrient absorption (Espe *et al.*, 1999; Hevrøy *et al.*, 2005). Indeed, Mendes *et al.* (2024b) have documented that tilapia fed with ECO and ORG diets presented reduced digestibility and feed utilisation efficiency, which suggests that the poor growth performance observed in our study was not solely due to reduced feed intake but also to lower nutrient absorption.

In conclusion, while sustainable diets formulated with functional ingredients such as quinoa and spirulina showed promising results for enhancing the welfare and stress resilience of Nile tilapia, challenges related to growth performance remain significant. The reduced weight gain of tilapia fed with alternative diets suggests the need for further refinement either of feeding strategies and/or diet formulations, thereby enhancing feed intake and growth rates. It would be valuable to investigate whether the observed low palatability persists when sustainable diets are introduced from the earliest stages of development. Despite these challenges, fish fed with sustainable diets did not lose weight and showed basal cortisol levels and mortality rates similar to those observed in the control group. This result may underscore the potential of these diets to contribute to sustainable aquaculture practices. Future research should focus on optimising the formulation of these diets to balance palatability and nutritional value, ensuring both the health and growth of tilapia (Mendes *et al.*, 2024a; Mendes *et al.*, 2024b). Lastly, although many commercial diets of omnivorous species such as tilapia contain very low levels of fishmeal and fish oil (Oliva-Teles *et al.*, 2022), it is essential to strive towards still minimising their inclusion and continue exploring alternative components that further reduce the carbon footprint associated with aquafeed production, such as recycled and by-products from other industries (Clark and Tilman, 2017; MacLeod *et al.*, 2020; Zhang *et al.*, 2024). This approach will further enhance the sustainability of tilapia farming, contributing to more environmentally friendly aquaculture practices.

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2. Welfare outcomes of Nile tilapia (*Oreochromis niloticus*) fed a novel spirulina-based diet

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Abstract

Aquaculture demands alternative feed sources that reduce reliance on fish meal and fish oil and promote sustainable farming practices. However, formulating diets free of those components that maintain optimal growth and health remains a challenge in aquaculture. While most studies on sustainable diets focus on digestibility and muscle nutrient composition, few explore diet effects on welfare indicators such as stress response, stress recovery and aggression. This study assessed the impact of a novel spirulina-based diet (Easyfeed®) on the welfare of Nile tilapia (*Oreochromis niloticus*). To this end, juvenile fish were divided into two dietary groups: a control group fed with a commercial diet specifically designed for tilapia and an experimental group fed with the Easyfeed diet, formulated according to organic standards. Over 30 days, growth performances were evaluated for each diet group. Additionally, different behavioural tests were conducted to assess acute stress response (i.e., diving test), stress recovery (i.e., feed intake test) and aggression (i.e., mirror test). Results indicated that tilapia fed with Easyfeed showed growth performances similar to those observed in the control group. However, the Easyfeed group found a lower feed conversion ratio and a lower specific growth rate. This suggested more efficient feed use but slower daily growth in those tilapia fed with the alternative diet. Moreover, behavioural assays revealed that tilapia fed with Easyfeed exhibited reduced stress response to the diving test, evidenced by increased activity behaviour. Conversely, no significant differences were found in the feed intake and mirror tests. Our findings generally indicated that the spirulina-based diet supports proper growth and reduces stress in Nile tilapia, suggesting potential welfare benefits. Nonetheless, the slower growth rate and economic aspects of using organic feed ingredients require consideration, and further long-term studies are necessary to fully evaluate the impacts on fish health and performance.

Introduction

Fish meal and fish oil have long been fundamental components of aquaculture feeds, providing essential nutrients that promote growth and health in farmed fish (Tacon and Metian, 2008). However, the increasing demand for these resources has raised concerns about their sustainability. The reliance on wild-caught fish for meal and oil not only depletes natural fish stocks but also poses environmental and economic challenges (Naylor *et al.*, 2009). Consequently, the inclusion of these ingredients has been drastically reduced in recent years, leading to an active search for alternative and sustainable ingredients that can ensure the growth and health of farmed fish while mitigating environmental impacts (Bandara *et al.*, 2018; Gasco *et al.*, 2018; Oliva-Teles *et al.*, 2022).

Although efforts to replace fish meal and oil are ongoing, achieving complete substitution remains a significant challenge. Many studies have shown that total replacement with alternative ingredients can lead to reduced growth rates and compromised health, especially in carnivorous species which have higher protein requirements (Desai *et al.*, 2012; Gatlin *et al.*, 2007; Krogdahl *et al.*, 2010; National Research Council, 2011; Oliva-Teles *et al.*, 2015). Furthermore, restricting the use of traditional raw materials often results in increased costs and a reduced, potentially more variable, supply base (Kaushik and Troell, 2010). This evidence suggests that formulating novel aquafeeds involves multiple considerations to achieve success and profitability in the aquaculture market.

However, this challenge is more feasible in omnivorous and herbivorous species such as carp (*Cyprinus carpio*), African catfish (*Clarias gariepinus*), channel catfish (*Ictalurus punctatus*) or Nile tilapia (*Oreochromis niloticus*) (Oliva-Teles *et al.*, 2022). These species have relatively lower protein requirements (i.e., from 25% to 35%) and have shown positive results when replacing fish meal and oil at higher levels, even up to 100%

(Elesho *et al.*, 2021; Kari *et al.*, 2020; Gaylord and Rawles, 2005; Sarker *et al.*, 2016). Nevertheless, most evidence of sustainable diets has focused on the effects on growth, digestibility, and muscle nutrient composition (Naylor *et al.*, 2009), with few examining their impact on broader aspects of fish welfare, such as stress response, aggression and overall well-being (Ciji and Akhtar, 2021).

The microalgae Spirulina *Arthrospira platensis* has gained significant recognition in the aquaculture industry due to its high protein content, essential amino acids, and bioactive compounds that can enhance fish health and immunity without affecting growth performances (Becker, 2013; Olvera-Novoa *et al.*, 1998; Mohammadiazarm *et al.*, 2021; Teimouri *et al.*, 2013). However, despite several reports in mammals supporting reduced anxiety and stress upon spirulina uptake (Basavarajappa *et al.*, 2023; Moradi *et al.*, 2021; Moradi-Kor *et al.*, 2020), there is a lack of information regarding its effect on fish stress responses (de Mattos *et al.*, 2019).

In this study, we tested the impact of a novel and sustainable spirulina-based diet (hereafter, Easyfeed) on the welfare of Nile tilapia. This diet was formulated according to organic standards and the nutritional requirements of the species. To this end, tilapia juveniles were fed the Easyfeed diet for 30 days, and their growth performance was assessed. Furthermore, the effect of experimental diets on acute stress response, aggression and stress recovery was assessed by exposing fish to different behavioural assays. The control experiment consisted of tilapia fed with a commercial diet with similar nutritional composition and specifically designed for Nile tilapia.

Materials and methods

Experimental groups and housing

Juvenile tilapia (2-3 months old; n = 90) were reared in the fish facility of the University of Murcia (Murcia, Spain) at 26 ± 1 ºC in 11 L housing tanks (n= 15 fish/tank). Housing tanks were randomly assigned to one of two experimental groups: the control or the Easyfeed group (n= 3 tanks/group). All housing tanks were equipped with constant aeration and supplied with filtered freshwater from mechanical and biological filters. The housing room was exposed to a 12:12 light-dark artificial photoperiod (AquaRay, 6500 K). To simulate the typical conditions of commercial facilities, housing tanks were kept barren and without physical enrichment.

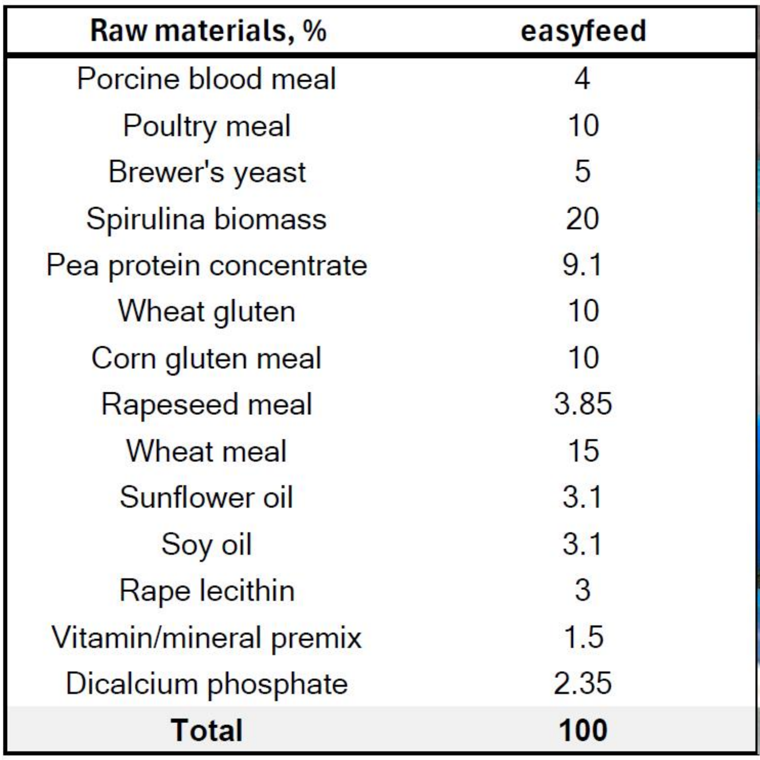
Fish from both experimental groups were fed once daily at 3% of tank biomass for 14 days before the experiment began. This was done to habituate tilapia to experimental diets. After habituation, fish were first weighed and feed was administered at 3% of initial biomass from day 0 to day 15. On day 15, fish weight was measured and feed was administered at 3% of the updated biomass until the end of the experiment (day 30).

Experimental diets

SPAROS Lda. (Olhão, Portugal) formulated and produced the Easyfeed diet according to the nutritional requirements of Nile tilapia (NRC, 2011). The selection of raw materials and feed additives was done in accordance with the organic standards of the European Commission Regulation (EC) Nº 710/2009 of 5 August 2009 (amending Regulation (EC) Nº 889/2008) for the implementation of Council Regulation (EC) Nº 834/2007. This information is summarised in Table 1.

The Easyfeed diet was manufactured by cold extrusion process, in which powder ingredients were mixed according to the target formulation in a double-paddle mixer (model RM90, MAINCA Spain) and ground (below 100 µm) in a micro pulveriser hammer mill (model SH1, Hosokawa-Alpine, Germany). Subsequently, the oils were added to the mixtures, which were humidified with 20-25% water and agglomerated by a low-shear and low-temperature extrusion process (ITALPLAST, Italy). Extruded pellets (0.5-0.8 and 0.8-1.2 mm) were dried in a vibrating fluid bed dryer (model DR100, TGC Extrusion, France). After drying, feed pellets were sieved (Vibroest, Russel-Finex, UK) to refine particle size ranges (0.5-0.8 mm diameter). Diets were packed in sealed aluminium bags and stored at room temperature, but in a cool and aerated emplacement.

The control diet consisted of a commercial aquafeed specifically produced for Nile tilapia (Aquate®, Alltech Coppens, Germany), with similar granulometry and nutritional composition to those obtained for the Easyfeed diet (Table 2).



Raw materials, %	easyfeed
Porcine blood meal	4
Poultry meal	10
Brewer's yeast	5
Spirulina biomass	20
Pea protein concentrate	9.1
Wheat gluten	10
Corn gluten meal	10
Rapeseed meal	3.85
Wheat meal	15
Sunflower oil	3.1
Soy oil	3.1
Rape lecithin	3
Vitamin/mineral premix	1.5
Dicalcium phosphate	2.35
Total	**100**

Table 1: Formulation of the Easyfeed diet.

	Unit	Easyfeed 0.5-0.8mm	control 0.5-0.8 mm
Moisture	g/100 g	5.3 ± 0.5	-
Ash	g/100 g	8.0 ± 0.3	7.8
Crude protein (N x 6.25)	g/100 g	47.2 ± 1.2	45

Total fat	g/100 g	13.1 ± 0.8	11
Crude fibre	g/100 g	2.1 ± 0.7	1
Starch	g/100 g	15.3 ± 0.5	-
Gross energy	MJ/kg	20.8 ± 0.2	20.1

Table 2: Nutritional composition of experimental diets. Data are presented as mean ± standard deviation (n=2).

Welfare indicators

The welfare of juvenile tilapia fed with both experimental diets was assessed by growth performance parameters (average fish weight, feed conversion ratio, specific growth rate), acute stress responses (diving test and feed intake recovery test) and aggression levels (mirror test). The experimental design is summarised in Figure 1.

For the growth performance, the total biomass of each experimental tank was weighed at the beginning (initial) and at the end (final) of the experimental trial, which lasted 30 days (n=15 fish/tank; n=3 tanks/diet). Thereafter, average fish weight, feed conversion ratio (FCR) and specific growth rate (SGR) were calculated for each experimental group using the following formula:

$$\mathrm{SGR}(\%/\mathrm{day}) = \frac{(\ln(\mathrm{Final\ weight}) - \ln(\mathrm{Initial\ weight})}{Days}$$

$$\mathrm{FCR} = \frac{Weight\ of\ feed\ consumed}{Weight\ gain}$$

The acute stress response of fish was behaviourally assessed by exposing individuals from each diet group (n=12 fish/diet) to the diving test assay. This behavioural test consisted of releasing the experimental subject in a novel environment (glass rectangular arena: 25 × 15 × 15 cm) and recording its spontaneous behaviour for 10 minutes. This procedure is commonly used to assess stress responses in teleost species (Blaser and Rosemberg, 2012). The experimental arena was illuminated from above with a white LED strip (AquaRay, 6500 K), and video recordings were captured by an ELP full-HD camera located 40 cm from the front of the arena. A computer running the Ethovision XT®

software performed the live-tracking of fish behaviour while different well-established fish anxiety-like behaviours were measured (Collier *et al.*, 2017). These included the time spent in the lower half of the arena (bottom dwelling), the time spent motionless (freezing; threshold = movement speed lower than 1 cm/s), the mean distance travelled (activity) and the angular velocity of the path (erratic movement).

The mirror test consisted of individually exposing 10 fish from each diet group to a novel environment (glass rectangular arena: 25 × 15 × 15 cm) that presented a mirror (15x15 cm) in one of the tank sides (n=10 fish/group). Once tilapia was released in the novel arena, its spontaneous behaviour was recorded for 10 minutes with the ELP camera. Then, the experimental observer manually counted agonistic behaviour, following those behaviours described by Barreto *et al.* (2009). These included the number of bites directed towards the fish's mirror image (i.e., bite) and the latency to the first bite attack (i.e., latency to attack).

Lastly, the feed intake recovery test consisted of individually exposing fish (n=10 fish/diet) to a net-chasing stressor for 2 minutes (i.e., chasing the experimental subject with a fish net in a 2L tank) and, consecutively, exposing the fish to a novel environment (glass rectangular arena: 25 × 15 × 15 cm). Once released in the novel arena, three pellets were administered by an automatic feeder and fish behaviour was recorded for 10 minutes. The latency to take the first and all pellets by experimental subjects was measured in each trial. Pellets were administered according to each diet group (i.e., control or Easyfeed pellet). Feed intake has been established as a welfare indicator across fish species (Huntingford *et al.*, 2006; Martins *et al.*, 2012). Furthermore, previous studies have shown that confinement and handling stressors for 2-6 minutes are sufficient to trigger fish stress responses, including in feeding behaviour (de Abreu *et al.*, 2020; Moscicki and Hurd, 2015; Pickering *et al.*, 1981; Ramsay *et al.*, 2009).

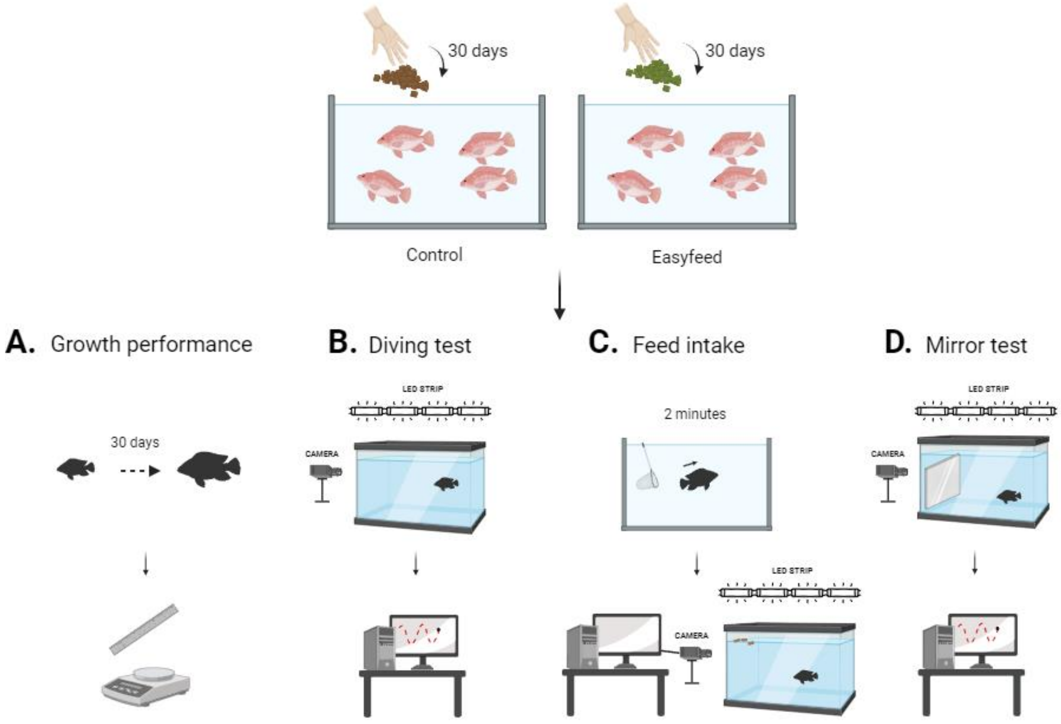


Figure 1: Illustrative scheme of the experimental design. Juvenile tilapia were fed for 30 days with a control or a spirulina-based diet (i.e., Easyfeed) (n= 3 tanks/diet group; 15 fish/tank). After the diet treatment, different welfare indicators were assessed to examine the impact of experimental diets. **A**. Growth performance (n=3 tanks/group). **B**. Diving test (n=12 fish/group). **C**. Feed intake recovery test (n=10 fish/group). **D**. Mirror test (n= 10 fish/group).

Statistical analysis

Statistical analysis was performed in R Statistical software version 4.0.1 (The R foundation for Statistical Computing Vienna Austria http://www.r-project.org). To analyse the effect of experimental diets (2 levels: Easyfeed, control) on stress and aggressive behaviour, multiple two-tailed *t-tests* were performed. A two-way analysis of variance (ANOVA II) was conducted to detect significant differences in the average weight of fish from the different experimental groups. The ANOVA II considered the diet group (2 levels) and the experiment stage (2 levels: initial, final) as fixed factors and the experimental tank as random factor. Normality was verified by the Shapiro-Wilks test and QQ plot. Descriptive statistics is represented in the text as mean ± standard error and the significance level was set at $p < 0.05$. Additionally, one-tailed and two-tailed *t-tests* were conducted to determine whether the bottom dwelling was driven by stress or by random movements across the diving test arena (i.e., expected value for random movements: 50%) and initial differences in weight between experimental groups, correspondingly.

Ethical note

The present research was conducted in the Department of Physiology facilities of the University of Murcia (Spain). Fish were reared following Spanish legislation on Animal Welfare and Laboratory Practices. Experimental protocols were performed following the Guidelines of the European Union (2010/63/UE) and Spanish legislation (RD 53/2013 and Law 32/2007) for the use of laboratory animals. They were also approved by the Committee of the University of Murcia on Ethics and Animal Welfare (A13230303).

Results

Tilapia fed with the control diet registered an initial average weight of 7.85 ± 1.58 g, whereas tilapia fed with the Easyfeed diet documented an average of 7.73 ± 0.89 g. These average values indicated similar initial weights for both experimental groups (*t*-test: $t_4 = 0.06$, $p = 0.94$). At the end of the experiment, tilapia from the control group exhibited an average fish weight of 22.50 ± 4.28 g while tilapia from the Easyfeed group registered 16.85 ± 1.40 g. The ANOVA test on these variables detected a significant effect of the stage of the experiment on the average weight of tilapia (ANOVA: $F_{1,4} = 74.20$, $p < 0.01$) but did not did so for the experimental diet (ANOVA: $F_{1,4} = 0.83$, $p = 0.41$). This indicated that tilapia significantly increased their average weight at the end of the experiment regardless the experimental diet (Figure 2A). Furthermore, no significant interaction between stage and diet factors was observed in the growth performance (ANOVA: $F_{1,4} = 4.01$, $p = 0.11$). Moreover, both feed conversion ratio (FCR) and specific growth rate

(SGR) found increased values in the control group when compared to the Easyfeed group (FCR: *t-test*, $t_4 = 4.23$, $p = 0.01$, Figure 2B; SGR: *t-test*, $t_4 = 7.60$, $p < 0.01$, Figure 2C).

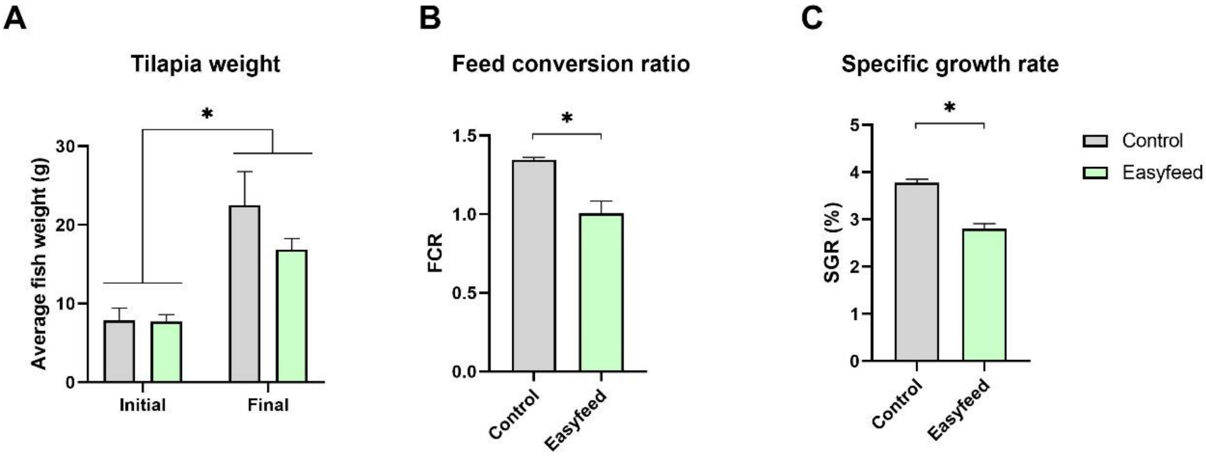


Figure 2: Growth performance indicators of juvenile Nile tilapia fed with control (grey) and Easyfeed (green) diets. **A.** Fish weight. **B.** Feed conversion ratio. **C.** Specific growth rate. Data are presented as mean ± standard error and asterisks indicate statistical differences between experimental groups.

Furthermore, t-test analyses on the variables examined in the diving test did not find significant differences between experimental groups in bottom dwelling (t-test: $t_{22} = 0.05$, $p = 0.95$, Figure 3A), freezing (t-test: $t_{22} = 1.77$, $p = 0.08$, Figure 3C) and erratic movement (t-test: $t_{22} = 1.60$, $p = 0.12$, Figure 3D) behaviours. However, tilapia from both experimental groups exhibited the expected bottom dwelling response to the diving test (i.e., more time in the bottom of the tank than expected by random movements across the arena) (t-test: control: $t_{11} = 2.57$, $p = 0.02$; Easyfeed: $t_{11} = 3.48$, $p < 0.01$) and significant differences between them were observed in activity behaviour (t-test: $t_{22} = 2.85$, $p < 0.01$), indicating more distance travelled in the Easyfeed group than in the control (Figure 3B).

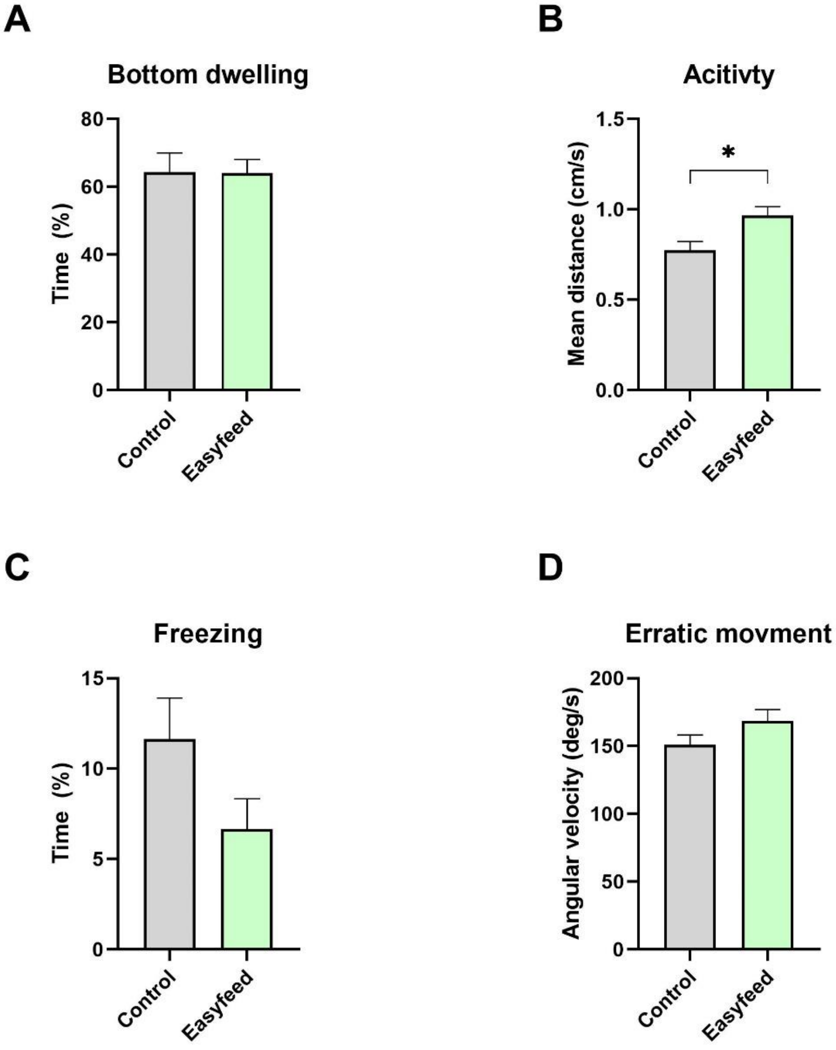


Figure 3: Behavioural performance of juvenile Nile tilapia fed with control (grey) and Easyfeed (green) diets in the diving test (n=12 fish/group). Data are presented as mean ± standard error and asterisks indicate statistical differences between experimental groups.

The mirror test did not register significant differences between experimental groups in any of the analysed variables such as latency to attack (t-test: $t_{22} = 0.18$, $p = 0.85$, Figure 34) and bite (t-test: $t_{10} = 0.37$, $p = 0.71$, Figure 4B) behaviours. Similarly, the feed intake recovery test revealed no significant differences between experimental groups in the latency to take the first (t-test: $t_{22} = 0.97$, $p = 0.33$, Figure 5A) and all (t-test: $t_{19} = 0.99$, $p = 0.33$, Figure 5B) pellets.

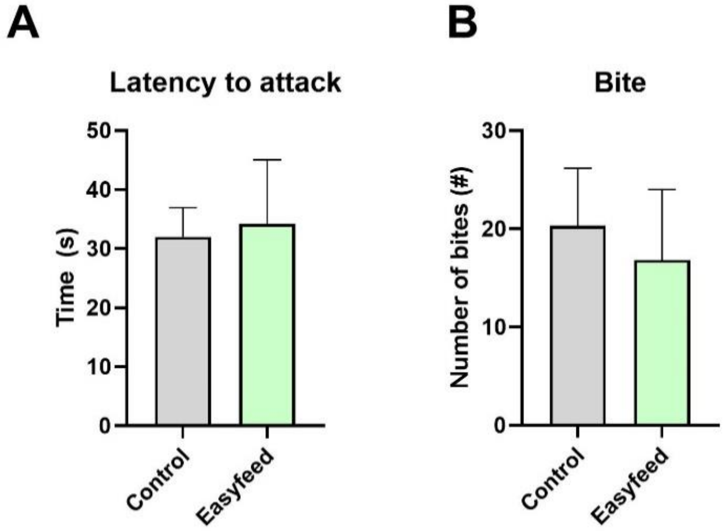


Figure 4: Behavioural performance of juvenile Nile tilapia fed with control (grey) and Easyfeed (green) diets in the mirror test (n= 10 fish/group). **A.** Latency to attack and **B.** Bite behaviours. Data are presented as mean ± standard error.

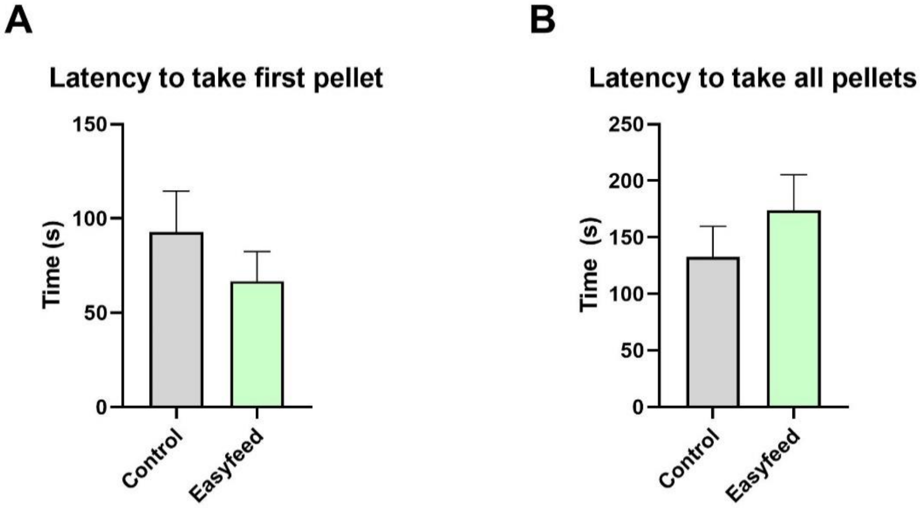


Figure 5: Behavioural performance of juvenile Nile tilapia fed with control (grey) and Easyfeed (green) diets in the feed intake recovery test (n= 10 fish/group). **A.** Latency to take first pellet. **B.** Latency to take all pellets. Data are presented as mean ± standard error.

Discussion

This study explored the effect of a novel spirulina-enriched diet on Nile tilapia welfare. Results indicated that tilapia fed with the Easyfeed diet for 30 days exhibited a similar growth performance to those obtained in tilapia fed with a commercial diet specifically designed for the species. Furthermore, the differences observed in the diving test suggested a slight reduction in the stress response of those fish fed with Easyfeed. Considering that this novel formulated feed is free of fish meal and fish oil and contains alternative raw materials that comply with organic standards, these findings support the robustness of sustainable diets on the growth performance and welfare of freshwater-farmed species such as Nile tilapia.

Notably, although tilapia fed with Easyfeed documented no significant differences in growth performance compared to the control diet, the feed conversion ratio (FCR) and specific growth rate (SGR) presented seemingly contradictory results. The lower FCR observed in the Easyfeed group suggests that these fish required less feed to achieve the same weight gain as those on the control diet. On the other hand, the lower SGR found in the Easyfeed group indicated a slower daily growth rate compared to the control group despite the same amount of feed was provided. One possible explanation for this discrepancy is that the Easyfeed diet might promote a more efficient nutrient assimilation process, allowing fish to maintain weight gain with less feed intake, but at a slower growth rate. This could be advantageous in reducing feed costs and waste, which aligns with sustainable aquaculture practices. Nevertheless, the slower growth rate might be a concern in commercial settings where rapid growth is often a priority. Although the differences in growth performance were not significant by day 30, it is possible that these differences could become more pronounced over longer periods, potentially impacting overall production efficiency. Future studies should explore the long-term impacts of such diets on growth efficiency and overall production economics to assess their viability fully.

Despite expectations of observing a significant effect on aggression due to the high spirulina content (i.e., 20%) of the Easyfeed diet (Demelash, 2018; Winberg *et al.*, 2001; Wolkers *et al.*, 2012), no significant differences were observed in the mirror test. This suggests that the spirulina-based diet did not influence aggressive behaviour under tested conditions. Conversely, although the stress recovery test did not show differences in feeding behaviour between diet groups, significant differences were observed in the acute stress response to the diving test. Tilapia fed with the Easyfeed diet exhibited longer average distances travelled than control fish in the diving test arena. Increased activity has been previously established as a stress indicator in Nile tilapia, including in novel environment paradigms such as the open field test (Barreto *et al.*, 2010; Pintos *et al.*, 2024). Therefore, our results suggested a reduced acute stress response in tilapia fed with Easyfeed.

The findings from this study align with previous research indicating the potential benefits of sustainable diets in aquaculture. Previous research has documented that several alternative ingredients, such as microalgae, positively influenced fish welfare by enhancing immune responses, reducing disease susceptibility, and promoting better overall health (Becker, 2013; Shah *et al.*, 2018; Xu *et al.*, 2014; Zhang *et al.*, 2014). Moreover, when combined with other alternative ingredients, microalgae can significantly contribute to the sustainability of aquaculture operations by reducing the reliance on fish meal and fish oil (Nagappan *et al.*, 2021; Naylor *et al.*, 2009; Hemaiswarya *et al.*, 2011; Tacon and Metian, 2008). Furthermore, spirulina has previously shown welfare benefits in Nile tilapia when included in their feed. For instance, Olvera-Novoa *et al.* (1998) demonstrated that spirulina-enriched diets could improve immune parameters and disease resistance in tilapia fry, which is crucial for maintaining fish health in intensive farming systems. While our study suggests that Easyfeed diet can reduce stress responses, further investigations on physiological and molecular stress indicators should be needed to confirm these findings.

In conclusion, this study provides valuable insights into the potential benefits of including spirulina in sustainable aquafeed formulations, especially for Nile tilapia. Our findings revealed proper growth performances and potential welfare benefits in fish fed with the Easyfeed diet. However, the economic feasibility of formulating alternative aquafeeds that comply with organic standards should also be carefully analysed to ensure their long-term viability within the industry (Ahmad *et al.*, 2022). Furthermore, long-term studies must be carried out to fully understand the impact of this diet on fish health and welfare. As the aquaculture industry moves towards more sustainable practices, the development and optimisation of sustainable diets will be crucial for balancing economic viability with environmental and animal welfare goals.

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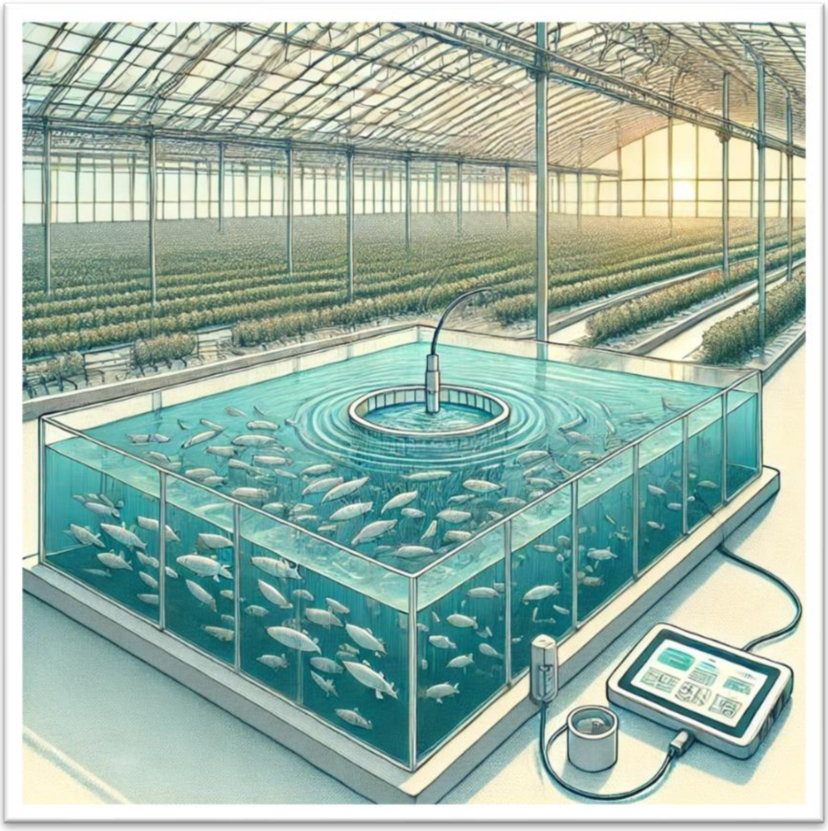
Chapter 4.

From the laboratory to the farm: daily variations in water quality parameters of decoupled aquaponic systems



1. Daily variation of water nitrogen compounds in decoupled aquaponics: a case study in Nile tilapia (*Oreochromis niloticus*) and cucumber (*Cucumis sativus*)

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Abstract

Aquaponics is an innovative and sustainable method for food production that combines aquaculture and hydroponics, utilising fish waste as nutrients for plant growth. However, the initial investment required for infrastructure, technology, and expertise is significant, and there are still many challenges in optimising resource use and system efficiency to make aquaponics economically viable on a larger scale. Hence, this study investigated daily variations of nitrate and nitrite water levels in a decoupled aquaponic farm, comprising a recirculating aquaculture system (RAS) for Nile tilapia (*Oreochromis niloticus*) farming and a nutrient film technique (NFT) system for cultivating cucumber crops (*Cucumis sativus*). Over a 72-hour period, water samples were continuously collected every 2 hours using a real-time water analyser. Our results showed that nitrate levels significantly increased over the three-day period in the RAS. On the contrary RAS nitrite levels decreased over the days. Contrary to expectations, no significant daily rhythms were observed for either nitrates or nitrites. In the NFT system, nitrate levels exhibited daily rhythms, and an overall increase was found over the days, while nitrite levels decreased progressively. Despite the challenges of managing industrial-scale systems, our findings suggest that carefully timing water exchanges between the aquaculture and hydroponic components can potentially improve nutrient management and enhance overall system efficiency in decoupled aquaponics. Tailoring interventions to align with these specific dynamics could lead to more sustainable and effective aquaponic operations.

Introduction

Aquaponics has emerged as an innovative and sustainable method for food production, combining fish farming (i.e., aquaculture) and soilless plant cultivation (i.e., hydroponics). This integrated system offers an efficient and ecological solution for producing both animal protein and plant-based food by utilising fish waste as essential nutrients for plants, and plants as natural biofilters that help maintain fish water quality. Consequently, aquaponics reduces the need for chemical fertilisers and minimises water usage, making this food production system an environmentally friendly alternative to conventional farming practices (Lennard and Goddek, 2019). However, aquaponic systems require significant investment in infrastructure, technology, and expertise. The initial costs can be high, and the economic returns are typically realised over longer periods (Love *et al.*, 2015; Goddek *et al.*, 2019). For this reason, research is committed to developing strategies that optimise resource use and improve the productivity of these systems to enhance their economic viability and sustainability (Goddek *et al.*, 2015; Ondruška *et al.*, 2022).

In recirculated aquaculture systems (hereafter, RAS), nitrite and nitrate levels are carefully monitored and controlled to ensure the health and welfare of the fish. Nitrite poses a significant threat to fish health if allowed to accumulate at high concentrations, as it can interfere with the ability of fish blood to carry oxygen, leading to suffocation (Kroupova *et al.*, 2005; Lewis and Morris, 1986; Xu *et al.*, 2022). Moreover, elevated nitrate levels can also be detrimental, potentially causing issues such as reduced growth rates, compromised immune function, and toxicity in visceral organs in fish (Monsees *et al.*, 2017; Presa *et al.*, 2022; Shimura *et al.*, 2004). Conversely, in hydroponics, nitrite and nitrate serve as essential nutrients for plant growth and development. Plant crops thrive on these nitrogen compounds, utilising them for vital processes such as protein synthesis (Bose and Srivastava, 2001; Crawford and Glass, 1998; Yoneyama *et al.*, 1980). Therefore, by absorbing and assimilating nitrite and nitrate from the water, plants can improve fish water quality by removing these potentially harmful nitrogen compounds.

The concept of decoupled aquaponic systems introduces a novel approach to aquaponics, wherein the aquaculture and hydroponic components are operated independently. This separation allows for independent management and optimisation of each component, potentially leading to better control over water quality and resource utilisation (Goddek *et al.*, 2019). One promising strategy for optimising resource use in decoupled aquaponics is investigating daily variations in water quality parameters, a research topic largely overlooked within this field. It is known that fish exhibit daily rhythms in urine excretion, which would affect the release of nitrogenous waste into the water (Gelineu *et al.*, 1998; Kajimura *et al.*, 2002). Similarly, plants have circadian rhythms that control nutrient uptake, including nitrate absorption, which varies throughout the day (Gutiérrez *et al.*, 2008). Therefore, understanding how water quality (i.e., nitrite and nitrate levels) varies across the 24-h cycle in aquaponics can provide valuable insights into the optimal timing for water exchanges and other management practices. By synchronising these activities with the daily rhythms of the system, it may be possible to enhance nutrient utilisation, improve water quality, and reduce the overall environmental footprint.

In this study, we conduct a pilot experiment to investigate daily variations in the water quality of an industrial-scale decoupled aquaponic system (Tilamur®, Murcia, Spain) farming Nile tilapia (*Oreochromis niloticus*) and cucumber (*Cucumis sativus*). This exploratory approach aimed to provide preliminary insights into the dynamics of nitrate and nitrite levels, measured continuously over 72 hours using a real-time water quality analyser. The water sampling was done simultaneously in both aquaculture and hydroponic systems. Thereafter, the existence of daily rhythms in nitrate and nitrite water levels was determined in each system as well as the variation of those levels across sampling days. As part of the industrial doctoral project, this pilot experiment was designed to inform future studies and identify practical management strategies that could enhance the efficiency and sustainability of large-scale aquaponic operations.

Material and methods

Recirculating aquaculture system: experimental subjects and design

Adult Nile tilapia fish (n=750-800) were reared in RAS containing eight 2.1 m^3 tanks with a 90 kg/m^3 stocking density at Tilamur facilities (Murcia, Spain; Figure 1). Fish were fed a commercial diet specifically produced for the species (Aquate®, Alltech Coppens, Germany) once daily at 09.00 a.m. (i.e., ZT1 = one hour after light on). The tanks were maintained at constant temperature (28 ± 1 °C) and exposed to a 13:11 h light-dark (LD) artificial photoperiod.

After the weekly system water change (i.e., 10%) for maintenance procedures, water samples were collected and analysed over 72 consecutive hours every 2 hours by the Real-time Nitrate & Nitrite Analyser (Aquamonitrix®, Ireland). This is a high-tech system for instantaneous, controlled, and internet-stored water sampling. The collection of samples was performed using a probe placed inside housing tanks, which automatically sent water samples to the analyser every 2h. The probe was placed in the housing tank that received water from the biofilter.

Hydroponic system: experimental subjects and design

Cucumbers *Cucumis sativus* crops (n= 380) were planted in a Nutrient Film Technique (NFT) hydroponic system. NFT systems were located in the 600 m^2 Tilamur greenhouse, which was exposed to natural light-dark cycles (approximately 11:13h LD – September/October 2023; www.tutiempo.net). After 20 days, nitrate and nitrite circulating levels were analysed over 72 consecutive hours, during the same days evaluated in the RAS. The data collection was also facilitated by the Real-time Nitrate & Nitrite Analyser (Aquamonitrix®, Ireland) and samples were also taken every 2 hours. No water changes were made during the sampling days, and samples were collected from the central repository of the NFT system (Figure 2).

Figure 1: Picture of Tilamur's **A.** Recirculating aquaculture system containing Nile tilapia housing tanks and **B.** Hydroponic system containing cucumber crops.

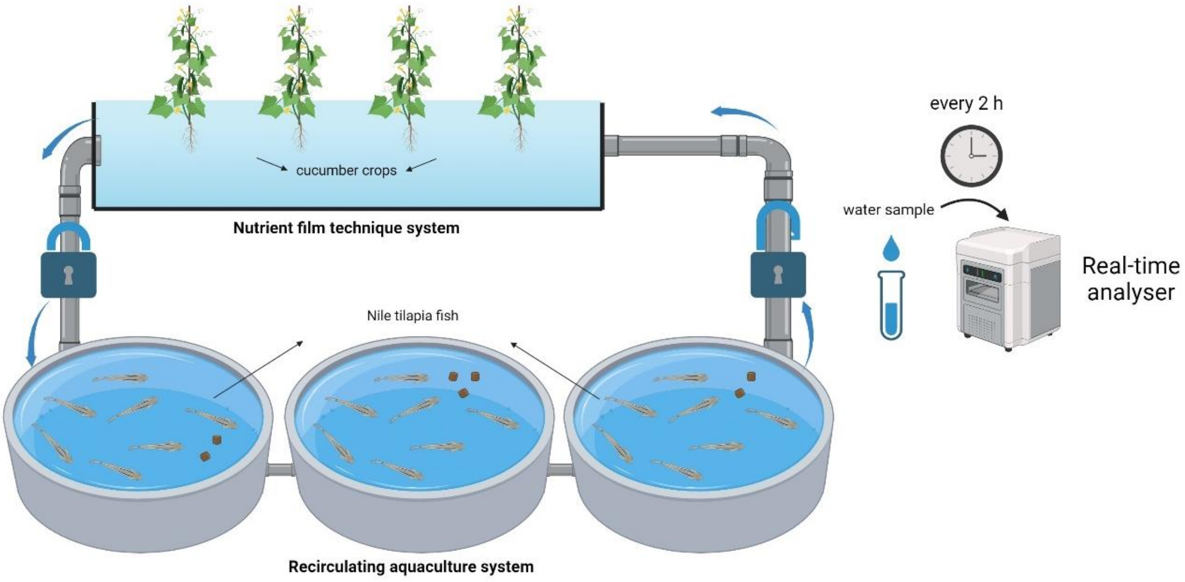

Figure 2: Illustrative scheme of the experimental design. Adult Nile tilapia (n= 750-800) and cucumbers *Cucumis sativus* crops (n= 380) were maintained in decoupled aquaponics within a recirculating aquaculture system and a nutrient film technique system, correspondingly. Water samples from each system were collected every 2 h over a 72 period. Nitrate and nitrite levels were measured by a Real-Time Analyser (Aquamonitrix®, Ireland).

Statistical analysis

Daily variations of nitrate and nitrite levels were studied in both RAS and hydroponic systems by the Cosinor analysis, using collected data from the 72-h sampling period. Cosinor analysis employs least-squares regressions to model cosine curves, which are useful to describe daily variations (Nelson *et al.*, 1979), and estimate a rhythm through a zero-amplitude test. In this analysis, a $p < 0.05$ constitutes evidence for a statistically significant rhythm in the given period of time under consideration (i.e., 24 h). This analysis employs least-squares regressions to model cosine curves, which are useful to describe circadian variations.

Furthermore, differences in overall levels of nutrients (i.e., nitrite and nitrate) between sampling days were studied by means of one-way analysis of variance (ANOVA I). This model considered the sampled day (3 levels: 1, 2 and 3) as a fixed factor. If necessary, post-hoc comparisons were made using the Tukey HSD test. Normality was tested by the Shapiro-Wilks test. All parameters which did not meet normality were transformed by square (RAS nitrate) and logarithmic (NFT nitrate) functions to improve model fitting.

Results

Water quality analyses revealed that, after the weekly water change, nitrate levels significantly varied over the days in tilapia RAS (ANOVA: $F_{2,31} = 18.31$, $p < 0.01$). Furthermore, post-hoc analyses found that nitrate levels on day 3 were higher than those registered on days 2 and 1 (Tukey: Figure 3A). Similarly, a significant impact of the day was found in nitrite levels (ANOVA: $F_{2,31} = 10.99$, $p < 0.01$), however indicating that nitrites on day 3 were lower than those registered on days 2 and 1 (Tukey: Figure 3D). Furthermore, and contrary to our expectations, neither nitrates nor nitrites showed daily rhythms in the RAS (Cosinor, nitrates: $p = 0.37$; nitrites: $p = 0.17$; Figure 3B and 3D).

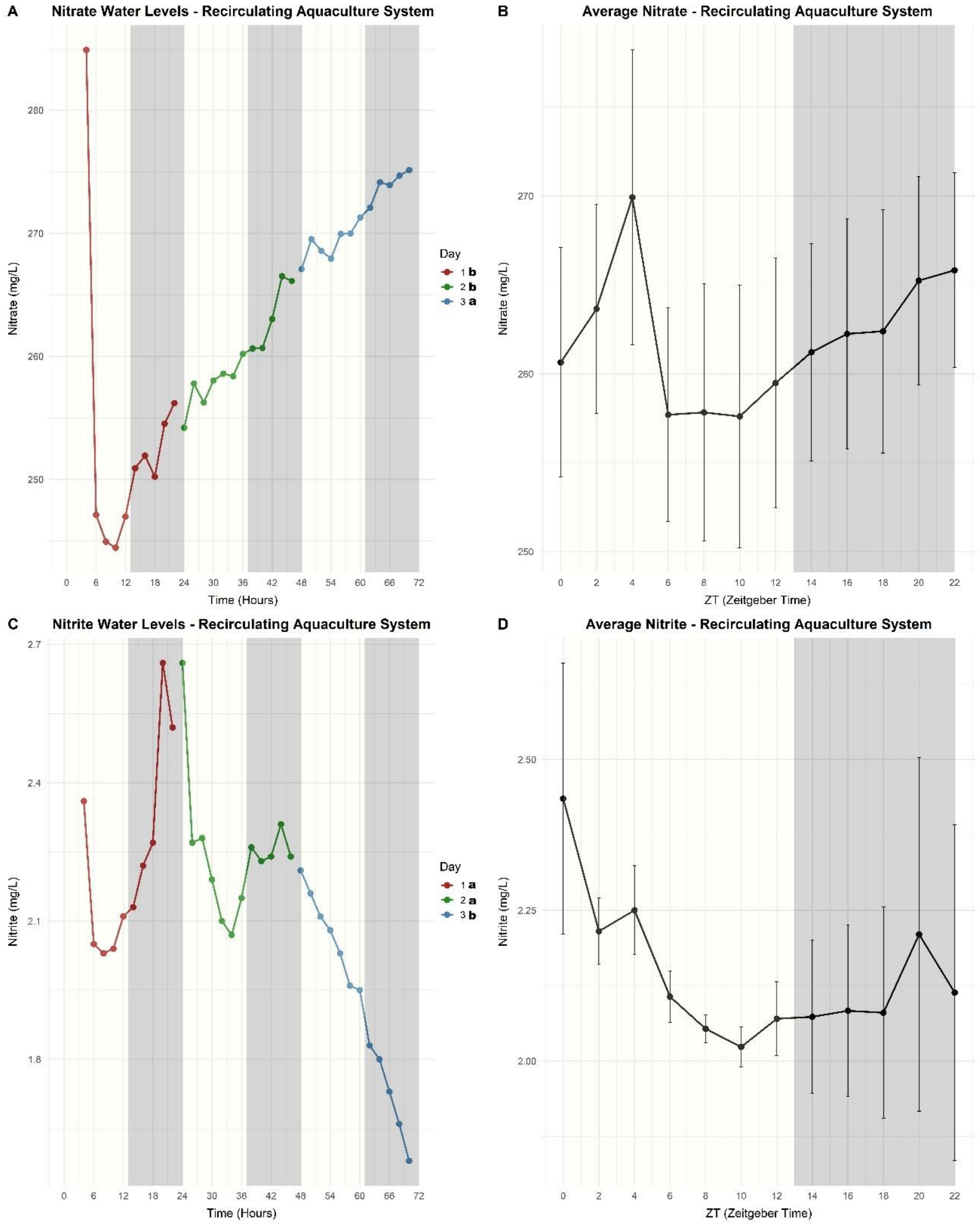

Figure 3: Nitrate and nitrite water levels in the Nile tilapia *Oreochromis niloticus* Recirculating Aquaculture System. **A.** Nitrate levels throughout three sampled days (day 1 = red; day 2 = green; day 3 = blue). **B.** Average nitrate levels throughout the . **C.** Nitrite levels throughout three sampled days (day 1 = red; day 2 = green; day 3 = blue). **D.** Average nitrite levels throughout the day. Data are presented as single data points in **A** and **C**, and as mean ± standard error in **B** and **D**. Different letters indicate significant differences in nitrite and nitrate levels among sampled days. Yellow and grey background colours represent light and dark phases, respectively.

In the NFT system, water quality analyses revealed that both nitrates and nitrites were significantly affected by the day (ANOVA: nitrate $F_{2,31} = 8.37$; nitrite $F_{2,31} = 79.40$; $p < 0.01$). For nitrate levels, post-hoc analyses registered higher values on day 3 compared to day 1 (Tukey: Figure 4A). Contrary, the Tukey HSD test registered lower nitrite levels within each successive day (Tukey: Figure 4C). In this case, daily rhythms were found for nitrates (Cosinor, $p < 0.01$; Figure 4B), but not for nitrites (Cosinor, $p = 0.14$; Figure 4D), indicating an acrophase close to the middle of the dark phase (ZT= 20.68).

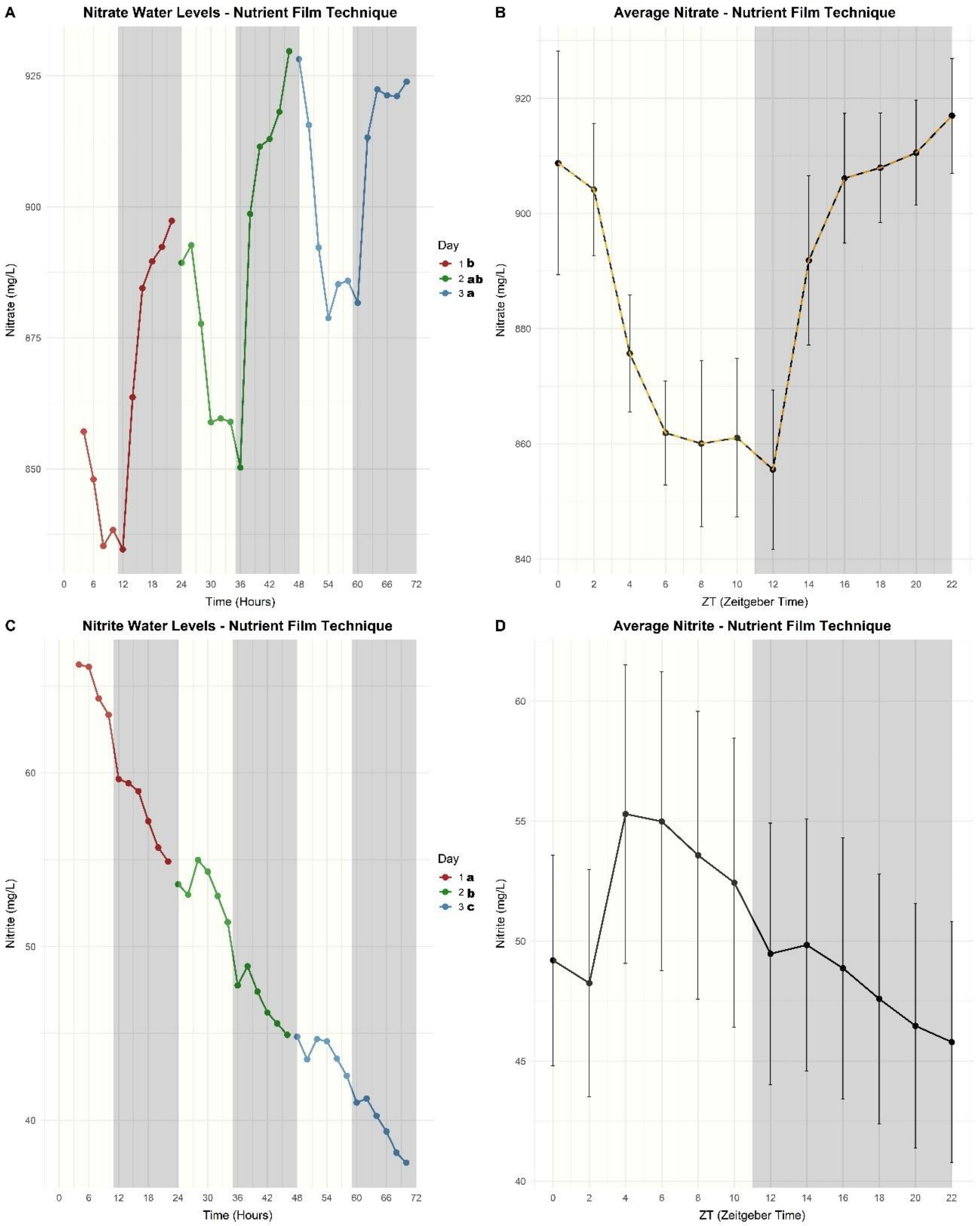

Figure 4: Nitrate and nitrite water levels in the cucumber *Cucumis sativus* Nutrient Film Technique system. **A.** Nitrate levels throughout three sampled days (day 1 = red; day 2 = green; day 3 = blue). **B.** Average nitrate levels throughout the day. **C.** Nitrite levels throughout three sampled days (day 1 = red; day 2 = green; day 3 = blue). **D.** Average nitrite levels throughout the day. Data are presented as single data points in **A** and **C**, and as mean ± standard error in **B** and **D**. Different letters indicate significant differences in nitrite and nitrate levels among sampled days. The dotted orange line indicates significant daily rhythms in the average nitrate levels by the Cosinor analysis. Yellow and grey background colours represent light and dark phases, respectively.

Discussion

This study examined daily variations in nitrate and nitrite levels in a decoupled aquaponic farm within a recirculating aquaculture system (RAS) for Nile tilapia and a nutrient film technique (NFT) system for cucumber. Our findings provide valuable insights into the temporal dynamics of water nitrogenous compounds (i.e., nitrate and nitrite) in both aquaculture and hydroponic components of aquaponics, highlighting key differences between these systems and potential implications for improving water management.

Our results showed that nitrate levels significantly increased in the RAS over the course of three days, with the highest concentrations observed on day 3. This increase is likely due to the accumulation of nitrogenous waste from fish metabolism and feeding (Brownell, 1980; Hrubec *et al.*, 1996), which the biofilter had not fully processed. The rise in nitrate levels over time suggests that the biofilter efficiency in converting nitrites to nitrates may have varied, possibly due to changes in microbial activity or water flow rates within the system. It is however worth noting that nitrate concentrations remained below 500 mg/L during all sampling days, a recommended threshold for Nile tilapia (Monsees *et al.*, 2017). Even the nitrate levels observed on the first day, before the routine 10% water exchange, were below this critical value. This indicates that nitrate levels were within recommended limits for Nile tilapia health and welfare.

Nitrite levels however exhibited an opposite trend to those reported in nitrate, decreasing significantly by day 3. This reduction in nitrites could indicate an effective conversion process within the biofilter (Crab *et al.*, 2007; McGee and Cichra, 2000), despite the increasing nitrate levels. However, the absence of significant daily rhythms for both nitrates and nitrites in the RAS was unexpected. Previous studies conducted under controlled laboratory conditions have reported daily fluctuations in fish excretion rates (Gelineu *et al.*, 1998; Kajimura *et al.*, 2002), leading us to anticipate similar patterns in our study. One possible explanation for this discrepancy is possibly linked to the scale and complexity of industrial aquaculture operations compared to laboratory settings. In a commercial fish farm, such as the one in our study, the high stocking densities, interactions between large numbers of fish, and the variability in environmental conditions could obscure or dampen the circadian rhythms observed in more controlled environments. Additionally, factors such as the continuous operation of mechanical and biological filtration systems, variations in water flow, and the potential for fluctuations in oxygen levels and other water quality parameters might further influence the expression of these rhythms. On the other hand, similar to that observed in nitrates, those nitrite values registered during sampling days were lower than those recommended for Nile tilapia (Yanbo *et al.*, 2006), further suggesting adequate water quality in the RAS.

In the NFT system, we also found a significant impact of the sampling day on both nitrate and nitrite levels. Similarly, nitrate levels increased over the days, peaking on day 3, while nitrite levels decreased progressively over the same period. The increase in nitrate levels over time may seem counterintuitive at first, especially considering that plants are actively absorbing these nutrients for growth. However, the daily rhythm found in nitrate water levels suggested that nitrate concentration may vary according to circadian biological processes such as photosynthesis, transpiration and respiration rates (Cseresnyés *et al.*, 2024; Hastings *et al.*, 1961; Hennessey and Field, 1991; Huck *et al.*, 1962; Gessler *et al.*, 2017). As plants transpire and photosynthesise, particularly during the daylight hours, they consume significant amounts of water from the hydroponic solution. This reduction in water volume could lead to a relative concentration of nitrates in the remaining solution, thereby increasing the measurable nitrate levels in the water. Additionally, the circadian rhythms of the plants likely influence both water uptake and nutrient absorption rates, potentially leading to periods where water is absorbed faster than nitrates can be utilised (i.e., dark phase), contributing to the observed increase in nitrate concentration.

The lack of a significant daily rhythm in nitrite levels in the NFT system suggested that cucumber crops primarily absorb nitrates rather than nitrites, which aligns with the preference of most plants for nitrate as their main nitrogen source (Crawford, 1995; Miller *et al.*, 2007). Nitrite, being a more reactive and less stable form of nitrogen, is typically present in lower concentrations in hydroponic solutions, as it is often rapidly converted to nitrate by nitrifying bacteria present in the biofilm within the system (Helmer *et al.*, 1999; Li *et al.*, 2019; Peng and Zhu, 2006). These bacteria play a crucial role in maintaining the nitrogen cycle in hydroponic environments by oxidising nitrite to nitrate, which can then be readily absorbed by plants. This ongoing microbial conversion of nitrites to nitrates could further contribute to the observed increase in nitrate levels, particularly if the nitrification rate exceeds the rate at which plants absorb the available nitrates. Additionally, since the absorption and conversion processes might be occurring at a relatively constant rate throughout the day, this could explain the lack of a discernible daily rhythm in nitrite levels, as opposed to the rhythm observed for nitrates.

Overall, our study explored the daily fluctuation of nitrate and nitrite water levels in a decoupled aquaponic system, with distinct patterns emerging between the RAS and NFT components. Our findings emphasise the importance of considering temporal dynamics in water quality management and suggest potential strategies for enhancing the sustainability and productivity of aquaponics. For example, knowing that nitrate levels tend to increase in the NFT system later in the day, it might be beneficial to reduce the frequency or volume of nutrient-rich water transferred from the RAS during these peak periods to prevent potential nutrient overload. This strategy could help maintain optimal water quality for both fish and plants, ensuring that nutrient levels align with the specific needs and absorption rates of each species. However, it is crucial to consider the specific species involved, as their nutritional requirements and tolerance to water quality parameters may vary. Furthermore, future studies should consider longer sampling durations and the inclusion of additional water quality parameters, such as ammonia and dissolved oxygen, to provide a more comprehensive understanding of the system's dynamics. Moreover, exploring the effects of varying light-dark cycles, feeding times, and biofilter configurations on daily rhythms in water quality could yield insights into optimising the design and operation of aquaponic systems.

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General discussion

This doctoral thesis explored how to improve the welfare assessment of fish species by utilising a variety of non-invasive welfare indicators, with a primary focus on behavioural traits. Furthermore, this research investigated different management strategies to enhance fish welfare, such as considering the time of day at which individuals are disturbed, their developmental stage, and the role of social interactions in gregarious and solitary species. Additionally, this thesis also assessed the impact of newly formulated sustainable diets on the overall well-being of Nile tilapia, one of the most widely farmed species globally. Lastly, the thesis extended its scope to an industrial setting by assessing the daily variations in water quality within a large-scale decoupled aquaponic system, providing insights into how water management can be optimised to support sustainable production practices. By integrating these approaches and studying a diverse range of both model and farmed species, this research offers valuable insights for improving welfare assessment and the living conditions of fish through a more accurate and thorough understanding of species-specific needs and stress indicators.

In the first chapter, our objective was to identify stress-sensitive behaviours in farmed species, both seawater and freshwater, that currently lack extensive research on their behavioural biology and/or welfare indicators. This knowledge gap has been critical to ensuring proper assessments, as it limits the ability to accurately evaluate and manage the welfare of these species in aquaculture practices (Bui *et al.*, 2019; Martins *et al.*, 2012; Saraiva *et al.*, 2019). Therefore, through a series of experiments, we aimed to fill this gap by exploring species-specific behavioural responses to various stressors, including novel environment exposures, social isolation, salinity changes and/or confinement events. Our findings critically revealed that behavioural stress responses are highly species-specific, cautioning on generalising welfare indicators across fish species as it could lead to inaccurate assessments and potential welfare risks.

For instance, our study in turbot notably evidenced that behaviours typically linked with stress-induced responses in teleost species such as zebrafish, Nile tilapia, goldfish or guppy (Maximino *et al.*, 2010b; Thompson *et al.*, 2016; Tran and Gerlai, 2016) would possibly indicate positive welfare states in turbot. Rather than preferring dark environments or seeking the bottom of the tank, this flatfish species displayed increased light-seeking behaviour and spent more time away from the bottom of the arena when stressed. Although there are no studies exclusively focused on stress behaviour in turbot, previous evidence suggested that both observed behaviours are indeed associated with poor welfare (i.e., lower growth rate, feed intake and metabolic rates) and high stress levels in flatfish species (Björnsson, 1994; Holm *et al.*, 1998; Li *et al.*, 2016). These findings collectively suggest that these avoidance behaviours may act as anxiety-like behaviours in turbot, and these tests (i.e., diving test and light-dark preference test) as valuable non-invasive methods to assess stress in this flatfish species.

Similarly, although avoidance behaviours were less evident, our study on the gilthead seabream revealed potential behavioural traits as stress markers, such as freezing and erratic swimming patterns. Previous studies have recently explored the behavioural performance of the gilthead seabream in novel environment paradigms, such as the open field test (Sánchez-Muros *et al.*, 2017). However, to the best of our knowledge, this is the first time that seabream behaviour has been explored in the diving test. Indeed, our findings suggested that this behavioural paradigm could serve as an effective tool for evaluating stress in seabream. Hence, these insights may contribute to expanding the available methods for assessing welfare in gilthead seabream and identifying potential behavioural indicators of stress specific to this species. Both turbot and gilthead seabream share the characteristics of being key seawater species in European aquaculture (Lei *et al.*, 2010; Person-Le Ruyet *et al.*, 2002; Seginer, 2016), yet there is a noticeable gap in comprehensive studies on their behavioural biology and welfare indicators. This commonality underscores the critical need for further research to develop reliable, species-specific and non-invasive indicators that can improve welfare management and support sustainable practices in these farmed species.

The other study conducted in this chapter has focused on freshwater aquaculture species such as Nile tilapia and koi carp, both described as social species (Bajer *et al.*, 2010; Bajer *et al.*, 2011; Gonçalves-de-Freitas *et al.*, 2019). Critically, our results indicated that both tilapia and carp exhibited social buffering effects, showing decreased anxiety-like behaviours in the presence of at least one conspecific. However, behavioural indicators demonstrating this effect varied between species. Notably, even in cases where the same indicators were supported, the direction of the anxiolytic effect was the opposite. For instance, in Nile tilapia, reduced stress was indicated by increased activity levels in the presence of conspecifics, while in koi carp, the same reduced stress was associated with decreased activity. This variation highlights the complex nature of social interactions in modulating stress responses and underscores the need for species-specific approaches when assessing and managing the welfare of fish.

Overall, the findings collected in *Chapter 1* provide valuable insights into the behavioural repertoire of economically important farmed species. Additionally, our results strongly support the use of behavioural indicators to measure stress and highlight the potential value of behavioural assays for assessing welfare non-invasively. In recent years, fish behaviour has become increasingly recognised as a reliable indicator of welfare (Browning, 2023; Cavallino *et al.*, 2023; Martins *et al.*, 2012; Toni *et al.*, 2019). Indeed, the practical application of fish behaviour in aquaculture settings can create low-stress methods for fish husbandry, reduce disease susceptibility or even optimise feeding practices, ultimately improving fish productivity (Macaulay *et al.*, 2021). However, further species-specific research is needed to deepen our understanding of the behavioural repertoire of farmed species and effectively integrate animal behaviour knowledge into farm management practices.

In the second chapter, the objective was to investigate whether the behavioural stress response does follow a daily rhythm in fish, and to explore the potential implications that this rhythmicity might have for fish welfare. To address this, we first conducted studies on a model species such as the zebrafish. Our findings revealed that adult zebrafish exhibit daily rhythmicity in their basal behaviour, as well as in their behavioural stress response to novelty. Specifically, our study showed that zebrafish anxiety-like behaviours — such as thigmotaxis, activity, and freezing — varied significantly throughout the day. Considering that these behaviours are well-established indicators of anxiety in zebrafish and other teleost species such as medaka or guppy (Champagne *et al.*, 2010; Hallgren *et al.*, 2011; Lucon-Xiccato *et al.*, 2022; Schnörr *et al.*, 2012; Volkova *et al.*, 2012), this variation is critical to consider when assessing stress, as it suggests that the time of day can remarkably influence the outcomes of behavioural tests. However, the variation observed in some of these indicators did not seem to be solely attributable to stress responses. For example, activity and freezing rhythms suggested that these daily changes might be driven by the inherent behavioural rhythms of fish, rather than exclusively by stress responses. Conversely, the rhythmic patterns observed in thigmotaxis behaviour were more attributable to stress in our experiment, indicating a stronger stress response during the night compared to the day in zebrafish. This distinction is important because it highlights the need to account for both stress-induced and rhythm-induced variations in behaviour when evaluating the welfare of fish. Additionally, we observed that some of these stress-related behaviours (i.e., freezing) varied differently depending on whether the individuals were exposed to a novel environment individually or in groups. This result agrees with the effect of social companions on fish behaviour observed in *Chapter 1* (i.e., Nile tilapia and koi carp). Taking this collectively, our results indicate that social context, combined with the time of day, plays a crucial role in shaping the stress responses of fish and should be considered to improve fish husbandry practices.

The implications of these findings are relevant for fish welfare. If stress responses are more pronounced at night, as our zebrafish results suggest, it may be beneficial to adjust management practices in fish husbandry to minimise stressors during this period. For instance, reducing handling or other potentially stressful activities during fish resting periods could help mitigate stress and improve the overall welfare of individuals. This hypothesis has previously been supported by physiological studies in some aquaculture species, such as the sole *Solea senegalensis* (López-Olmeda *et al.*, 2013) or the gilthead seabream *Sparus aurata* (Vera *et al.*, 2014).

Building on these insights, our next studies extended the investigation to commercially important farmed species, specifically tench and Nile tilapia. We aimed to determine whether the daily modulation of stress responses observed in zebrafish also occurs in these farmed species, and to explore how these rhythms might differ between species with contrasting activity patterns (i.e., diurnal and nocturnal). Our results further supported our previous hypothesis, showing that behavioural stress indicators peaked during the resting phase of each species and were reduced during their active phase. Specifically, in Nile tilapia, a diurnal species, stress responses were more pronounced during the night, while in tench, a nocturnal species, these responses were increased during the day. However, the behavioural indicators revealing these time-dependent effects did not completely overlap between species, further supporting our previous results on highly species-specific stress indicators. These findings collectively reinforce the idea that stress responses in fish are closely tied to their natural activity patterns, highlighting the importance of aligning management practices with these rhythms to optimise animal welfare in captivity (Sánchez-Vázquez *et al.*, 2019).

Contrary to that observed in Nile tilapia, previous studies have reported shy and solitary behaviours in tench (Kennedy and Fitzmaurice, 1970; Vanovac *et al.*, 2021). This evidence suggested that tench might not benefit from social interactions in the same way as more social species. However, our next study aimed to investigate whether social buffering could still occur at specific times of the day, considering the observed influence of daily rhythms on stress modulation in this species. Therefore, we compared the effects of conspecifics on individual behaviour throughout the day in species with opposed activity patterns and apparently distinct social behaviour, such as Nile tilapia and tench. This was tested in fish exposed to a novel environment after chronic isolation (i.e., 24h) or in groups. Surprisingly, we found that both species exhibited significant social buffering, demonstrating reduced anxiety-like behaviours in the presence of conspecifics. However, the daily modulation of this effect differed between species. In Nile tilapia, social buffering varied between day and night following distinct behavioural traits. In contrast, tench exhibited a consistent social buffering effect across the 24-h cycle. On one hand, in agreement with results of Chapter 1, this finding further highlighted the species-specific modulation of fish stress responses. On the other hand, it supported that even solitary species may benefit from conspecifics, a phenomenon reported in a limited number of mammal and fish species (Dalerum *et al.*, 2006; Dunlap *et al.*, 2021; Makuya *et al.*, 2024). Indeed, this suggests that solitary behaviour does not necessarily indicate a lack of sociality, as visual and olfactory stimuli alone can be sufficient to provide a sense of social environment and attenuate stress (Gilmour and Bard, 2022; Makuya and

Schradin, 2024; Pintos *et al.*, 2021). Consequently, our findings support that social enrichment strategies should also be explored in non-strictly social species, as they may reveal unexpected benefits for improving welfare practices in fish. Furthermore, this comparative study showed that the stress-sensitive behaviours observed during chronic social isolation were consistent with those identified in the other experimental chapters under acute stress conditions (i.e., *Chapter 1.2* and *Chapter 2.2*). This consistency further highlights the reliability of behavioural indicators in assessing stress, suggesting their potential as robust welfare indicators for both acute and chronic stress scenarios.

Another factor that has been shown to affect stress responses in fish is the age of the individuals, with evidence suggesting that younger or older fish may experience higher stress levels depending on the species (Aponte and Rutherford, 2019; Barcellos *et al.*, 2012; Henríquez-Martínez *et al.*, 2022; Mariën *et al.*, 2024; Polverino *et al.*, 2016). Given these findings, we sought to investigate how age might influence the daily modulation of stress indicators, a topic that has not been studied to date in fish. For this, we conducted behavioural studies on well-established model species (i.e., zebrafish) and paradigms (i.e., open field and diving tests). Our research revealed that both juvenile and adult zebrafish exhibited daily rhythms in their stress responses, but the overall response and daily patterns differed significantly between age groups. Hence, our findings suggested that juvenile zebrafish may be more sensitive to stressors than adults, and that the time of day differently modulated stress responses according to the age of individuals. This agrees with previous studies supporting distinct stress sensitivity across ontogeny in fish by behavioural and physiological indicators (Auperin and Geslin, 2008; Barcellos *et al.*, 2012; Koakoski *et al.*, 2012; Moreira *et al.*, 2019).

Overall, *Chapter 2* underscores the importance of considering the time of day to mitigate stress in fish, a factor that was consistently observed across different species.

Additionally, this research highlights how daily rhythms interact with other intrinsic factors of species, such as social interactions or the age of individuals, further emphasising the need to account for these variables when designing welfare standards in aquaculture. By understanding and integrating time-dependent variations into management practices, it is possible to develop more effective and tailored approaches to enhance fish welfare in captivity.

In the third chapter, the focus of the thesis shifted towards promoting sustainability in aquaculture, using the Nile tilapia as the experimental model, a species whose stress behaviours had been extensively studied in the previous chapters. Nile tilapia is one of the most widely farmed species globally (FAO, 2024). Its aquaculture production surpassed that of salmonids in 2005 and, by 2018, it had firmly established itself as the second most important farmed finfish species group worldwide (Miao and Wang, 2020; FAO, 2022). Despite its global significance for aquaculture, the sustainability of tilapia farming is not uniformly guaranteed across all producing countries (Debnath *et al.*, 2023; Gule and Geremew, 2022). This is mostly due to the conditions and farming practices in which tilapia is raised, where animal welfare is not always ensured, and disease outbreaks are frequent (Ndashe *et al.*, 2023; Obirikorang *et al.*, 2019; Paredes-Trujillo *et al.*, 2016). In recent years, the aquaculture industry has been shifting towards more sustainable practices, particularly in the field of fish nutrition. This transition is primarily centred on developing alternative diets that reduce reliance on fish meal and fish oil (Bandara, 2018; Ghamkhar and Hicks, 2021; Nagappan *et al.*, 2021; Oliva-Teles *et al.*, 2022), thereby minimising environmental impact, even in omnivorous species like tilapia (Figueiredo-Silva *et al.*, 2015; Furuya *et al.*, 2004; Suloma *et al.*, 2014; Teodósio *et al.*, 2020; Trosvik *et al.*, 2012). This opens opportunities to explore feed formulations that also enhance stress resilience and improve welfare by including functional ingredients (Encarnação, 2016; Nagarajan *et al.*, 2021; Olmos-Soto *et al.*, 2015). For this reason, in this third chapter, we explored the impact of newly formulated sustainable feeds on the welfare of Nile tilapia.

The first study within this chapter evaluated the effects of sustainable diets incorporating emergent, circular by-driven and low carbon-footprint ingredients (i.e., spirulina, quinoa, insect-meal, corn gluten meal and rice bran) on distinct welfare indicators of juvenile Nile tilapia. The results highlighted some of the challenges associated with these novel formulations, particularly in terms of palatability and growth performances. Fish fed the eco-efficient and organic diets exhibited reduced weight gain compared to those on the control diet, likely due to lower palatability and acceptance. Despite these challenges, the eco-efficient diet showed a promising reduction in the acute stress response, as measured by the open field test, suggesting potential benefits for stress resilience in tilapia. These findings underscore the need for further refinement of sustainable diets to achieve a balance between promoting stress resilience and ensuring adequate growth. Moreover, it highlights the importance of conducting long-term studies to determine whether the initial challenges related to palatability and feed intake can be mitigated over time, potentially leading to improved growth outcomes as fish adapt to these novel diets.

To further explore the potential effects of ingredients such as spirulina, we tested another sustainable feed formula in younger tilapia individuals habituated for longer periods to the experimental diet. In this second study, we aimed to determine whether early exposure and a longer acclimation period to an enriched spirulina diet could enhance palatability, support better growth outcomes, and reveal potential effects on stress resilience that may have been hindered in the previous experiment. Notably, this new aquafeed (i.e., Easyfeed®) revealed similar growth performances to those found in fish fed with a commercial diet. Moreover, fish fed the Easyfeed diet exhibited reduced acute stress responses, further highlighting the potential welfare benefits of including spirulina in tilapia aquafeeds. These findings suggested that the challenges associated with the palatability of alternative diets might be effectively addressed by considering longer habituation periods to novel feeds and the developmental stage at which feeds are introduced in feeding schedules.

In the fourth chapter, the research centred on the dynamics of water quality in a decoupled aquaponic system, where Nile tilapia (*Oreochromis niloticus*) was cultivated alongside cucumber (*Cucumis sativus*) crops. This study represented the industrial experience of my doctoral project, where we aimed to explore how resource use could be optimised on a large-scale aquaponics farm. One of the biggest concerns of sustainable aquaculture development is the solid waste generated by fish farming (Bueno *et al.*, 2017; Bueno *et al.*, 2023). Aquaponics offers a promising solution by integrating aquaculture and hydroponics, where fish waste is repurposed as a nutrient source for plant growth. However, there is still a need to better optimise processes at larger scales to offset the significant initial investments and accelerate economic returns (Goddek *et al.*, 2015; Ondruška *et al.*, 2022). In this context, our study aimed to describe the daily variations in water nitrogen compounds — specifically nitrate and nitrite levels — within a decoupled aquaponic farm, to identify better opportunities and practical applications for improving system efficiency, viability and sustainability through targeted interventions.

Our research within this chapter focused on monitoring nitrate and nitrite levels over a 72-hour period, with water samples collected every 2 hours using a real-time analyser. Contrary to our expectations, we did not observe significant daily rhythms in nitrate and nitrite water levels in the recirculating aquaculture system (RAS). Previous studies in laboratory settings have observed daily rhythms in the urine and nitrogen excretion of fish (Kajimura *et al.*, 2002; Lu *et al.*, 2013; Saha *et al.*, 1988), possibly influenced by feeding schedules and regimes (Gelineu *et al.*, 1998). However, these rhythms may have been dampened in the RAS water by several factors unique to large-scale operations, as discussed in that chapter. The main implication of our finding is that the water output from the RAS to the nutrient film technique (NFT) system is likely to exhibit only gradual changes in nitrate and nitrite levels over time, rather than fluctuating within a daily cycle.

In contrast to the findings in the RAS, our study did observe significant daily rhythms in nitrate levels within the NFT system used for cultivating cucumber crops. This aligns with previous laboratory studies that have demonstrated daily rhythms in nitrogen uptake in various plant species, including cucumber (Hansen, 1980; Imsande and Touraine, 1994; Keltjens and Nijenstein, 1987; Pearson and Steer, 1977; Yoneyama *et al.*, 1980). These studies typically report that nitrate uptake often peaks during specific times of the day, driven by circadian rhythms that regulate nutrient absorption and metabolic processes in plants. Our results suggest a similar pattern, with higher nitrate absorption during the light phase (i.e., lower nitrate water levels) and reduced uptake during the dark phase (i.e., higher nitrate water levels). However, the multitude of variables co-existing in an industrial aquaponic system complicates drawing definitive conclusions about the exact mechanisms driving these rhythms. Factors such as the activity of microbial communities on roots and system surfaces (Cafà *et al.*, 2018; Chave *et al.*, 2008; Guevara *et al.*, 2024; Sheridan *et al.*, 2017), daily rhythms in other biological processes such as photosynthesis and respiration (Endo and Ikusima, 1989; Hansen, 1980; Gessler *et al.*, 2017), or even daily fluctuations in environmental photoperiod and temperature likely influence nitrogen cycles, making it difficult to determine to what extent the variations in nitrogen levels in the water are solely attributable to the uptake of these compounds as nutrients.

Despite these complexities, the major finding is that circulating NFT water is subjected to significant nitrate-level fluctuations. Understanding and leveraging these natural rhythms could be crucial for optimising resource use. By timing nutrient delivery and water exchanges to align with peak absorption periods, it may be possible to improve the efficiency of nutrient utilisation, reduce waste, and enhance the overall sustainability of the system.

In conclusion, this doctoral thesis contributes to the advancement of fish welfare assessment by providing valuable insights into behavioural indicators of stress for a wide range of model and farmed fish species. Accordingly, it supports distinct behavioural assays to evaluate fish stress responses without exposing individuals to unnecessary harmful stimulus, promoting non-invasive methods to assess animal welfare and more ethical farming practices. Furthermore, this thesis explores various strategies to minimise stress in fish and thereby to improve their living conditions, either by considering the time of day at which fish are exposed to stressors, social enrichment strategies and/or the age of individuals. Additionally, our research on alternative aquafeeds further contributes to sustainable farming by supporting organic feed sources and reducing the use of fishmeal and fish oil. Lastly, the final experimental chapter aimed to bridge the gap between academia and industry and proposed practical applications in large-scale industrial settings. Overall, this doctoral thesis tackles different challenges currently faced by the fish farming industry and supports sustainable strategies to address them. Moreover, in an industry where over 500 aquatic species are farmed, this thesis highlights the urgent need for further study the welfare needs of aquatic farmed species and develop species-specific and appropriate welfare standards. This would be necessary for ensuring the long-term viability and sustainability of an industry that is playing a fundamental role in global food security.

Conclusions

1. Behavioural assays that exploit fish avoidance behaviours as stress indicators are valuable non-invasive methods for assessing stress responses and, thereby, the welfare of farmed species such as Nile tilapia, tench, koi carp, turbot and gilthead seabream. Our findings reveal that behavioural stress indicators are highly species-specific in fish, underscoring the need for further research on the behaviour of farmed species to ensure accurate welfare standards, welfare assessments, and improved farming conditions.

2. Both solitary (tench) and social (tilapia, koi carp and seabream) fish species exhibit social buffering effects, highlighting the role of social interactions in regulating stress responses and their importance in enhancing animal welfare. However, this buffering effect can vary throughout the day.

3. Behavioural stress responses exhibit daily rhythmicity in both model (zebrafish) and farmed (tilapia and tench) fish species. This indicates that fish welfare assessments must account for the time of day to avoid biased outcomes and provide accurate evaluations. Additionally, daily variations in stress responses are influenced by the species' activity patterns, with stress peaking during the resting phase and decreasing during the active phase. This implies that fish welfare might be improved by considering the time of day at which fish are handled. Furthermore, it highlights the importance of time-specific welfare strategies that consider species' circadian rhythms and support effective management practices.

4. Behavioural stress responses in zebrafish vary throughout the 24-hour cycle and differ based on the individual's age, highlighting the importance of considering both age and time of day when establishing welfare standards.

5. The inclusion of alternative feed ingredients, such as quinoa and spirulina, shows potential welfare benefits for Nile tilapia, particularly in enhancing their stress resilience.

However, challenges related to palatability and growth performance emphasise the need for further refinement of these formulations and feeding strategies.

6. Resource optimisation in decoupled aquaponics can be achieved by scheduling water exchanges according to daily fluctuations in water quality within both the aquaculture and hydroponic systems.

Conclusiones

1. Los ensayos comportamentales que utilizan comportamientos de evitación de como indicadores de estrés en peces son métodos no invasivos y valiosos para evaluar respuestas al estrés y, por lo tanto, el bienestar de especies de cultivo como la tilapia del Nilo, la tenca, la carpa koi, el rodaballo y la dorada. Nuestros hallazgos revelan que estos indicadores de estrés son altamente específicos para cada especie, lo que resalta la necesidad de profundizar en el estudio del comportamiento de las especies de cultivo para asegurar estándares de bienestar precisos, evaluaciones rigurosas y mejores condiciones de cultivo.

2. Tanto las especies de peces solitarias (como la tenca) como las sociales (como la tilapia, la carpa koi y la dorada) mostraron efectos de amortiguación social, lo que resalta el papel de las interacciones sociales en la regulación de las respuestas al estrés y su importancia para mejorar el bienestar animal. No obstante, este efecto puede variar a lo según la hora del día.

3. Las respuestas comportamentales al estrés muestran una ritmicidad diaria tanto en especies de peces modelo (pez cebra) como en especies de cultivo (tilapia y tenca). Esto indica que las evaluaciones del bienestar deben tener en cuenta la hora del día para evitar resultados sesgados y brindar evaluaciones precisas. Además, las variaciones diarias en las respuestas al estrés están influenciadas por los patrones de actividad de cada especie, con picos de estrés durante la fase de reposo y disminuyendo durante la fase de actividad. Esto sugiere que el bienestar de los peces podría mejorar considerando la hora del día en la que se realizan las prácticas de manipulación, y resalta la importancia de implementar estrategias de bienestar específicas en función del tiempo, que tomen en cuenta los ritmos circadianos de las especies y respalden prácticas de manejo efectivas.

4. Las respuestas comportamentales al estrés en el pez cebra varían a lo largo del ciclo de 24 horas y difieren según la edad del individuo, lo que subraya la importancia de considerar tanto la edad como la hora del día al establecer estándares de bienestar.

5. La incorporación de ingredientes alternativos en los piensos acuícolas, tales como la quinoa y la espirulina, demuestra beneficios potenciales para el bienestar de la tilapia del Nilo, especialmente al mejorar su resiliencia al estrés. Sin embargo, los desafíos relacionados con la palatabilidad y el rendimiento en el crecimiento ponen de manifiesto la necesidad de reforzar aún más las formulaciones y estrategias de alimentación.

6. La optimización de recursos en sistemas acuapónicos desacoplados puede lograrse programando los cambios de agua de acuerdo con las fluctuaciones diarias en la calidad del agua, tanto en los sistemas de acuicultura como en los sistemas hidropónicos.

Conclusioni

1. I test comportamentali che sfruttano i comportamenti di evitazione dei pesci come indicatori di stress sono metodi non invasivi e preziosi per valutare le risposte allo stress e, di conseguenza, il benessere di specie allevate come la tilapia del Nilo, la tinca, la carpa koi, il rombo e l'orata. I nostri risultati rivelano che tali indicatori di stress sono altamente specifici per ogni specie, sottolineando la necessità di approfondire lo studio del comportamento delle specie allevate per garantire standard di benessere accurati, valutazioni rigorose e migliori condizioni di allevamento.

2. Sia le specie di pesci solitarie (come la tinca) sia quelle sociali (come la tilapia, la carpa koi e l'orata) mostrano effetti di buffering sociale, evidenziando il ruolo delle interazioni sociali nella regolazione delle risposte allo stress e la loro importanza per migliorare il benessere animale. Tuttavia, questo effetto può variare a seconda dell'ora del giorno.

3. Le risposte comportamentali allo stress presentano una ritmicità giornaliera sia nelle specie modello (pesce zebra) sia in quelle allevate (tilapia e tinca). Ciò indica che le valutazioni del benessere devono tenere conto dell'ora del giorno per evitare risultati distorti e fornire valutazioni accurate. Inoltre, le variazioni giornaliere delle risposte allo stress sono influenzate dai pattern di attività specifici delle specie, con picchi di stress durante la fase di riposo e diminuzioni durante la fase di attività. Questo suggerisce che il benessere dei pesci potrebbe migliorare considerando l'orario delle pratiche di manipolazione, sottolineando l'importanza di strategie di benessere specifiche per fascia oraria che tengano conto dei ritmi circadiani delle specie e supportino pratiche di gestione efficaci.

4. Le risposte comportamentali allo stress nel pesce zebra variano nel corso del ciclo di 24 ore e differiscono in base all'età dell'individuo, evidenziando l'importanza di considerare sia l'età sia l'ora del giorno nel definire gli standard di benessere.

5. L'inclusione di ingredienti alternativi nei mangimi, come quinoa e spirulina, mostra potenziali benefici sul benessere della tilapia del Nilo, in particolare migliorando la lora resilienza allo stress. Tuttavia, le sfide legate alla palatabilità e alle prestazioni di crescita evidenziano la necessità di perfezionare ulteriormente queste formulazioni e le strategie di alimentazione.

6. L'ottimizzazione delle risorse in sistemi acquaponici disaccoppiati può essere ottenuta programmando i ricambi d'acqua in base alle fluttuazioni giornaliere della qualità dell'acqua sia nel sistema di acquacoltura sia in quello idroponico.

Annexes

- **Scientific publications**

Pintos, S., Lucon-Xiccato, T., Vera, L. M., & Bertolucci, C. (**2023**). Daily rhythms in the behavioural stress response of the zebrafish *Danio rerio*. *Physiology & Behavior*, 268, 114241. https://doi.org/10.1016/j.physbeh.2023.114241

Pintos, S., Lucon-Xiccato, T., Vera, L. M., Conceição, L., Bertolucci, C., Sánchez-Vázquez, J., & Rema, P. (**2024**). Social buffering of behavioural stress response in two fish species, Nile tilapia (*Oreochromis niloticus*) and koi carp (*Cyprinus carpio*). *Ethology*, *130*(7), e13464. https://doi.org/10.1111/eth.13464

- **Scientific publications in progress**

Pintos, S., De Alba G., Lucon-Xiccato, T., Geralda Campos, F., Sánchez-Vázquez, F.J., Bertolucci, C., Vera, L.M. Circadian modulation of behavioural stress indicators varies between diurnal and nocturnal fish species. Submitted to *Applied Animal Behaviour Science*

Pintos, S., Lucon-Xiccato, T., Vera, L.M., Sánchez-Vázquez F.J., Bertolucci, C. Circadian modulation of behavioural stress responses in zebrafish is age-dependent. Submitted to *Journal of Experimental Zoology Part A: Ecological and Integrative Physiology (requested a minor revision after first round of evaluation)*

Pintos, S., Lucon-Xiccato, T., De Alba G., Geralda Campos, F., Sánchez-Vázquez, F.J., Bertolucci, C., Vera, L.M. Is social buffering consistent across the time of the day? A study in a diurnal (Nile tilapia *Oreochromis niloticus*) and a nocturnal fish species (tench *Tinca tinca*). Submitted to *Integrative Zoology*

- **Conferences**

1. *7th European Student Conference on Behaviour & Cognition* (2021), Budapest, Hungary.
2. *European Aquaculture Conference* (2022), Rimini, Italy. **Oral exposition.**
3. *PhD days – University of Firenze* (2022), Florence, Italy. **Oral exposition.**
4. *5th International Student Course in Behavioural Biology* (2022), Paris, France.
5. *Join ITN meeting EasyTrain-Eatfish* (2022), Faro, Portugal. **Oral exposition.**
6. *14th International Congress on the Biology of Fish* (2022), Montpellier, France. **Poster.**
7. *Congreso Nacional de Acuicultura* (2022), Cadiz, Spain. **Oral exposition.**
8. *European Aquaculture Conference* (2023), Rimini, Italy. **Poster.**
9. *PhD days – University of Firenze* (2024), Florence, Italy. **Oral exposition.**
10. *3th Italian Zebrafish Meeting* (2024), Palermo, Sicily. **Poster.**
11. *European Aquaculture Conference* (2024), Copenhagen, Denmark. **Poster and booth expositor.**

- **Courses and workshops**

1. *EASYTRAIN 1st Training Module: "Basic and Industrial Aquaponics"* (2022), Murcia, Spain. **Oral exposition.**
2. *EASYTRAIN 2nd Training Module: "Fish nutrition and diet design"* (2022), Faro, Portugal. **Poster.**
3. *Join ITN meeting EasyTrain-Eatfish* (2022), Faro, Portugal. **Oral exposition.**
4. *EASYTRAIN 3rd Training Module: "Microalgae Biotechnology"* (2023), Ferrara, Italy. **Oral exposition.**
5. *Join ITN meeting EasyTrain-Eatfish-Rasopta* (2023), Vienna, Austria. **Oral exposition.**
6. *EASYTRAIN 4th Training Module: "Engineering and Business*" (2024), Eindhoven, Netherlands. **Oral exposition.**

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Summary in Spanish

En los últimos años, el bienestar animal ha recibido una creciente atención tanto en la sociedad como en la comunidad científica, impulsada por una mayor conciencia sobre las implicaciones éticas de las prácticas de producción animal. Esta preocupación ha derivado en 1) regulaciones más estrictas, 2) una demanda creciente por productos certificados en bienestar animal y 3) una sensibilización generalizada acerca de las condiciones de vida en la que los animales son cultivados en sistemas de producción intensiva. Aunque en animales terrestres se han logrado importantes avances en cuanto a sus necesidades en cautiverio y la protección legal de su bienestar, el bienestar de los animales acuáticos ha sido históricamente relegado y aún no se encuentra suficientemente protegido, tanto a nivel práctico como a nivel normativo.

En particular, los peces, que representan el mayor grupo de animales vertebrados cultivados, no suelen ser tenidos en cuenta en las discusiones sobre bienestar animal. Esta tendencia también se ha visto reflejada en la producción y literatura científica. Sin embargo, la evidencia acumulada en las últimas décadas ha demostrado de manera contundente que los peces son animales sintientes, capaces de experimentar dolor, miedo y estrés. Por lo tanto, a pesar de que su bienestar debería estar garantizado en acuicultura, la evidencia científica y los ambientes de cultivo demuestran lo contrario. En este contexto, la industria acuícola, una de las de mayor crecimiento a nivel global, enfrenta una presión cada vez mayor por parte de consumidores, investigadores y políticos para mejorar las condiciones de vida de los peces de cultivo.

A fin de avanzar en esa dirección, esta tesis doctoral tuvo como objetivo mejorar la evaluación del bienestar animal en peces mediante el uso de indicadores de estrés no invasivos, principalmente comportamentales, así como explorar estrategias prácticas de manejo que puedan contribuir a mejorar las condiciones de vida de los peces en cautiverio.

Esta tesis doctoral se organizó y estructuró en cuatro capítulos experimentales:

El **Capítulo 1** se centró en la identificación de indicadores comportamentales de estrés en especies de cultivo económicamente relevantes, pero escasamente estudiadas desde la perspectiva del bienestar y comportamiento animal. Estas especies incluyeron al rodaballo (*Scophthalmus maximus*), la tilapia del Nilo (*Oreochromis niloticus*), la carpa ‘koi’ (*Cyprinus carpio*) y la dorada (*Sparus aurata*). Mediante el uso de ensayos conductuales extensamente estudiados en especies modelo (como el test de campo abierto, de exploración o de preferencia luz/oscuridad), se evaluaron respuestas al estrés siguiendo indicadores comportamentales en estas especies de acuicultura. Estos tests comportamentales se basan en la observación de patrones de evitación innatos frente a estímulos novedosos o potencialmente amenazantes. Por ejemplo, el test de campo abierto permite evaluar la tendencia a explorar o evitar áreas abiertas del entorno, mientras que la prueba de luz/oscuridad se basa en la preferencia natural de muchos peces por entornos más oscuros. La modificación de estos patrones puede reflejar alteraciones en el estado emocional de los individuos (ansiedad/estrés), y por lo tanto, de su bienestar. Los resultados de este capítulo mostraron que las respuestas al estrés eran altamente especie-específicas, lo que evidencia la necesidad de desarrollar protocolos de evaluación y manejo adaptados a las particularidades de cada especie. Por ejemplo, mientras que en especies modelo (pez cebra) ciertos comportamientos están altamente asociados a estados de estrés, en el rodaballo estos mismos indicadores parecen estar asociados a menor estrés y un mayor bienestar. Estos hallazgos son especialmente relevantes ya que ponen de manifiesto los riesgos en extrapolar indicadores de bienestar entre especies de peces. Además, en este capítulo, se exploró el rol modulador de conespecíficos sobre la respuesta al estrés en la tilapia del Nilo (*Oreochromis niloticus*) y la carpa koi (*Cyprinus carpio*). Ambas especies mostraron un efecto de amortiguamiento social (social buffering, en inglés), aunque con diferencias marcadas en los indicadores implicados y en la dirección de los efectos. Por ejemplo, en la tilapia, la reducción del estrés se asoció con un aumento en la actividad locomotora y una reducción del comportamiento de congelamiento (freezing, en inglés). Por el contrario, en la carpa koi, menores niveles de estrés se asociaron con una menor actividad y más tiempo en el centro de la arena experimental. Esta variabilidad refuerza la idea de que las interacciones sociales son complejas, que los indicadores de estrés son altamente especie-específicos y que, por ende, es necesario comprender el comportamiento de cada especie para luego implementar estos conocimientos en ambientes de cultivo.

El **Capítulo 2** abordó la influencia de los ritmos circadianos sobre la respuesta al estrés en peces. En primera instancia, se estudió el pez cebra (Danio rerio), una especie modelo y diurna, y se observó que los comportamientos de estrés variaban significativamente a lo largo del día, siendo más intensos durante la noche. Estos resultados indicaron que la hora del día puede influir de forma determinante en 1) el bienestar de los peces y 2) los resultados de las pruebas de comportamiento, lo que tendría implicaciones biológicas y metodológicas importantes. En los siguientes experimentos se extendió el análisis a especies de cultivo con cronotipos opuestos: la tilapia (diurna) y la tenca (*Tinca tinca*, nocturna). En ambas, las respuestas al estrés se acentuaron durante sus respectivas fases de reposo, y se atenuaron durante sus fases de actividad. Este patrón refuerza la hipótesis de que los ritmos circadianos modulan las respuestas al estrés en peces y que, por tanto, las prácticas de manejo (como la manipulación, la alimentación o el transporte) deberían adaptarse a estos ritmos para minimizar el impacto negativo sobre el bienestar animal. Sin embargo, este capítulo vuelve a destacar que los indicadores de estrés pueden variar significativamente entre especies y, por ello, es importante profundizar este tipo de investigaciones.

Además, se exploró cómo factores como la edad de los peces o la presencia de conespecíficos podían interactuar con los ritmos circadianos para modular las respuestas al estrés. En el caso de la tenca, una especie tradicionalmente descrita como solitaria y nocturna, se observó que la presencia de otros individuos reducía consistentemente potenciales indicadores de estrés comportamental durante el ciclo de 24 horas. En cambio, en la tilapia, el efecto amortiguador de la presencia de individuos de la misma especie fue más marcado durante ciertas fases del día. Este estudio comparativo evidenció que incluso especies consideradas ‘poco sociales’ pueden beneficiarse del enriquecimiento social, y que estos efectos pueden estar modulados por la hora del día.

El **Capítulo 3** abordó la sostenibilidad en acuicultura desde la perspectiva de la alimentación de los peces. En este caso, se evaluó el efecto de dietas sostenibles formuladas con ingredientes alternativos (como la quinoa, la espirulina, harinas vegetales y subproductos provenientes de economías circulares) sobre el bienestar de la tilapia del Nilo. En una primera instancia, se observó que algunas formulaciones de piensos reducían ligeramente las respuestas al estrés, pero presentaban problemas de palatabilidad y comprometían el crecimiento de los peces. En un segundo experimento, con una dieta experimental distinta, se ajustó el periodo de adaptación de los individuos a las dietas y se testearon en estadios más tempranos del desarrollo. Los resultados evidenciaron tasas de crecimiento comparables a las observadas con una dieta comercial y una cierta resiliencia al estrés. Estos hallazgos sugieren que es posible desarrollar dietas funcionales sostenibles que mejoren el bienestar de los peces y que, a su vez, mantengan un crecimiento apropiado.

El Capítulo 4 trasladó la investigación a un sistema industrial de acuaponía desacoplado, evaluando las variaciones diarias de los compuestos nitrogenados (nitrato y nitrito) en el agua de cultivo de sistemas acuícolas e hidropónicos interconectados. Aunque no se detectaron ritmos diarios marcados en el sistema acuícola recirculante (“RAS”, por sus siglas en inglés), sí se observaron fluctuaciones significativas en el sistema hidropónico (nitratos). Este hallazgo destaca la importancia de sincronizar los sistemas acuapónicos desacoplados (intercambios de agua y/o el suministro de nutrientes) de manera tal de optimizar el uso de recursos y aumentar la eficiencia de los mismos.

En la **discusión general**, se integraron finalmente los aportes de los diferentes capítulos experimentales y los diferentes enfoques utilizados para mejorar la evaluación y gestión del bienestar animal en peces. Esta tesis doctoral combinó estudios experimentales en especies de peces modelo, especies de cultivo, especies de agua dulce y de agua salada, especies con diferente naturaleza social (gregarias y sociales) y con diversos cronotipos (diurnas y nocturnas). Por lo tanto, este proyecto de investigación no solo permitió generar conocimiento acerca del comportamiento animal de diferentes especies sino también respaldar prácticas de manejo que puedan ser aplicadas en ambientes de cultivo para mejorar el bienestar animal.

En primer lugar, los resultados obtenidos resaltaron la importancia de considerar el comportamiento como una herramienta clave para evaluar el bienestar de los peces de manera no invasiva, en contraposición a las técnicas tradicionales mayormente utilizadas en la literatura científica de peces (indicadores fisiológicos y moleculares de estrés). A su vez, las diferencias observadas entre especies, incluso ante los mismos estímulos estresantes (ambientes novedosos), demuestran que los protocolos y estándares generalizados pueden llevar a diagnósticos erróneos o incompletos. Por tanto, esta tesis defiende el desarrollo de indicadores y prácticas adaptadas a cada especie, teniendo en cuenta sus características biológicas, ecológicas y etológicas. En segundo lugar, se evidenció que las respuestas al estrés no son constantes, sino que están moduladas por factores como la hora del día, la edad del animal o el entorno social. Esta visión dinámica del estrés aporta una nueva perspectiva acerca del bienestar animal, proponiendo que la temporalidad (cronobiología) y/o el contexto social deben considerarse como variables centrales en el diseño de prácticas de manejo de animales y evaluación de bienestar. En tercer lugar, la incorporación de ingredientes alternativos en piensos acuícolas mostró que, a pesar de que existen desafíos en el desarrollo y fórmulas de piensos sostenibles, es posible avanzar hacia una acuicultura más respetuosa con el medio ambiente sin descuidar el bienestar animal.

Por último, al realizar estudios piloto en sistemas acuapónicos a escala industrial, se observó que es necesario tener en cuenta las dinámicas temporales de la calidad de agua entre sistemas (acuícolas e hidropónicos) en pos de mejorar el uso de recursos y la eficiencia de los mismos.

En **conclusión**, esta tesis doctoral ofrece contribuciones relevantes para la mejora del bienestar en acuicultura, tanto desde el punto de vista metodológico como práctico. Por un lado, aporta herramientas novedosas para evaluar el estrés en peces, fundamentales para el diseño de protocolos específicos y no invasivos para los animales. Por otro lado, propone estrategias innovadoras para mitigar el estrés a través del manejo de variables como la hora del día, el enriquecimiento social, la edad de los individuos o la alimentación funcional. En conjunto, estos hallazgos promueven prácticas de producción alineadas con las demandas actuales de sostenibilidad y bienestar animal. En una industria donde se cultivan más de 500 especies acuáticas bajo condiciones muy diversas, y donde en muchos casos se desconoce el repertorio comportamental de los animales cultivados, resulta crucial profundizar en la investigación sobre sus necesidades de bienestar en cautiverio. Sólo así será posible desarrollar prácticas más justas, efectivas y sustentables, capaces de garantizar la viabilidad a largo plazo de la acuicultura, una actividad que tiene un rol central en la seguridad alimentaria global.

Por último, cabe destacar que esta tesis se desarrolló en el marco de un doctorado europeo industrial basado en la colaboración entre universidades y empresas del sector acuícola (*easyTRAIN: eco-innovative training network*). Esto permitió no solo abordar cuestiones fundamentales desde un enfoque académico y científico, sino también conocer la realidad de la industria acuícola e intentar trasladar ese conocimiento a contextos de producción. En primer lugar, la etapa inicial de este programa doctoral involucró nueve meses en la Universidad de Ferrara (UNIFE, Italia). Posteriormente, se llevó a cabo una estancia de seis meses en SPAROS®, una empresa portuguesa especializada en nutrición y formulación de piensos para peces. Durante esos seis meses también se realizaron estancias cortas y complementarias en la Universidad de Trás-os-Montes e Alto Douro (UTAD). La tercera fase del doctorado, que duró 1 año, tuvo lugar en la empresa TILAMUR®, una compañía de acuicultura situada en el sureste de España y especializada en la producción de tilapia del Nilo mediante sistemas de acuaponía. Finalmente, los últimos nueve meses de esta tesis doctoral se realizaron en la Universidad de Murcia (UMU), donde se llevaron a cabo los análisis finales, la integración de resultados y la redacción de la tesis.