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**“Detecting aggressive behaviour patterns using video analysis in farmed rainbow trout (*Onchorynchus mykiss*) in recirculating aquaculture systems (RAS)”**



Universidade do Algarve

Faculdade de Ciências e Tecnologia

2023



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**“Detecting aggressive behaviour patterns using video analysis in farmed rainbow trout (*Onchorynchus mykiss*) in recirculating aquaculture systems (RAS)”**

Dissertation to obtain the grade of Master (MSc) in Aquaculture

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Alexandre Miguel Poeira José

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## ABSTRACT

In 2018, the European Union funded the iFishIENCi Horizon 2020 project to promote sustainable aquaculture production while improving aquafeed usage and reducing wastes. Given that feed ration and fish behaviour may have a significant impact on production efficiency and fish welfare, one aim of the iFishIENCi project was to incorporate automated feeding and behaviour assessments of juvenile rainbow trout (*Oncorhynchus mykiss*) to assess the relationship between aggressive behaviour frequency with different feed ratios (FR). Under the scope of this project, one-hundred rainbow trout were cultivated in a recirculating aquaculture system (RAS) and a feed availability/behavioural frequency experiment was conducted implementing three distinct feeding regimes. Feeding regimes intended to investigate aggression at different levels of feed availability: Minimum FR (1.99% total biomass), Recommended FR (2.44% total biomass) and Maximum FR (2.88% total biomass). Pursuing, circling, lateral display, and frontal confrontations were taken into consideration. Behaviour frequency was monitored during prefeeding and postfeeding events at 12-minute intervals. Significant influences of FR were between Minimum FR and Maximum FR in prefeeding events, and between Recommended FR and Maximum FR for postfeeding events. Mean frequency values recorded for Recommended FR peaked for prefeeding events with pursuing behaviours ( $\bar{x} = 252.0 \pm 210.4$ ), and with circling in postfeeding events ( $\bar{x} = 74.3 \pm 39.58$ ). The findings indicate FR influence in aggressive behaviour frequency, with Maximum FR being the optimum to minimize fish aggression. Further research is needed to continue to improve efficient aquaculture production and to develop new techniques to minimize fish aggression.

**Keywords:** Aquaculture, rainbow trout, fish behaviour, aggression, RAS, feeding regime

## RESUMO

Em 2018, a União Europeia financiou o projeto iFishIENCi Horizonte 2020 para promover a produção aquícola sustentável, melhorando simultaneamente a utilização de alimentos para animais aquáticos e reduzindo os resíduos. Dado que a ração alimentar e o comportamento dos peixes podem ter um impacto significativo na eficiência da produção e no bem-estar dos peixes, um dos objetivos do projeto iFishIENCi foi incorporar avaliações automatizadas da alimentação e do comportamento de trutas arco-íris juvenis (*Oncorhynchus mykiss*) para avaliar a relação entre a frequência do comportamento agressivo com diferentes rácios alimentares (FR). No âmbito deste projeto, foram cultivadas cem trutas-arco-íris num sistema de aquicultura recirculante (SRA) e foi conduzida uma experiência de disponibilidade alimentar/frequência comportamental implementando três regimes alimentares distintos. Regimes alimentares destinados a investigar agressões em diferentes níveis de disponibilidade de ração: FR Mínimo (1,99% de biomassa total), FR Recomendado (2,44% de biomassa total) e FR Máximo (2,88% de biomassa total). Perseguição, cerco, exibição lateral e confrontos frontais foram levados em consideração. A frequência do comportamento foi monitorizada durante os eventos pré e pós-alimentação em intervalos de 12 minutos. Influências significativas de FR foram entre FR Mínimo e FR Máximo em eventos pré-alimentação, e entre FR Recomendado e FR Máximo para eventos pós-alimentação. Os valores médios de frequência registados para FR recomendado atingiram o pico para eventos pré-alimentação com comportamentos de perseguição ( $\bar{X} = 252,0 \pm 210,4$ ) e com circulação em eventos pós-alimentação ( $\bar{X} = 74,3 \pm 39,58$ ). Os resultados indicam a influência do FR na frequência do comportamento agressivo, sendo o FR máximo o ideal para minimizar a agressão dos peixes. É necessária mais investigação para continuar a melhorar a produção aquícola eficiente e desenvolver novas técnicas para minimizar a agressão dos peixes.

**Palavras-chave:** Aquacultura, truta arco-íris, comportamento, RAS, regime alimentar

## **Acknowledgement**

Words cannot express my gratitude to my professor Dr<sup>a</sup>. Catarina Cortes Valente de Oliveira for having an invaluable patience throughout the development of this master's thesis. I would like to thank to Dr. Steven Prescott for his mentoring and for accepting me as his mentee, for making realise aquaculture is so much more than just fish production, helping me grow in the professional aspect.

I would like to thank the Erasmus+ programme from University of Algarve which allowed me to travel and experience in aquaculture in Europe. Additionally, I would also like to thank AquaBioTech Group for allowing my participation in the iFishIENCI Horizon 2020 project, expanding my horizons towards the aquaculture industry development, and to the Innovia and RDI departments for taking me in as a part of them.

Lastly, I am grateful to my family for supporting me during the three years of development of this thesis, especially my mother and brother who encourage me to take on the masters programme challenge in the first place. To my girlfriend Natalie Panasiak, who became my anchor in moments I wanted to give up and reminded me the importance of these project.

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## **ABBREVIATION INDEX**

ABT	AquaBioTech Group
AF	Automatic Feeder
AI	Artificial Intelligence
ALAN	Artificial Light at Night
AVG	Average
CI	Confidence Intervals
Df	Degrees of Freedom
DO	Dissolved Oxygen
FAWC	Farm Animal Welfare Committee
FCR	Feed Conversion Ratio
FR	Feeding Rate
FTS	Flow Through System
IP	Internet Protocol
LED	Light Emitting Diode
Max	Maximum
Min	Minimum
min	minutes
MIS	Mirror Image Stimulation
MP	Megapixel
RAS	Recirculation Aquaculture System
Rec	Recommended
RSPCA	Royal Society for the Preservation of Cruelty to Animals
SD	Standard Deviation
UV	Ultraviolet

# 1. INTRODUCTION

## 1.1 Recirculating Aquaculture Systems

Aquaculture is the breeding, rearing, and harvesting of fish, shellfish, algae, and other organisms in all types of water environments (NOAA, 2017). It involves the selective breeding of fish, either in freshwater or seawater, with the purpose of producing a food source for consumption (FAO, 2016). Diverse aquaculture practices are used world-wide in different environments (e.g., freshwater, brackish, and marine), and geographical locations (from freshwater to marine, and from polar to tropical waters), using different technologies and farming systems, and allows the production of a great variety of species (Ahmed and Turchini, 2021; FAO, 2016).

Aquaculture has been on the frontline of public concerns regarding sustainability (Martins *et al.*, 2010). Addressing some of these concerns, recirculation aquaculture systems (RAS) are systems in which water is (partially) re-used after undergoing waste treatment. RAS have greater control over production outcomes, and the productivity depends on many variables, such as, culture species, stocking densities, feeding rate, duration of production cycle, and other management aspects (Ahmed and Turchini, 2021).

RAS are aquaculture systems of emerging importance. To be operated economically, commercial RAS must have a certain density of fish to be profitable, and many researchers are currently conducting studies to determine if RAS is a viable form of intensive aquaculture (Jenner, 2010). A key benefit of RAS is that it enables fish to be reared in a controllable environment. This may afford the ability to produce fish in areas where existing conditions and availability of good quality water are unsuitable (Murray *et al.*, 2014). RAS may also be suitable for the maintenance of broodstock, rearing of seed, and production of smolts (in the case of salmon), and can thus support the cultivation of fish in other types of systems (e.g., open-cages/net-pens). Although RAS have been initially developed and are ideally suited to produce fresh and warm water fish species (e.g., channel catfish, rainbow trout, striped bass, and tilapia), it also offers potential to produce brackish and marine species (Helfrich and Libey, 1991).

Technology has made it feasible to produce seafood in coastal marine areas and the open ocean, as the demand for fish has soared (Moe Føre *et al.*, 2022). Aquaculture

is currently used to produce food and other commercial products, but has also other applications, such as habitat restoration and replenishment of wild stocks, and rebuild populations of threatened and endangered species (NOAA, 2017). Therefore, RAS may provide a substitute for conventional aquaculture by separating fish production from the maritime environment (O'Shea *et al.*, 2019). Despite RAS offering a greater opportunity for fish production, its current contribution for global aquaculture production is minor (Waite *et al.*, 2014). In fact, RAS have not yet been widely implemented due to a series of challenges at the social, economic, and technological levels (Ahmed and Turchini, 2021). For example, one of the constraints this system faces are the lack of space for expansion and new sites, mostly due to competition with other uses and interests (Badiola *et al.*, 2012). Caged-based and flow-through (FTS) systems, demonstrate some limitation regarding availability to resources such as freshwater, and concerns over pollution. (Ahmed and Turchini, 2021; Badiola *et al.*, 2012; Waite *et al.*, 2014). However, access to freshwater may be less of a concern in RAS. According to Bergheim *et al.*, 2013, on their on-going study in the aquaculture facilities in Norway, Canada, and Iceland, the water use of an FTS was substantially higher (52'000 litres), compared with the 780 litres used by the same facility in a RAS system when producing 1kg of fish. Furthermore, economic viability of RAS requires a long payback period. On average, it takes up to 8 years of production to turn into profit (Badiola *et al.*, 2012). Therefore, profitability determining factors of RAS such as productivity, initial investment, operating costs, and sale price of products helps determine its sustainability on a case-by-case basis (Ngoc *et al.*, 2016). The hefty initial capital expenditures required by RAS contribute to its sluggish adoption (Schneider *et al.*, 2006). To cover investment expenditures, high stocking densities and productivity are necessary. Consequently, welfare concerns may arise (Martins *et al.*, 2005; Van Dooren *et al.*, 2023).

## 1.2 Fish Behaviour

The study of stress control has long been a central issue in the analysis of fish behaviour (Colgan, 1973). Behavioural variations of fish are overly sensitive to stress factors, such as changes in environmental parameters (Papadakis *et al.*, 2012). In natural environments, the importance of strong fish habitat governance as a prerequisite for healthy fisheries is becoming globally recognized (Bundy *et al.*, 2016). This may be

especially important for farmed fish, which are exposed to a larger variety of dynamic and multifactor stress factors (Macaulay *et al.*, 2020).

Behaviour is the first visible change after exposure to a stressor (Kane *et al.*, 2004; Zhao *et al.*, 2016) and has proven to be a sensitive indicator of environmental conditions and animal welfare in ecosystems or controlled conditions (Sadoul *et al.*, 2014). Some fish species exhibit behaviours that are frequently regarded as stress indicators, because they indicate defence strategies that increase the likelihood of survival, such as swimming activity, group distribution, or space usage (Espmark and Baeverfjord, 2008). Because behavioural stress reactions are frequently instantaneous, they have the potential to serve as early indicators of deteriorating conditions (Dawkins, 2004; Kane *et al.*, 2004). Under certain circumstances, fish can be exposed to frequent and continued periods of stress, resulting in suppressed growth and diminished health. Artificial light at night (ALAN) can be one example of a stress factor (Ashley, 2007). The stress associated with the artificial lights have been recognized has potential factors to low performances, and behavioural changes (Hillyer *et al.*, 2021).

Regarding variations in an individual's response to stress, physiologists such as Gosling (2001) and Sih *et al.* (2004), tend to use the term coping style/mechanism. Coping styles often include both behavioural and physiological responses to unfavourable environments and stressors, such as feed deprivation and availability. In fish, the importance of understanding these mechanisms has gained increasing attention (Huntingford *et al.*, 2006; Koolhaas *et al.*, 1999). Moreover, Martins *et al.* (2011) showed that coping styles are predictive of how stimuli are appraised, supporting the inclusion of emotional or affective states. Although coping styles may have beneficial effects in both farmed and wild fish, certain coping styles may have negative consequences for aquaculture. This can cause aggressive behaviour and differences in risk taking among individuals, which may ultimately have an impact on production and compromise welfare (Øverli, 2007; Kane *et al.*, 2004).

Terminology remains one of the main challenges when addressing the topic of consistent individual variation in physiology and behaviour. A consensus is emerging that increased understanding of the consequences of stress-coping styles in aquaculture is important to safeguard a sustainable development of this industry (Castanheira *et al.*, 2015). Behaviourally speaking, proactive individuals are typically bolder and show an

active avoidance in response to challenges, usually these types of individuals are generally more aggressive and socially dominant towards conspecifics, and tend to follow behavioural routines (Laursen *et al.*, 2011).

Behavioural endpoints are valuable assessments to discern and evaluate effects of environmental stress. These points of exposure are important because they integrate several different factors that can connect the biochemical and physiological processes and can provide insight into the effects of environmental contamination inside the community level as well as in individual levels (Kane *et al.*, 2004). Significant advancements have been made in automated assessments (Huntingford *et al.*, 2012). Studies have been conducted using fish behaviour as a tool for analysing environmental risks, as well as their response to certain stimuli, such as chemical stress analyses or time spent engaging in a certain behaviour (Kane *et al.*, 2004). Because of the physical nature of light and its complex interactions with the environment, a variety of different properties of visible objects can be recognised (Guthrie, 1986).

While fish are one of the most cultivated of all vertebrate groups, incorporating their behaviour into production management has proved elusive (Macaulay *et al.*, 2020). As a consequence of water re-use and high stocking densities, RAS may lead to an accumulation of substances released by the fish into the water, e.g., cortisol and alarm pheromones (Martins *et al.*, 2010). It is therefore often difficult to predict how fish cultivated in these systems will react to different conditions and potential stressors (Stien *et al.*, 2007).

### 1.3 Behavioural and Welfare Indicators

Behavioural indicators may tell an observer about the welfare of the fish at the point of observation, having the advantage of being fast and easy to observe (Martins *et al.*, 2012). Furthermore, indicators provide an immediate indication of the state of the fish and is in most situations a non-invasive measure (Noble *et al.*, 2018). For example, high ventilation rates and gasping at the surface may indicate inadequate oxygen levels or damage to the respiratory system (Noble *et al.*, 2018). Feeding processes in aquaculture could also impact behavioural patterns (Lee *et al.*, 2013). Underfeeding could result in aggressive behaviours, and excess feeding may promote behaviours associated with

unfavourable environments, particularly in closed systems, e.g., increased nitrogen or decreased oxygen levels (Bergqvist and Gunnarsson, 2011; Gozdowska *et al.*, 2022).

Evaluating fish behaviour is one of the best welfare indicators available to the aquaculture industry and the only one where we have some degree of access to the subjective experience of the fish (Noble *et al.*, 2018). Fish can display a wide spectrum of behavioral patterns that emerge from complex interactions with their conspecifics and their environment (Brown, 2014; Assan *et al.*, 2021). Understanding how and why these behaviours arise is important and could be useful in different fields (Føre *et al.*, 2018; Sirovnik *et al.*, 2018; Thogerson *et al.*, 2009). A set of easily observed measurements known as operational welfare indicators (OWIs) were recently adopted to monitor fish behaviour and welfare in a non-destructive form (Noble *et al.*, 2018), of which, swimming behaviour is of particular importance for all aquaculture systems.

The welfare of an animal is a complex issue, and a unified definition is hard to find in the literature (Segner *et al.*, 2011). Fish welfare is a key issue in commercial farming and is central to many decisions that farmers take during their daily husbandry practices and longer-term production planning (Noble *et al.*, 2018). Animal based welfare indicators (WIs) are attributes from the animal itself that indicate that one or more welfare needs have not been fulfilled. They can be indicators of prior welfare problems e.g., results of previously poor nutrition or feeding response which can be identified by the condition factor of the fish or the degree of emaciation (Noble *et al.*, 2018).

The issue of farmed fish welfare has raised increasing public and scientific awareness over the last decade, and it has contributed to the development of new policies related to fish welfare (Martins *et al.*, 2011; Gilannejad *et al.*, 2021). Citing Dawkins (1990) “The primary basis for the concept of ‘animal welfare’ is the belief that animals are sentient beings with an ability to experience good or bad feelings or emotional states”, this meaning that depending on the environment provided to the fish in an enclosed system, the perception that the fish gets from its surroundings determines if they experience or not a proper non-stressful environment. Due to the fact that indicators of fish welfare are simpler to monitor than indications of fish aggression, the industry appears to place more emphasis on indicators than on techniques to create and maximize fish welfare (van den Boogaart *et al.*, 2023). Understanding how animal aggression and welfare are related encourages compassionate treatment, helps managers to create

successful management plans, and improves the welfare of animals in a variety of settings. Although implementing behaviour-based welfare measures in fish farming may bear a higher initial economic burden, the long-term advantages may outweigh the costs (Bernal-Higuera *et al.*, 2023). Furthermore, as technology and information transfer within the aquaculture sector develops, welfare solutions may become more available, affordable, and scalable (van den Boogaart *et al.*, 2023).

Fish must have the freedom to control their bodily movements, the ability to move away from danger and have buoyancy control (Stien *et al.*, 2013). The ability to move away from danger is a fundamental need for all animals, and to learn to predict danger and learn from aversive incidents. In fish farming, this is relevant to the ways in which fish are crowded and managed; farmers should pay attention to avoidance behaviour, increased oxygen consumption, catecholamine, cortisol and serotonin levels, all indicating stress and potential fear (Noble *et al.*, 2018).

Behaviour represents a reaction to the environment as fish perceive it and is therefore a key element of fish welfare (Martins *et al.*, 2012; van den Boogaart *et al.*, 2023). It is therefore essential to understand the species-specific biology before drawing any conclusions in relation to welfare (Dawkins, 2004). However, behaviour-based observations have some limitations. Different individuals within the same species may exhibit divergent manifestations towards stressors. Therefore, the assessment of welfare in a few individuals may not represent the average welfare of a group (Rousing *et al.*, 2001). Limitations of behavioural welfare indicators can also include that they are often variable over time and difficult to quantify, that whoever is observing and controlling the fish welfare and behaviour needs to have a sufficient skill set to observe the animals 'body language' and patterns is, and therefore able to identify the individual differences in behaviour within animal groups (Martins *et al.*, 2011). Nonetheless, behaviour observations can provide valuable insight into the well-being of farmed fish populations.

#### 1.4 Aggressive Behaviours

Aggressive behaviour is a trait commonly found in almost every animal species. Aggression is defined as "behaviour that actually or potentially causes harm to another animal," according to Damsgård and Huntingford (2012). In intensive aquaculture, despite the abundance of resources, aggressive behaviour is often observed and has some

serious implications (Damsgård & Huntingford 2012; Oikonomidou *et al.*, 2019). Usually, aggressive behaviours are displayed as non-contact interactions between individuals (Miczek *et al.*, 2001), however, there is a large scale of aggression registered among rainbow trout, when inserted in an intensive aquaculture environment (Oikonomidou *et al.*, 2019).

Pursuing is a non-contact aggressive behaviour observed in fish when placed under different stress situations. This behaviour can be classified as the most basic and simple form of aggressive behaviour amongst fish. In this action, one fish is seen to burst into swimming rapidly and directly towards another fish. Pursuing can be observed when an individual that initiates the chase is most likely a dominant or a “territory holding” fish. If a subordinate enters too close into the territory, the chase is initiated. This action is similar to the burst action. The main difference between the two is that in this case the burst is directed towards another fish and can lead to another aggressive behaviour, like circling (Miczek *et al.*, 2001; Noble *et al.*, 2011).

Circular interactions (Circling) are one of the many non-contact displays seen amongst fish, and specifically rainbow trout. Circling is recognized when individuals swim towards each other (or one dominant fish approached another fish) relatively rapidly, followed by the fish turning away from each other as they are about to come into contact. At that point, fish are then seen to swim in a tight circle, in a head to tail fashion, conducting repeated loops (Best *et al.*, 2023).

Lateral displays are another form of non-contact displays seen amongst aggressive behaviours. It can be observed when two fish swam towards one another, and before coming in contact, turn side to side with one another. The fish line themselves up horizontally against each other, facing either opposite directions or the same direction. The fish are then seen to beat their caudal fins in a rapid motion against one another. During this motion, the rest of the fish’s body is usually tense and does not move (Chiszar *et al.*, 1975; Oh *et al.*, 2019).

Frontal confrontations are aggressive actions happening when individuals are seen to swim towards one another and engage in a “face to face” confrontation while their fins are spread and mouth is open (Ramanzini *et al.*, 2018).

Aggressive interactions occur whenever the interests of two or more individuals’ conflict. The conflicts are more likely to occur when there is competition for a limited

resource (such as territory, food, mates, and a nesting site, etc, Xu *et al.*, 2021). Among factors affecting aggressive behaviour in intensively reared fish species, the feeding procedure is one of the most important (Pěnka *et al.*, 2023). Food is a valuable resource for fish in intensive aquaculture. Any factor that may alter food monopolisation may also increase competition and intensify aggression (Hubená *et al.*, 2021). One clear example of competition is the increase of aggressive behaviour during feeding, when low feeding rations are offered or when food is distributed in a localised way rather than dispersed over a larger surface (Oikonomidou *et al.*, 2019).

Fish present different aggressive traits for a number of reasons, such as, dominance, hierarchy, territory, as a defence mechanism, among others (Huntingford *et al.*, 2012; Newman, 1956). A hierarchy can be developed in a tank community when social fish use aggression to sort out their relative positions. After the dominance line is established, the aggression subsides (Reebs, 2008). Domination-subordination relations have been observed in many different species, however in salmonids it is clearer to visualize the dominance levels (Newman, 1956). A well-formed and stable hierarchy is a peaceful hierarchy that is beneficial for every being inside the environment, because if the fish have a continuous fighting behaviour, it becomes energetically costly, and can harm a good portion of the community (Reebs, 2008). However, the low-ranking individuals suffer the consequences of being in the low rankings, as their food supply gets limited, resulting in a slower growth rate of this fish. The hierarchy is established by the confrontation of two individuals in an aggressive interaction and, the winner is considered the dominant and the loser gets a lower dominance level (Brandão *et al.*, 2021; Damsgård & Huntingford, 2012; Preston *et al.*, 2014). Engaging in a fight usually is the last stage of confrontation. Threats and other displays of “warning” take part before the actual fight interaction. Only when these confrontations escalate to induce a high enough stress level, fish show a more aggressive behaviour.

The agonistic behaviours associated with the dominance hierarchy are dependent on several biotic and abiotic factors such as fish size or sex, the spatial and temporal distribution of food, feeding frequency and feed delivery rate (Martins *et al.*, 2011). Many studies in fishes (salmonids, specifically) were conducted to examine the implications of social ranking on foraging success, where they managed to link the rate of aggressiveness with the food intake (Oikonomidou *et al.*, 2019; Preston *et al.*, 2014). As a result, subordinate and low-ranking individuals adopt alternative behaviours in a way that allows

them to feed in the presence of the dominant fishes (Harwood *et al.*, 2002). Some strategies used by these individuals can be feeding at a different time, adopt sneak feeding strategies in the presence of dominant individuals to access a limited food resource (Preston *et al.*, 2014) or even occupying different niches within the same habitat (Kaspersson *et al.*, 2012).

To date, there are two possible ways to record and analyse the fish aggressive interactions. One of the methods is the Dyadic Fight protocol (DF). Dyads (couple/pair) of fish will compete for a limited resource (e.g., shelter, mate, and food) after a period of isolation. The dyadic battle simulates a realistic scenario in which a fish engages in combat with a genuine foe and either triumphs or fails. (Paull *et al.*, 2010). Mirror image stimulation (MIS) is the alternate approach for documenting aggressive behavior in fish. Mirror image stimulation tests have been used in different studies to help quantify the aggression in fish behaviour (Rowland, 1999). MIS tests can also be used to quantify other types of behaviour relating to animal temperament. In salmonids, such tests have compared aggression in fish from different habitats (Archard and Braithwaite, 2011). Moretz *et al.* (2017) compared the behaviour of zebrafish with MIS and concluded that by using this type of evaluation it was possible to see a clear behavioural difference between different strains.

## 1.5 Rainbow Trout

The rainbow trout (*Oncorhynchus mykiss*) (Fig. 1) is an important cold-water aquaculture species worldwide with excellent nutritional value (Ren *et al.*, 2022; Shekarabi *et al.*, 2022). Common causes for stressful conditions in rainbow trout cultivation include high stocking density, poor hygiene, and malnourishment, making the trout more susceptible to pathogens and potentially adversely affecting growth, performance, and flesh quality (Rashidian *et al.*, 2022).



Figure 1: Rainbow trout (*Oncorhynchus mykiss*), retrieved from Washington Department of Fish and Wildlife, accessed on May 28th, 2023

In rainbow trout the magnitude of the cortisol response to stress is an individual characteristic which is stable over time, with a moderate to high degree of heritability (Øverli *et al.*, 2005). The tight relationship between stress-coping style and social stress is demonstrated both by the influence of previous social interactions on physiology and aggressive behaviour, and by how the capacity to react to stressful conditions with adaptive neuroendocrine responses predicts social position (Hoglund *et al.* 2008). In wild rainbow trout, the juveniles are commonly more intensely territorial animals due to their residence in stream habitats (Pottinger and Carrick, 1999). Biting, mouth fighting, pursuing and aggressive nipping are some observable interactions between fish when territory or other resources come into question (Brandão *et al.*, 2021; Huntingford *et al.*, 2012; Martins *et al.*, 2010).

El-Haroun *et al.*, (2009) conducted some studies on rainbow trout, where different dietary levels were implemented to investigate the correlation between aggression and feeding in this species, as well as performance. According to this study, trout are more prone to reduced growth, and the diploid salmonids registered a higher level of aggression during feeding. Surface feeding response and behavioural interactions can therefore be influenced by the ploidy level of fish (Preston *et al.*, 2014).

Food restriction increases aggression, competitive ability and predation risk in rainbow trout (Petrauskiene, 2002). The tendency of individuals to be aggressive is an important behavioural axis, that often correlates across contexts, as well as with other behavioural axes such as boldness and activity (Archard and Braithwaite, 2011). While many studies show a decrease in aggression as food abundance increases, there are fewer

studies contrasting the hypothesis of an increase in aggression as food abundance initially increases (Toobaie and Grant, 2013).

## 1.6 Video Analysis

Camera-based methodologies for aquatic monitoring have been drawing increasing attention as they enable a non-extractive and non-lethal approach to studying fish behaviour. Fish detection, linkage of the identified fish locations across successive frames (fish tracking), and extraction of swimming feature characteristics such as speed, direction, and amplitude are the three steps that may be used to perform individual-level movement analysis., etc (Georgopoulou *et al.*, 2021). Video recording techniques have been used for the remote observation in real-time, and also for offline fish activity, aggressiveness, and behaviours through video playback (Kane *et al.*, 2004; Stien *et al.*, 2007; Zhao *et al* 2016).

Due to the limitations for analysing fish behaviour, fish farmers must rely on monitoring of behavioural indicators such as swimming and feeding behaviour, visible health indicators such as injuries and mortalities and of easily measurable environmental parameters such as temperature and oxygen (Stien *et al.*, 2007). One of the main benefits of video systems is that fish behavior may be observed without a person being present in close proximity to the experimental area (Papadakis *et al.*, 2012). Specific approaches have been used to automate behavioural investigations to respond to a particular biological topic, demonstrating how challenging it is to create a more universal instrument (Papadakis *et al.*, 2012). Video analysis can be used to assess and verify the vertical fish distribution in tanks (Stien *et al.*, 2007), or advanced motion analysis like image processing systems can be used to quantify and identify fish behaviour (Delcourt *et al.*, 2009; Kato *et al.*, 2004). Moreover, the behaviour of fish is more complex because the apparent image of the fish is highly variable and the direction and speed of fish displacements can change very quickly (Delcourt *et al.*, 2009).

There is an emerging interest in the use of Internet-of-Things and Artificial Intelligence (AI) based approaches towards increasing the efficiency of feeding practices in aquaculture. Monitoring of fish behaviour may prove to be an important component of this process. Behavioural indicators of hunger state may be useful to provide information about the most appropriate time to offer feed.

## 1.7 Aims and Objectives

Aquaculture plays a crucial role in meeting the global demand for seafood while still addressing the challenges presented worldwide (overfishing, illegal capture, etc.). Recently, sustainability is a significant matter addressed in the aquaculture industry that was introduced to optimize production and mitigate certain issues in fish production, such as aggressiveness among individuals, growth issues and feeding efficiency. In this context, the present study aims to investigate the interplay between feed availability and fish aggressiveness, as these factors are recognized as key determinants of aquaculture performance. Understanding how these factors interact and influence the growth and overall health rainbow trout development is essential for designing efficient and responsible aquaculture practices that can sustainably support the burgeoning human population's nutritional needs.

The project will have a focus on investigating feeding patterns and responses of juvenile rainbow trouts in relation to different feed ratios available. With this, we aim to identify the impact of feed availability in aggressive behaviours presented by *O. mykiss*, in RAS. Project objectives bounded were:

1. Determine the relation between feed availability and frequency of aggressiveness displays by rainbow trout, as three different ratios of feed will be given to the fish.
2. Determine the relation between the different percentages of feed given with the frequency of aggressive behaviours.
3. Examine the influence of different daily feeding periods in behavioural frequency.
4. Investigate biomass influence on behaviour visibility to allow a reliable data retrieval and proper management.
5. Use the acquired data to promote and develop a rainbow trout aggression index to help farmers and assess fish welfare and production efficiency which could then be expanded to other RAS produced fish species.

The project was conducted in the wet laboratory INNOVIA facilities at AquaBioTech Group in Malta. This project was completed under the framework of the EU-funded Horizon 2020 iFishIENCi project. The iFishIENCi project aims to improve

production methods and feeding efficiency, as well as sustainability of the aquaculture industry, through developing and demonstrating a disruptive Internet of Things (IoT)/ AI based innovations with smart precision feeding solutions while addressing the fish quality of commercially important species.

## 2. MATERIAL AND METHODS

### 2.1. Recirculating Aquaculture System

In this project the Recirculation Aquaculture System (RAS) presented in Fig. 2 was used for rainbow trout husbandry. The system is designed at a commercially relevant scale and is part of the onsite husbandry and research facilities managed by AquaBioTech Group (ABT). The characteristics of the tanks such as shape, volume and surface area can have an influence on water circulation, aeration dispersion and fish behaviour. The system used by ABT is composed of 12 (twelve) round 1500L fiberglass tanks, inserted in a freshwater recirculating system. The tanks were filled at half of their capacity (0.75m<sup>3</sup>/750L) to avoid fish escaping throughout the study.

A 3,000 L fiberglass sump (Aquacirc, Mosta, Malta), and 97 m<sup>3</sup>/h drum filter (Rotoclean 60 Drum Filter, Faivre, Baume-les-Dames, France) were used to maintain water quality. A sump is an accessory tank in which mechanical equipment is kept and is especially important for larger tanks, where hang on skimmers and reactors provide adequate filtration. Having a sump integrated into a RAS helps to control the water volume in the system, making it more stable and less prone to fluctuations of pH and salinity, build-up of nitrogen, chlorine, and ammonia, and the unintentional introduction of foreign substances in the system. This mitigation is done by plastic pellets (biomedia) via biochemical filtration.

Adjusting the flow rate above or below the estimated turnover rate is frequently necessary due to oxygen demand. The fish density in this project was 100 rainbow trout per tank maintained at  $\approx 13 \text{ kg/m}^3$  at an initial average weight of  $\approx 69 \text{ g/fish}$ . The flow rate was maintained at approximately 1'200 L/h (Summerfelt *et al.*, 2004), to sustain adequate oxygenation and circulation for the population. Given that tank shape was circular, water spin rate inside the tank was also taken in consideration. Circular tanks are designed to

distribute oxygen evenly throughout the water column (Mjåtveit *et al.*, 2022). Aeration was provided by a saturation cone, which injected oxygen at 0.6 kg/h, which was distributed to individual tanks through air diffusers. Aeration systems used aeration stones to distribute diffused air at the bottom of the tanks, creating an upward water movement, bringing colder, cleaner, oxygen-rich water to the fish. Similar to the aeration system, an emergency oxygenation system distributes diffused liquid oxygen into the tanks, when levels of dissolved oxygen in the water drop below 6 mg/L. Adding liquid oxygen directly into the tanks can quickly improve fish health and stimulate feeding. Dissolved oxygen (DO) is one of the most critical parameters for aquaculture, as it is vital for all living organisms (Chatziantoniou *et al.*, 2022).

Disinfection of recirculating water from the individual tanks is especially important for a RAS system, where the majority of the water used is recycled, and to maintain the volume, fresh water is topped up in the system. For this, a combination of two methods was used: a low concentration of ozone (O<sub>3</sub>), injected into the water (30g/h), mixed with 225W dual ultraviolet (UV) spectrum UVA/UVB irradiation, for ozone and UV disinfection. (MR-1 220PP, UltraAqua, Aalborg Øst, Denmark). Using ultraviolet lights for the water treatment reduces bacterial concentration and controls pathogens in aquaculture production systems (Stevčić *et al.*, 2019). Microorganisms including disease-causing bacteria are eliminated (Ahmad *et al.*, 2022), or the DNA of the organisms is significantly damaged when exposed to an adequate dose of UV light. Ozone can also improve the quality of aquaculture production water by helping improve solids settling (Cui *et al.*, 2023) and by reducing nitrite-nitrogen (NO<sub>2</sub>-N), fine particulate matter, and microbial activity. Ozone nanobubbles are introduced into the water, which lowers the bacterial burden, increases the dissolved oxygen content, and modifies the fish's innate immune system (Dien *et al.*, 2022).

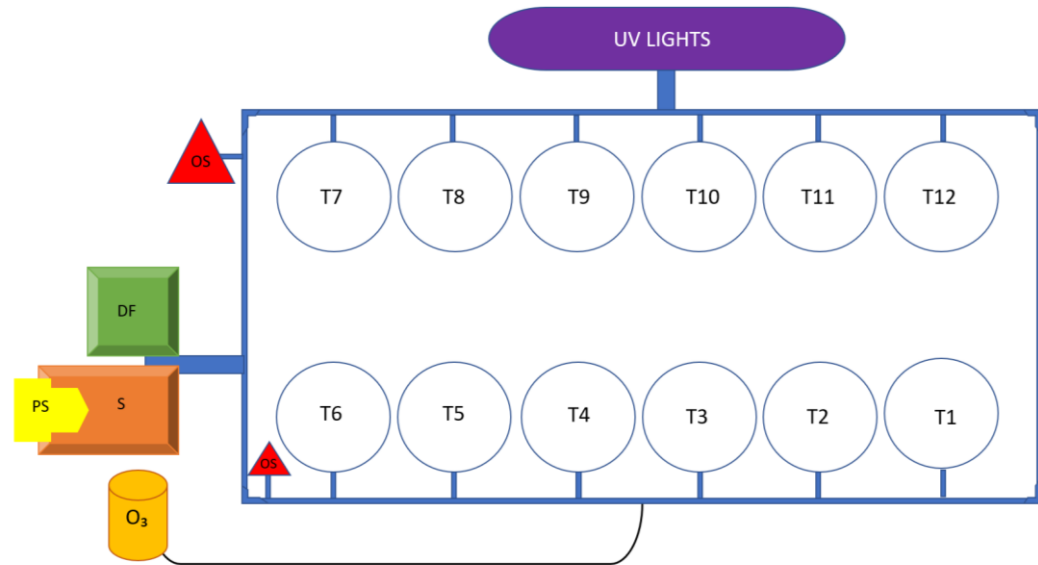


Figure 2: Diagram of experimental rainbow trout RAS System layout, with 12 tanks. ultraviolet (UV) and ozone (O<sub>3</sub>) disinfection systems, oxygen saturation cones (OS), mechanical drum filter (DF), sump (S), protein skimmer (PS).

Despite tank availability, only 3 (three) tanks were used for this project: Tanks 2, 10 and 12 from the layout shown in the image above (Fig. 2) This was due to the overhead cameras being installed for those three tanks.

## 2.2. Camera Specifications

To analyse fish behaviour during the 3 stages of this project, Internet Protocol (IP) pan-tilt-zoom cameras by Imenco Smart Solutions (Imenco Morus Surveillance Camera) and JideTech Manufacturers (JIDETECH 5MP POE PTZ CAMERA, Shenzhen JideTech Development Co. Ltd.) were used. The cameras had a 5 megapixel (MP) and 20x optical zoom, ensuring a good level of definition. Cameras were suspended above the tank and were attached to the ceiling, except the camera for Tank 2, that was at a different height due to the tank position in the bay. A lens, sensor, video amplifier, microprocessor, and network interface that links the camera to the network are all included in IP cameras. The sensor of the camera defines image resolution. For the trials recording we used a lens resolution of 4.7-94 mm, which gives a wide focal area image (Fig. 3, retrieved from Dahua NVR/DVR Quick User Guide - Jim's Security, Cazanis 2020). The camera sensor was calibrated to 5 MP and the video footage amplification was set in standard zoom.

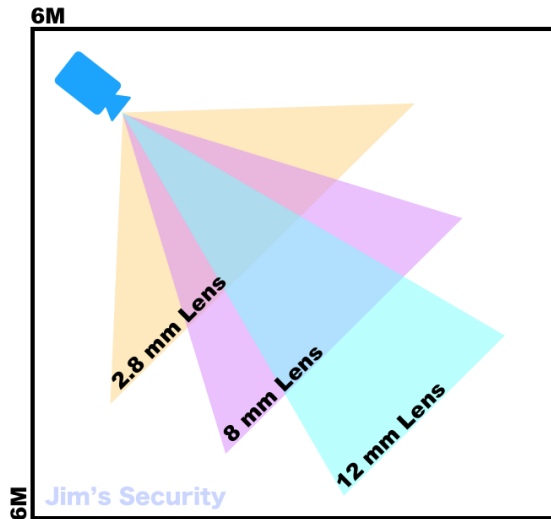


Figure 3: Example of the lens resolution for the IP cameras, Retrieved from Dahua NVR/DVR Quick User Guide - Jim's Security, Cazanis 2020.

The IP cameras were supported by a network video recorder (HIKVision Embedded NVR DS-7600, Hangzhou Hikvision Digital Technology Co. Ltd, Hangzhou, China). Overhead LED (Fig. 4) lights (36 LED Waterproof High- 18 Density Cultivation Light, TMC LED, Guan Tai Co., LTD, Taipei City, Taiwan) were also placed above each tank, providing illumination set to a 12h light/12h dark photoperiod. Throughout the experiment, the illumination glare was decreased to optimize the recording and visibility, however some glare was inevitable.

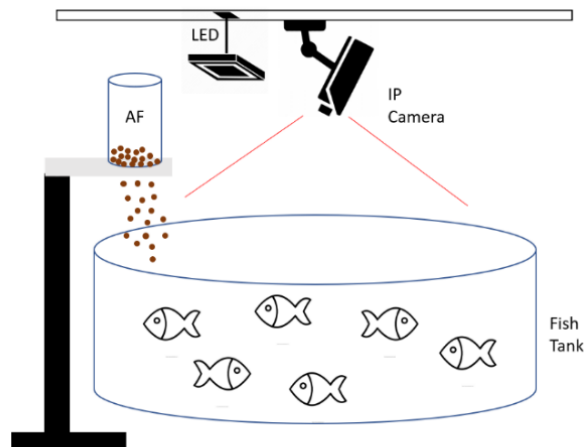


Figure 4: Scheme of camera setup used to capture fish behavioural activity with an overhead Internet Protocol (IP) camera, LED light with a 12/12 photoperiod, and an automatic feeder (AF)

### 2.3. Experimental Diet

The diet used for this project was developed by AllTech Coppens (AllTech Coppens 3.0 mm SUPREME-22, AllTech Coppens, Leende, Netherlands), and was chosen because it is commonly used in industrial scale trout production. Table 1 summarizes Supreme-22 3mm feed nutritional data (AllTech Coppens, accessed 2023).

*Table 1: Experimental Diet Nutritional Data*

<b>Nutritional Data</b>				
<b>Protein %</b>	<b>Fat %</b>	<b>Crude fibre %</b>	<b>Ash %</b>	<b>Total phosphorous %</b>
43-45	20-23	1.0-2.0	4.0-8.0	0.90

### 2.4. Preliminary Visibility Experiment Design

An experimental plan of six weeks was designed to investigate the relationship between different feeding rates with aggressive behavioural occurrences. An acclimation period to the feeding regime and to the tanks was implemented during the first week of the experimental plan. A preliminary visibility experiment was implemented during the second week of the experimental plan. The main fish behaviour experiments were conducted during the remaining four weeks of the plan.

The first week, starting on April 7<sup>th</sup>, was used only for the acclimation of fish to the tanks and to the recommended feeding rate (2.44% of tank biomass). The fish were fed by an automatic feeder that was programmed to deliver the feed at approximately 15 minute intervals, four times per day (9am, 12pm, 3pm and 5pm). During this week, three tanks were used to evaluate three different fish densities to determine which density is most feasible for visual identification of fish behaviour. ABT technicians moved the fish to Tanks 2, 10 and 12, with 4.91 kg/m<sup>3</sup>, 9.86 kg/m<sup>3</sup> and 14.74 kg/m<sup>3</sup>, respectively. On April 12<sup>th</sup>, a visibility test was conducted to determine which fish density would be used for the main trials. Fish behaviours were recorded at prefeeding, during feeding and postfeeding. Videos were reviewed manually, without the use of additional software and behaviours were not recorded. Ideal density was determined by the frequency of interactions between individuals a minimal amount of overlapping of the fish, which can impair the visibility of the behaviours.

## 2.5. Main Behaviour Experiments Design

In the following week, on April 18<sup>th</sup>, the first fish movement and weighing were performed. Fish were then acclimated to minimal feeding at a reduced feeding rate (FR) (1.99% of biomass) for one week leading up to the first trial of the experiment. This stage started on April 25<sup>th</sup>. Fish were fed the minimal FR and behaviour was recorded 30 minutes (min) before feeding, 15 min during feeding, and 30 min after feeding. Immediately after this week, fish were weighed and then acclimated for one week to the feeding rate of the next phase. Implementation of the second trial of the experiment occurred on May 2<sup>nd</sup>, where the fish were fed the recommended FR of 2.44% of biomass, and behaviour was recorded before, during, and after feeding, using the same time intervals. The same weighing followed by a one week acclimation period to maximum FR was done afterwards. Finally, on May 10<sup>th</sup>, for the third trial fish were fed a maximum FR of 2.88% of the biomass and behaviours recorded as described above. A complete summary of the experimental plan and procedures is provided in Table 2. During the whole experiment fish were weighed 4 times in total.

*Table 2: Different stages experimental procedures*

<b>EXPERIMENTAL PROCEDURES</b>		
<b>TRIAL 1</b>	<b>TRIAL 2</b>	<b>TRIAL 3</b>
<b>Minimal feeding rate</b>	<b>Recommended feeding rate</b>	<b>Maximum feeding rate</b>
<b>1.99%</b>	<b>2.44%</b>	<b>2.88%</b>
1.) Movement and weighing of experimental fish at Day 0.	1.) One week acclimation to recommended feeding rate.	1.) One week acclimation to maximum feeding rate.
2.) One week acclimation to minimal feeding rate.	2.) Record 30 min before feeding event.	2.) Record 30 min before feeding event.
3.) Record 30 min before feeding event.	3.) Record 15 minutes during the feeding event.	3.) Record 15 minutes during the feeding event.
4.) Record 15 minutes during the feeding event.	4.) Record 30 minutes after the feeding event.	4.) Record 30 minutes after the feeding event.
5.) Record 30 after the feeding event.	5.) Weighing of experimental fish.	5.) Final weighing of experimental fish at end of trial.
6.) Weighing of experimental fish.		

Table 3: Amount of feed given according with the feeding regime per tank.

<b>Diet: AllTech Coppens Supreme-22 3.0 mm</b>	<b>% Biomass/Day</b>	<b>Amount of Feed Per Day (g)</b>		
		<b>Tank 2</b>	<b>Tank 10</b>	<b>Tank 12</b>
<b>Minimum feeding regime</b>	1.99% (Low FCR)	137	137	139
<b>Recommended feeding regime</b>	2.44% (Average of 2 diets)	191	193	184
<b>Maximum feeding regime</b>	2.88% (Optimal Growth)	282	264	261

Table 3 elucidates the amount of the feed given throughout the different trials of the main experiment. Minimum feeding regime and Maximum feeding regime are percentages given by the experimental diet supplier (AllTech Coppens), as advice, depending on the needs of the producers. Minimum FR was designed as a low FCR (Feed Conversion Ratio) diet to farmers looking to minimize feed waste. Maximum FR was designed for farmers looking to achieve maximum possible growth (AllTech Coppens, accessed 2023). Recommended feeding regime was calculated independently as mean value between the supplier suggested Minimum FR and Maximum FR.

## 2.6. Evaluation of Behaviour Parameters

All behaviour parameters were evaluated by manual observation, using the video behaviour registration software CowLog (CowLog 3.0.2; Pastell, 2016), to help record and count the behaviours in the four feeding periods the feed was delivered (9am, 12pm, 3pm and 5pm). Shortcuts to quantify the behaviours observed were created in CowLog. Each time one of the evaluated behaviours was observed, a button for the shortcut was pressed which created a record in the behaviour software. After all behaviours in a given video were recorded, an automatic Excel sheet was created and downloaded from CowLog.

Individual behaviours were then grouped in terms of the frequencies of their occurrence before and after the feeding event. Videos recorded during the feeding events were discarded due to an elevated agglomeration of fish, which did not allow any possible evaluation of the behaviours (Fig. 5).

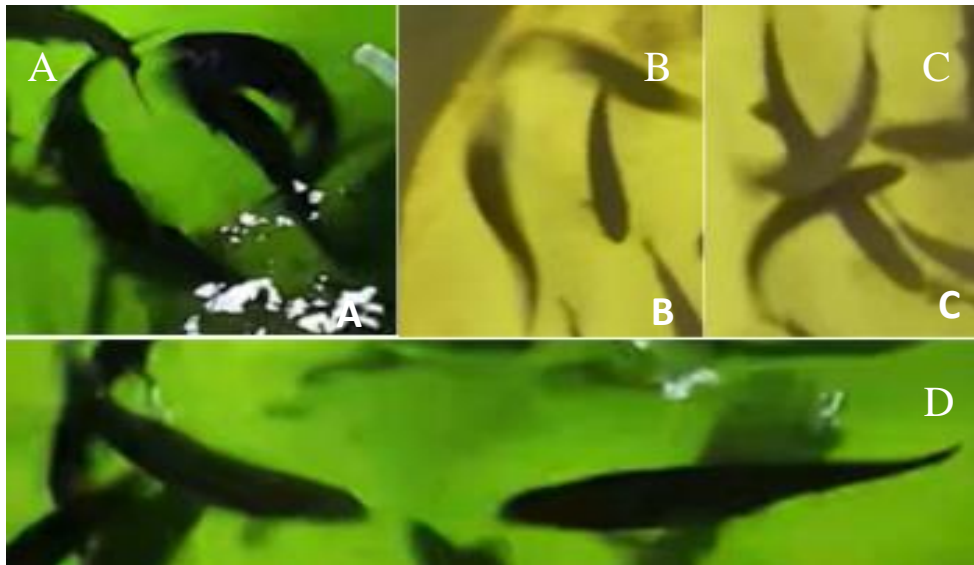


Figure 5: During feeding, fish agglomeration did not allow a proper analysis of the behaviours

Various behaviours are considered as baseline or normal for rainbow trout by many different authors, however normal behaviours were not considered in the behavioural observation and analysis of this study. The most common behaviours described as normal in the scientific literature for *O. mykiss* include constant swimming, bursting, bottom searching and static swimming (A. P. Farrell, 2008). Constant swimming is defined as the act of swimming in the same direction inside the tank throughout the day (against the tank current). Static swimming is categorized as swimming with no movement, also against the waterflow of tanks. Burst swimming involves rapid, sprint activity (for predator avoidance or advancement over rapids) that lasts for 20 s or less (Bellringer *et al.*, 2014).

Behaviours labelled as aggressive were considered for video observation. These included pursuing, circular interaction, lateral displays, and frontal confrontations. Aggressive behaviours were analysed according to the period either before or after the feeding event. Circling (Fig. 6A) was recorded when individuals were seen to swim towards each other (or one dominant fish approached another fish) relatively rapidly, followed by the fish turning away from each other as they are about to come into contact. Pursuing (Fig. 6B) behaviour can be classified as the most basic and simple form of aggressive behaviour amongst fish. This behaviour was observed when a fish burst into swim rapidly and directly towards another fish. This action is similar to the burst action. Lateral displays (Fig. 6C) were recorded when two fish swam towards one another, and before coming in contact, turned side to side with one another. During recording it was

observed individuals beat their caudal fins in a rapid motion against one another's. Frontal confrontations (Fig. 6D) were the fourth considered form of non-contact behaviours amongst the trout. In this action, individuals were seen to swim towards one another.



*Figure 6: Images exemplifying each studied behaviour: A - Circling movement; B - Pursuing interaction between two individuals; C - Lateral display; D - Frontal confrontation*

Taking all these definitions into consideration, by using CowLog 3.0.2, all visible aggressive behaviours were counted and evaluated, taking also into consideration the times of occurrences of the described behaviours.

## 2.7. Video processing

The workflow for processing behaviour videos involved multiple steps. Initially the pre- and post- feeding videos were clipped into two segments by using the video editing software OpenShot (OpenShot Video Editor, Version 2.6.1; OpenShot Studios LLC, 2022). The video analysis was done by eye tracking, during the 12 minutes before feeding and, the first 12 minutes post-feeding, having four sections per period of feeding with 2 minutes length, giving a total of 72 behavioural clips. To maintain the same time limits for the prefeeding videos and the postfeeding videos, the sections length selected were [-12min until 0min], for pre feeding and [0min until 12min], for post feeding, where the feeding period was considered as the 0 minutes.

For the analyses of the resulting clips, the behaviour analysis software CowLog was used (Fig. 7). This software allows the creation of shortcuts for the behaviours,

permitting a faster registration of the identified behaviours in the videos. CowLog, after each coding, creates a detailed and automatic excel sheet, allowing a better organization of the behaviours. Fish behaviours were then organized according to the time limits they were recorded and graphics showing the percentage of behaviours per each time limit as well as graphics demonstrating the frequency of each behaviour were generated.

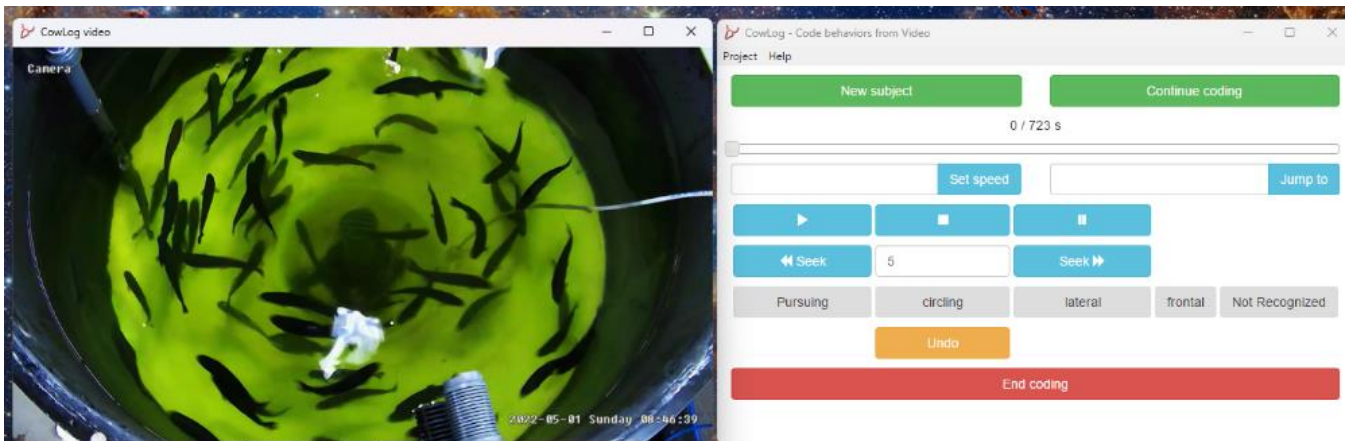


Figure 7: Exemplification of the behaviour registration.

## 2.8. Statistical Analysis

All statistical analysis were conducted using Microsoft Excel for Windows 11 and Rstudio (Rstudio version 2023.03.0 "Cherry Blossom", R version 4.2.3 "Shortstop beagle") programs. A total of 8 tables were gathered to evaluate the mean values of each behaviour at different feeding moments (pre-feed and post-feed) and assessing the correspondent standard deviation (SD). A Levene's Test was performed to assess the homogeneity of variances for both variables – feed ratio and behaviour. One-way ANOVA and Multi-way ANOVA analyses were used to observe differences between trials with normally distributed data. When homogenous of variances was not confirmed, a Kruskal-Wallis test was performed, to search for differences between the two groups, namely feeding periods and feeding regimes (Zar, 2018).

### 3. RESULTS

#### 3.1. Preliminary visibility trial

The visibility test was performed after the acclimatation of the fish stock, and 9.86 kg/m<sup>3</sup> was determined to be the highest fish density that allowed an accurate visibility of the studied behaviours. The lowest density (4.91 kg/m<sup>3</sup>) did not result in a proper quantity of behaviours to perform the statistical tests and the highest density (14.74 kg/m<sup>3</sup>) did not allow an accurate visibility of behaviours, due to the overlapping of individuals in the tank, thus these densities were discarded.

100 fish/tank corresponding to 9.86 kg/m<sup>3</sup> was determined as the best option, due to having maximum visibility with the maximum number of fish per tank.

#### 3.2. Biomass sampling

Fish biomass was monitored throughout the whole experiment. Table 4 shows fish mean weight and total biomass evolution across the three trials, resulting from 4 different biometric samplings: 1) before the beginning of the trials, on April 18<sup>th</sup>, where all 100 fish were sampled; 2) before the first trial, on April 25<sup>th</sup>, where 42 fish were weighed in T2 (78.57g/fish), 59 fish weighed in T10 (79.36g/fish) and 48 fish weighed in T12 (75.67g/fish).; 3) before the second trial on May 2<sup>nd</sup> where 50 fish were weighed in T2, T10 and T12, resulting in an average (AVG) weight per fish of 97.84g, 91.80g and 90.52g, respectively; and 4) At the end of the trials, on May 10<sup>th</sup>, where AVG weight was 119.32g/fish for T2, 117.13g/fish for T10 and 116.47g/fish for T12. There was an overall AVG weight increase across the different stages (Fig. 8). By the end of the experiment fish weight increased, on average, 1.7 times, where in T2 the increase verified was 1.73, 1.71 for T10 and 1.67 for T12.

*Table 4: Biomass Sampling Results with the corresponding Feeding Rates (FR) applied in the different days of the experiment, with the first day not having a feeding rate implemented (NA).*

Sampling Date Tank	18-Apr-22 (NA)			25-Apr-22 (1.99%FR)			02-May-22 (2.44%FR)			10-May-22 (2.88%FR)		
	2	10	12	2	10	12	2	10	12	2	10	12
Total Number of fish	100	100	100	100	100	100	100	100	100	100	100	100
Number of sampled fish	100	100	100	42	59	48	50	50	50	62	46	55

Sampled Biomass (g)	6880	6860	6962	3300	4682	3632	4892	4590	4526	7398	5388	6406
Total Biomass (g)	6880	6860	6962	7857	7936	7567	9784	9180	9052	11932	11713	11647
Average weight per fish (g)	68,80	68,60	69,62	78,57	79,36	75,67	97,84	91,80	90,52	119,32	117,13	116,47
Density (kg/m <sup>3</sup> )	9,83	9,80	9,95	11,22	11,34	10,81	13,98	13,11	12,93	17,05	16,73	16,64

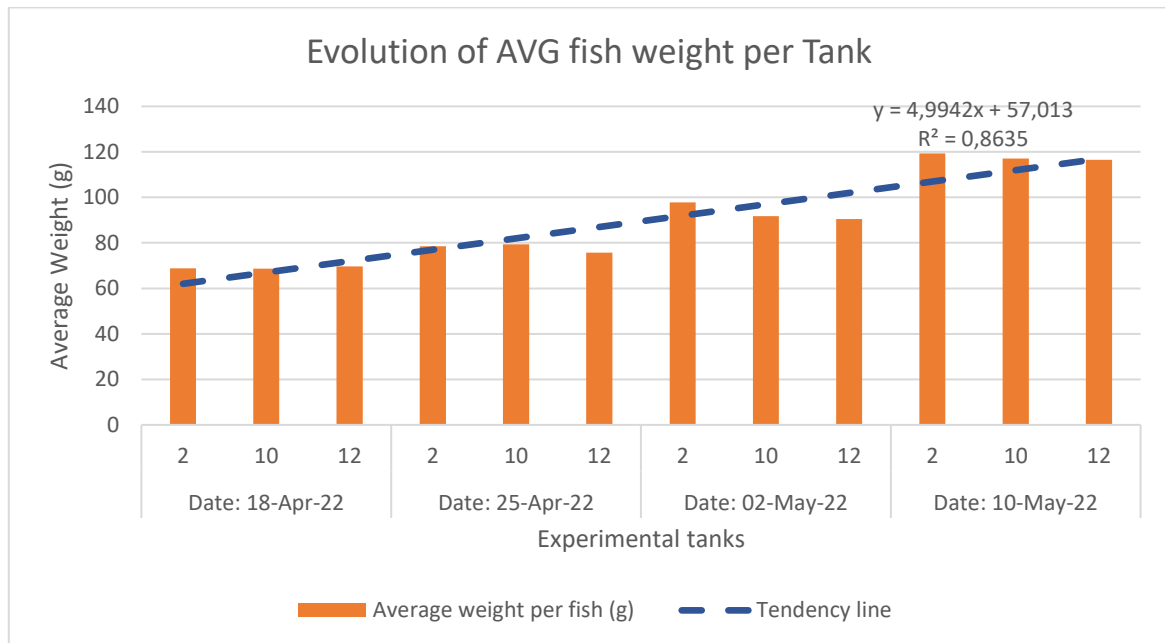


Figure 8: Evolution of average fish weight per tank (2, 10 and 12), throughout the experiment.

### 3.3. Behaviour frequency

Pursuing, circling, lateral display and frontal confrontations were the aggressive behaviours monitored during this project. In the following subsections, the results for each behaviour are depicted, for both periods of pre and postfeeding. Minimum FR (1.99 %) is represented in the figures in blue, recommended FR (2.44%) trial, in orange, and maximum FR (2.88%) in grey.

#### 3.3.1. Pursuing

The pursuing behaviour frequencies during the prefeeding period (Fig. 9A) did not show homogeneity of variances ( $p < 0.05$ ). Pursuing behaviour observed for the minimum FR kept a stable frequency during the entire experiment, having a slight, but not statistically significant, increase by the end of the feeding periods (9am  $\bar{x} = 169.7 \pm$

38.6; 12pm  $\bar{x} = 163.7 \pm 52.0$ ; 3pm  $\bar{x} = 178 \pm 14.0$ ; 5pm  $\bar{x} = 192.3 \pm 40.5$ ). This behaviour, in the recommended FR reached over 250 mean occurrences recorded, presenting a trend of increase along the day (9am  $\bar{x} = 161.7 \pm 27.0$ ; 12pm  $\bar{x} = 164.3 \pm 7.4$ ; 3pm  $\bar{x} = 205.7 \pm 69.6$ ; 5pm  $\bar{x} = 252.0 \pm 210.4$ ). As for the maximum FR, a similar number of occurrences of this behaviour was observed throughout the four feeding times, presenting maximum values at 3pm (9am  $\bar{x} = 127.0 \pm 20.0$ ; 12pm  $\bar{x} = 145.0 \pm 20.0$ ; 3pm  $\bar{x} = 163.0 \pm 18.5$ ; 5pm  $\bar{x} = 114.7 \pm 68.2$ ). The Kruskal-Wallis test showed that FR had an influence on behaviour occurrences ( $p < 0.05$ ). The post-hoc test (Dunn test) revealed a significant difference between the maximum FR and the minimum FR.

Postfeeding pursuing behaviour frequencies (Fig. 9B), recorded under minimum FR, had similar values overall, presenting a slight decrease at 5pm (9am  $\bar{x} = 131.3 \pm 28.9$ ; 12pm  $\bar{x} = 134.3 \pm 16.5$ ; 3pm  $\bar{x} = 134.0 \pm 12.8$ ; 5pm  $\bar{x} = 113.7 \pm 22.0$ ). Postfeeding pursuing behaviours showed a stable number of frequencies, for recommended FR, with maximum values at 3pm (9am  $\bar{x} = 128.0 \pm 8.89$ ; 12pm  $\bar{x} = 123.0 \pm 5.20$ ; 3pm  $\bar{x} = 134.3 \pm 51.73$ ; 5pm  $\bar{x} = 119.7 \pm 44.38$ ). For the last FR, maximum, pursuing had lower frequencies at the feeding event at the end of the day, at 5pm (9am  $\bar{x} = 99.3 \pm 3.06$ ; 12pm  $\bar{x} = 88.7 \pm 13.32$ ; 3pm  $\bar{x} = 101.7 \pm 22.68$ ; 5pm  $\bar{x} = 50.7 \pm 32.32$ ). The frequencies of this behaviour showed no homogeneity of variances ( $p < 0.05$ ) and data presented statistical differences, between recommended and maximum FR, as well as between minimum FR and maximum FR (Kruskal-Wallis, Dunn's test,  $p < 0.05$ ).

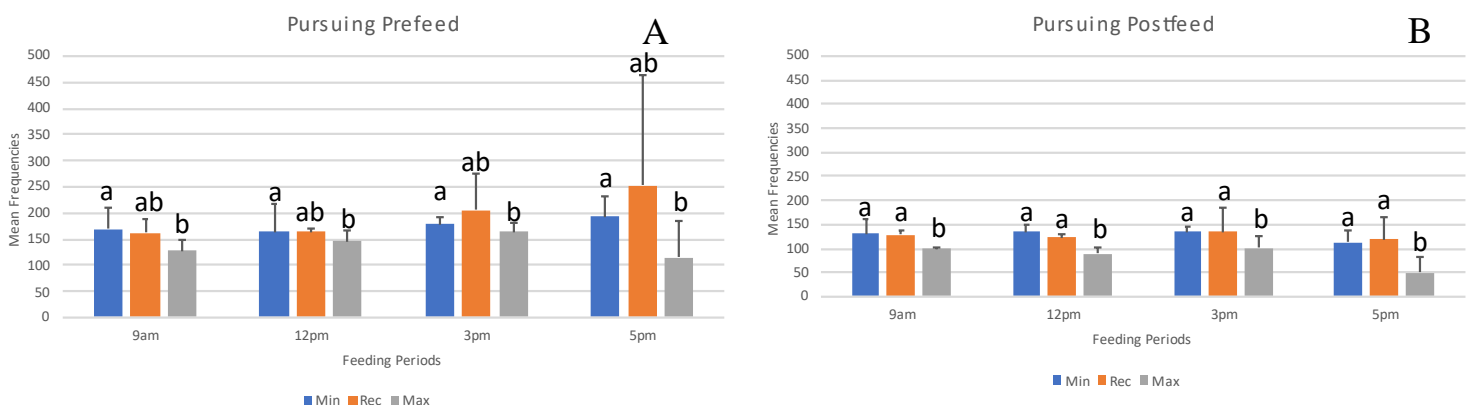


Figure 9: Mean values of pursuing behaviour frequencies throughout the 4 feeding moments of the day, during pre-feeding (A) and post feeding (B) events; Min = minimum feeding rate (1.99%), Rec = recommended feeding rate (2.44%), Max = maximum feeding rate (2.88%). Different lower case letters (a,b) indicate significant statistical differences between FR (Kruskal-Wallis; Dunn's test =  $p < 0.05$ ).

### 3.3.2. Circling

Prefeed circling behaviour frequencies (Fig. 10A), did not show homogeneity of variances. This behaviour presented a similar pattern as the previous one (pursuing), increasing slightly towards the end of the day both at minimum FR (9am  $\bar{X} = 98.7 \pm 48.1$ ; 12pm  $\bar{X} = 93.3 \pm 49.8$ ; 3pm  $\bar{X} = 106.7 \pm 54.0$ ; 5pm  $\bar{X} = 113.3 \pm 52.5$ ) and at recommended FR (9am  $\bar{X} = 106.3 \pm 27.4$ ; 12pm  $\bar{X} = 104.0 \pm 16.1$ ; 3pm  $\bar{X} = 123.0 \pm 54.0$ ; 5pm  $\bar{X} = 154.0 \pm 127.5$ ). Also at maximum FR, this behaviour had a similar variation of frequencies as the pursuing behaviour, with a maximum value observed at 3pm (9am  $\bar{X} = 60.7 \pm 19.9$ ; 12pm  $\bar{X} = 71.3 \pm 28.0$ ; 3pm  $\bar{X} = 88.3 \pm 21.0$ ; 5pm  $\bar{X} = 69.0 \pm 66.2$ ). Kruskal-Wallis and Dunn's tests indicated statistical differences between recommended and maximum FR ( $p < 0.05$ ).

During the postfeeding events (Fig 10B), circling behaviour at minimum FR showed a tendency to decrease towards the end of the day (9am  $\bar{X} = 88.3 \pm 17.7$ ; 12am  $\bar{X} = 94.3 \pm 21.9$ ; 3pm  $\bar{X} = 82.3 \pm 27.0$ ; 5pm  $\bar{X} = 74.7 \pm 3.2$ ). Regarding the recommended FR, circling maintained its mean frequencies throughout the feeding times (9am  $\bar{X} = 64.0 \pm 4.36$ ; 12pm  $\bar{X} = 60.7 \pm 7.23$ ; 3pm  $\bar{X} = 74.3 \pm 39.58$ ; 5pm  $\bar{X} = 64.3 \pm 29.14$ ). At a maximum FR, circling behaviours presented a similar profile as pursuing, decreasing at 5pm (9am  $\bar{X} = 60.3 \pm 14.01$ ; 12pm  $\bar{X} = 47.7 \pm 12.90$ ; 3pm  $\bar{X} = 56.0 \pm 8.72$ ; 5pm  $\bar{X} = 26.7 \pm 26.50$ ). Statistical tests performed on these postfeeding behaviours showed no homogeneity of variances ( $p < 0.05$ ) and exposed significant statistical differences ( $p < 0.05$ ), between recommended and maximum FR.

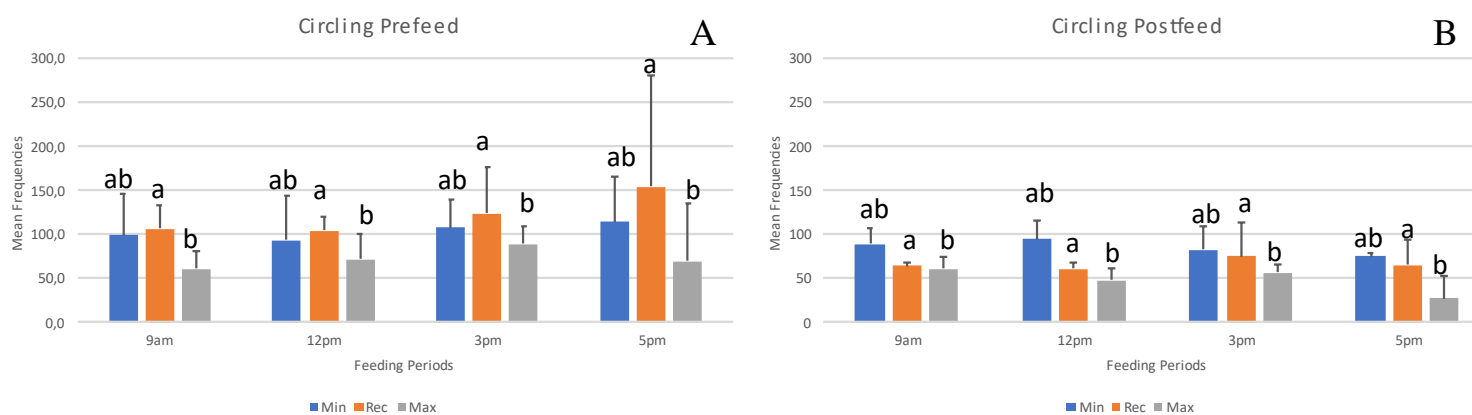


Figure 10: Means of circling behaviour frequencies throughout the feeding periods in prefeeding (A) and postfeeding (B) events; Min = minimum feeding rate (1.99%), Rec = recommended feeding rate (2.44%), Max = maximum feeding rate (2.88%). Different lower case letters (a,b) indicate significant statistical differences between FR. (Kruskal-Wallis; Dunn's test =  $p < 0.05$ ).

### 3.3.3. Lateral Display

For the lateral display behaviour, during prefeeding events (Fig.11A), the lowest values were registered at 12pm under minimum FR (9am  $\bar{X} = 15.0 \pm 2.6$ ; 12pm  $\bar{X} = 8.7 \pm 2.5$ ; 3pm  $\bar{X} = 14.7 \pm 0.6$ ; 5pm  $\bar{X} = 18.3 \pm 6.1$ ). Under recommended FR, lateral display frequencies were higher at 3 and 5pm, in comparison with the other time points (9am  $\bar{X} = 10.7 \pm 4.9$ ; 12pm  $\bar{X} = 8.3 \pm 4.0$ ; 3pm  $\bar{X} = 16.0 \pm 16.1$ ; 5pm  $\bar{X} = 16.3 \pm 18.8$ ). Under maximum FR, this behaviour had a higher mean frequency at 3pm and presenting a substantial decrease along the day, till 5pm (9am  $\bar{X} = 8.3 \pm 2.1$ ; 12pm  $\bar{X} = 10.7 \pm 1.2$ ; 3pm  $\bar{X} = 11.7 \pm 4.2$ ; 5pm  $\bar{X} = 5.7 \pm 7.2$ ). In terms of statistical analysis this behaviour did not show homogeneity of variances neither significant difference among feeding regimes or feeding time points (Kruskal-Wallis,  $p > 0.05$ ).

Regarding lateral displays during postfeeding events (Fig. 11B), the frequencies were consistent in the entire experiment under minimum FR, only presenting a minimal decrease at the end of the day (9am  $\bar{X} = 8.3 \pm 3.2$ ; 12pm  $\bar{X} = 8.3 \pm 3.5$ ; 3pm  $\bar{X} = 8.0 \pm 7.5$ ; 5pm  $\bar{X} = 7.0 \pm 6.6$ ). The mean frequency under recommended FR (Fig. 10B), showed a maximum value at 12pm (9am  $\bar{X} = 4.3 \pm 1.15$ ; 12pm  $\bar{X} = 8.0 \pm 2.65$ ; 3pm  $\bar{X} = 5.7 \pm 2.89$ ; 5pm  $\bar{X} = 3.3 \pm 2.31$ ), the same happening for maximum FR (9am  $\bar{X} = 3.3 \pm 2.52$ ; 12pm  $\bar{X} = 5.3 \pm 2.08$ ; 3pm  $\bar{X} = 4.3 \pm 2.08$ ; 5pm  $\bar{X} = 1.7 \pm 1.53$ ). Similar, to the prefeeding events, in postfeeding statistical analysis lateral displays did not show homogeneity of variances (Levene's test,  $p < 0.05$ ) and significance within the feed ratio (Kruskal-Wallis,  $p < 0.05$ ).

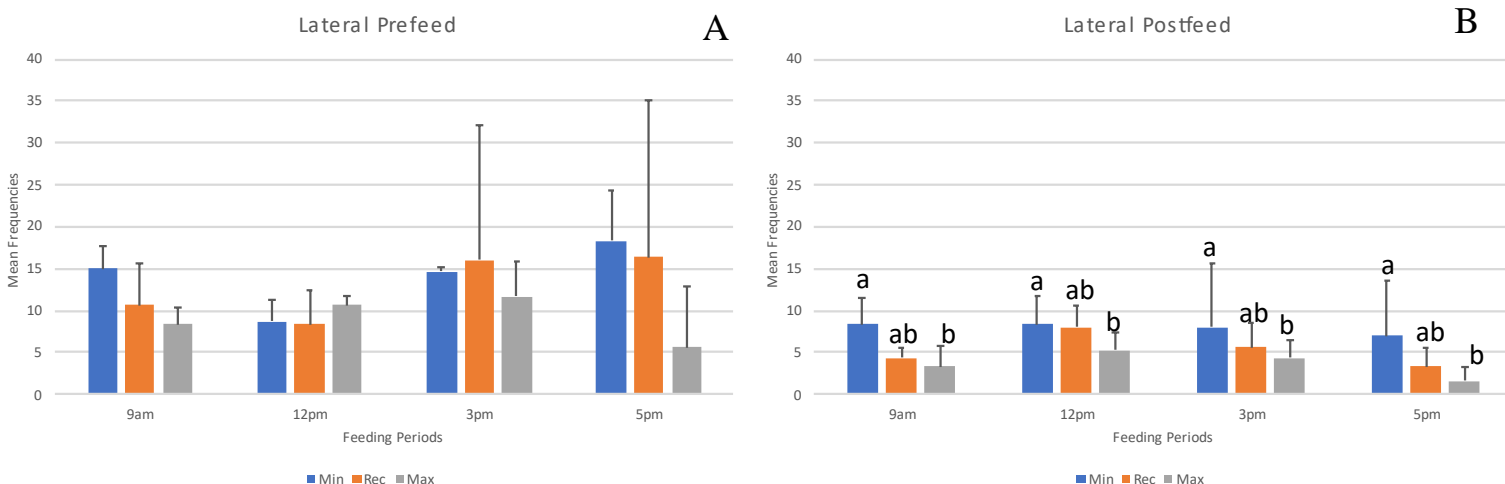


Figure 11: Means of lateral display behaviour frequencies throughout the feeding periods in prefeeding (A) and postfeeding (B) events; Min = minimum feeding rate (1.99%), Rec = recommended feeding rate (2.44%), Max = maximum feeding rate (2.88%). Different lower case letters (a,b) indicate significant statistical differences between FR (Prefeed Kruskal-Wallis, Dunn's test =  $p > 0.05$ , Postfeed Kruskal-Wallis; Dunn's test =  $p < 0.05$ ).

### 3.3.4. Frontal Confrontations

Frontal confrontation was in general the behaviour presenting the lowest frequencies. During prefeeding (Fig 12A), and under minimum FR the highest values were observed at 5pm (9am  $\bar{X} = 0.7 \pm 0.6$ ; 12pm  $\bar{X} = 0.3 \pm 0.6$ ; 3pm  $\bar{X} = 0.7 \pm 1.2$ ; 5pm  $\bar{X} = 1.0 \pm 0.0$ ). During recommended FR period, no occurrences were seen at 9am, and an increase from 12pm until 5pm was registered (12pm  $\bar{X} = 0.7 \pm 0.6$ ; 3pm  $\bar{X} = 1.7 \pm 2.9$ ; 5pm  $\bar{X} = 2.0 \pm 3.5$ ). As for the maximum FR, frontal confrontation had no occurrences at 9am and 5pm, and frequencies were low on the other time points (12pm  $\bar{X} = 1.0 \pm 1.0$ ; 3pm  $\bar{X} = 0.3 \pm 0.6$ ). By performing the statistical analysis, no homogeneity of variances was verified in the data ( $p < 0.05$ ) and no significant differences were observed among FRs (Kruskal-Wallis,  $p > 0.05$ ).

Postfeeding frontal confrontations (Fig. 12B) did not register any events in minimum FR, while at recommended FR this behaviour presented higher frequencies at 12pm (9am  $\bar{X} = 0.3 \pm 0.58$ ; 12pm  $\bar{X} = 1.3 \pm 1.53$ ; 3pm  $\bar{X} = 0.7 \pm 0.58$ ; 5pm  $\bar{X} = 0.7 \pm 1.15$ ). At maximum FR, frontal confrontations only occurred at the 9am feeding period (9am  $\bar{X} = 0.3 \pm 0.58$ ). All statistical analysis showed no homogeneity of variances in the data ( $p < 0.05$ ), and when performing a Kruskal-Wallis test, significant differences among feeding ratios ( $p < 0.05$ ) were seen. The behaviours registered under recommended FR were proven to be significantly higher the registered under the other two FRs ( $p < 0.05$ ). There was no relation between feeding period and frequency of behaviours.

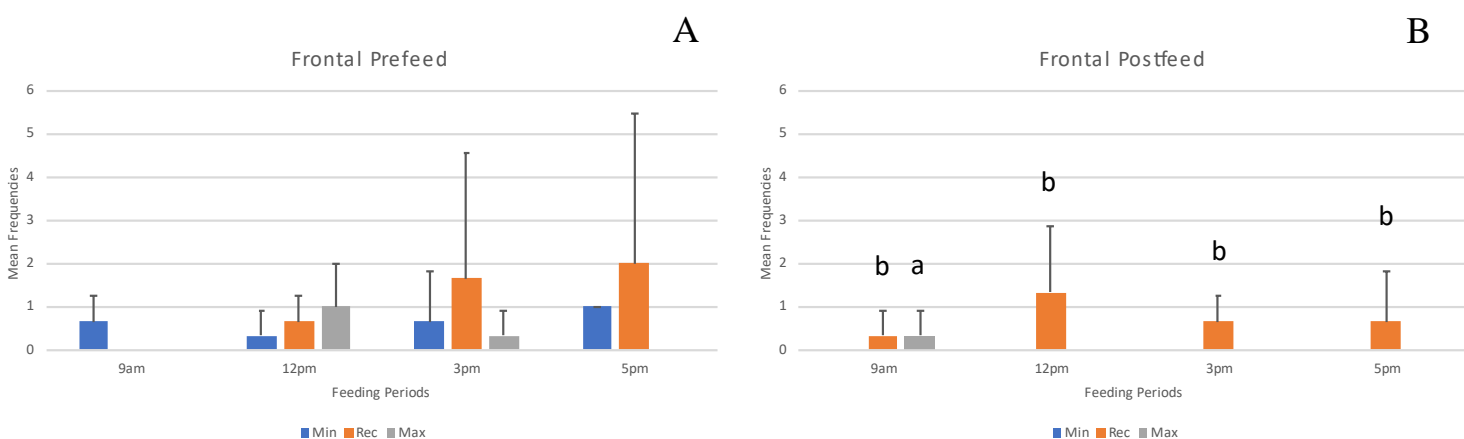


Figure 12: Means of frontal confrontation behaviour frequencies throughout the feeding periods in prefeeding (A) and postfeeding (B) events; Min = minimum feeding rate (1.99%), Rec = recommended feeding rate (2.44%), Max = maximum feeding rate (2.88%). Different lower case letters (a,b) indicate significant statistical differences between FR (Prefeed Kruskal-Wallis, Dunn's test =  $p > 0.05$ , Postfeed Kruskal-Wallis, Dunn's test =  $p < 0.05$ ).

## 4. DISCUSSION

The present research had the objective of studying the impact of feeding ratio on fish aggressiveness in rainbow trout, using behaviour-based video analysis in a commercially relevant scale RAS. With the feed ratio trials performed in rainbow trout, it was expected to obtain a decrease in aggressiveness, along with the increase in FR and further feeding periods. In addition, it was expected that observations of different aggressive behaviours would be more frequent in the pre-feeding periods than in the post-feeding periods, in relation to the hunger levels of the fish and the availability of the feed.

Overall, the findings of this study showed that FR had an influence on fish aggressiveness and behaviour in RAS cultivated rainbow trout. Mean frequencies of aggressive behaviours displayed expected results, with some discrepancies in several of the recorded behaviours throughout the various feeding regimes. Significant influences for prefeeding events were verified between minimum FR and maximum FR, as well between recommended FR and maximum FR. Regarding postfeeding events the most significant influence of FR was between recommended FR and maximum FR, for pursuing, circling and frontal confrontation. The feeding periods did not present any influence in the frequency of any behaviours. Comparing postfeeding recommended and maximum FR, maximum FR displayed a positive influence in behaviours, with mean frequencies being lower in this feed ratio.

Statistical results showed that in prefeeding events, except for lateral displays and frontal confrontations, behaviours were influenced by FR. In the same way, postfeeding behaviour frequencies were influenced by quantity of feed available. In general, all behaviours observed showed to be influenced by feed availability, with circling being the behaviour most influenced by the FR. These results are in agreement with Zimmermann *et al* (2012), who concluded that feed availability influenced farmed Atlantic cod (*Gadus morhua*) interactions within sea cages. Similar results were also obtained by Pang *et al* (2022) and Ros *et al* (2006), using catfish (*Silurus meridionalis*), in agreement with our study. Pang *et al* (2022) measured metabolic response under different dietary plans, with different feeding periods. Their findings suggested an augmented agitation with decreased FRs originated lower metabolic responses, e.g., less aggression towards others. As for the investigation conducted by Ros *et al* (2006), aggression results similar to our

project were obtained with Mozambique tilapia (*Oreochromis mossambicus*), however, in these authors experiment oxygen consumption was also taken in consideration.

Overall, the prefeeding results showed a tendency of increase in aggressiveness along the different feeding times, as well as a tendency to increase as FR increased. Additionally, it was possible to observe that the recommended FR presented the highest increase in aggressive behaviours, when considering the prefeeding events. Postfeeding had an opposite fluctuation, showing a general decrease in aggressive behaviours, with higher mean frequencies registered at 9am feeding period and decreasing until the 5pm feeding period, for all three percentages of feeding ratio.

It is important to note that some of this decrease in observed behaviours was due to the standard operating procedures of the system. During the minimum FR trial, at the 12pm feeding period, tank oxygen levels declined, and the emergency oxygen system was enabled. This caused a reduction in visibility and therefore a reduction in recorded behaviours. When comparing all FRs, the recommended FR had the highest occurrences of aggressive behaviours across the whole experiment, with the exception of lateral display, which revealed a higher frequency in minimum FR, both in prefeeding and postfeeding events. This shows that when rainbow trout were previously subjected to a low FR, an increase in FR enhances aggression and competition for food. In contrast, when the fish are submitted to a maximum FR after a recommended FR, mean aggressive behaviour frequency tended to decrease overall, both in prefeed and postfeed periods. Consequently, such contrasts between these two FRs show that the highest percentage of feed is more adequate to fulfil rainbow trout satiation and to reduce aggression levels in recirculatory systems. Typically, fish fed with a restricted dietary intake, respond behaviourally different to different levels of hunger. When food is available, fish begin to feed more voraciously and then gradually slow down or stop as hunger wanes (Assan *et al.*, 2021). Thus, maximum FR seems to be an optimum FR to minimize aggressive behavioural frequencies, when compared with the proposed recommended FR.

Results in postfeed showed a trend to decrease aggressive behaviour frequency with the increase in FR, along the daily feeding times, in accordance with a study conducted by Zhao *et al* (2016) where the feeding intensity and aggression of tilapia was evaluated over 3 feeding periods. It was concluded that fish tend to have reduced aggression towards others in the same environment when fed to satiation. Similarly,

Oikonomidou *et al* (2019) performed a study using European seabass and gilthead seabream to evaluate aggression in relation to feed availability. In the end, the higher aggressiveness ratios were observed when the stock was fed at a lower FR. In our results, trout fed at maximum FR showed to be more satisfied with the amount of feed available, therefore, presented a less aggressive behaviour towards other individuals in the same tank.

Moving forward with the evolution of these type of experiments to improve aquaculture production and to reduce harmful behaviours in the stock is necessary. Naturally, visibility takes a crucial role in the observation of fish behaviours. Throughout the whole experiment, camera visibility was a challenge due to the cameras' framerate and lens footage reach. Therefore, for further research, improved cameras with better framerate and lens reach should be used to promote a cleaner image and facilitate observation. Moreover, the placement of emergency oxygen injectors and automatic feeders can also be improved in future investigations. Regarding emergency oxygen in particular, the placement must be strategic to allow a better visibility, especially when injectors are enabled and functioning for extended periods of time.

Manley *et al* (2015), referred in his study the importance of an established feeding frequency to maintain the welfare of the tank population. Feeding rates can have a significant impact on aggressive fish behaviour in aquaculture systems. In general, fish tend to become more aggressive when they are hungry or when they perceive a limited access to food resources (Baras and Jobling, 2002). On the other hand, overfeeding can also lead to aggressive behaviours, especially if it leads to waste and a deterioration of water quality. Therefore, it is important to balance feeding rates with the nutritional requirements of the fish and the capacity of the aquaculture system to manage the waste.

Pursuing and circling were the most frequent aggressive behaviours recorded throughout the trials, and frontal confrontations the least frequent. Pursuing and circling are known as the most common aggressive behaviours observed when fish are inserted in an environment with limited food availability (Zimmermann *et al.*, 2012). Such results can provide important data to the development of an AI algorithm that would be able to understand these behaviours and automatically dispense feed to avoid an increase in aggression and preserve animal welfare. Similar information regarding fish behaviour is currently being used to develop an algorithm under the Smart Remote Access Server

(SMARTTRAS) and iBOSS outputs for the iFishIENCi project. By collecting and analysing fish behaviour data, researchers and producers can gain valuable insights into various aspects of animal well-being, allowing farmers to respond accordingly, ensuring optimized feeding and reduced feed waste.

Feeding regimes play an important role in fish aggression in aquaculture systems. Therefore, providing an adequate and consistent supply of food is essential in reducing aggression among fish (Pěnka *et al.*, 2023; An *et al.*, 2021). Timed feeding involves providing fish with a set amount of food at specific times throughout the day, which help reduce waste and improve overall feeding efficiency (Gilannejad *et al.*, 2021). As seen by our results, at higher feed availability (maximum FR), less aggressiveness from the trout was registered towards the end of the of the feeding times, agreeing with previous studies, already conducted with other fish species like Atlantic salmon (*Salmo salar*) (Behrend *et al.*, 2022).

To address the aquaculture industry's need for operational optimisation, the iFishIENCi project (Horizon 2020 project funded by the European Union) aims to improve the cycle of fish production through the development and demonstration of smart feeding approaches in a diverse range of aquaculture contexts. The iFishIENCi project consists of 16 interdisciplinary industry and academic partners interested in reducing aquaculture generated wastes through the use of IOT and AI technology. By utilising state-of-the-art sensors enabling biology online (i.e., fish behaviour, growth, welfare, health and environmental microbiome), water quality monitoring to improve decision-making and smart feeding control, the iFishIENCi Biology Online Steering System (iBOSS) aims to employ intelligent fish behaviour tools to anticipate fish feeding, health, and welfare (iFishIENCi, 2020). The main goal of quantification analysis is to automate many tasks, including biomass estimation and aggressive behaviour monitoring (An *et al.*, 2021). This is one of the reasons why iFishIENCi aims to develop an AI capable of automatically detecting various fish behaviours to optimize feed availability.

In addition, there is ongoing research exploring the use of natural compounds and other substances that may help to reduce aggression in fish. For example, a study by Hubená *et al.* (2021) showed that adding plant extracts to fish feeds, such as methamphetamine and tramadol, can reduce aggression and improve overall fish health

in the first two weeks of usage. However, after that initial period, there is a tendency of aggressiveness increase as a consequence of those extracts.

Animal aggression occurs in most farming fields. In the poultry industry, feeding-related aggression has been extensively studied in farmed hens (Sirovnik *et al.*, 2018, Thogerson *et al.*, 2009). Overall, studies concluded that restricting animal's access to feed tended to increase aggression in a closed environment. Despite studies of aggression related with feed availability being performed in different species (fish, pigs, poultry, , etc.), the same tendency is obtained across the farming industries. The exhibition of hostility towards individuals in the same environment increases when animals are subjected to a feeding schedule or a restriction of feed (Peden *et al.*, 2018).

For the monitoring of fish wellbeing there are currently many associations providing welfare recommendations, such as the Royal Society for the Prevention of Cruelty to Animals (RSPCA) welfare criteria (RSPCA, 2018). Inadequate stocking densities and transportation methods can increase fish cortisol levels and overall stress (Gozdowska *et al.*, 2022). Taking into consideration that good animal welfare reduces aggressive interactions and, therefore, reduces costs in treatment methods and applied nutrition, the evolution of farming techniques that prevent such behaviours have helped the aquaculture industry to reduce costs and increase effectiveness on large scale production (Fernandes *et al.*, 2021). Although improving animal welfare has high costs to implement the needed technology and improve labour (Fernandes *et al.*, 2021), on the long-term it compensates by presenting a better product quality (Sandøe *et al.*, 2020). On a financial level, fish aggression in aquaculture farming can lead to significant economic losses, such as reduced growth rates (Jørgensen *et al.*, 2017), increased mortality rates (van den Boogart *et al.*, 2023; Ul Hassan *et al.*, 2021), and lower quality products (Oikonomidou *et al.*, 2019; Zhao *et al.*, 2016). It is important for farmers to implement strategies to manage aggression, such as providing adequate space and environmental enrichment for the fish, and carefully monitoring feeding regimes to reduce competition for food.

Managing the farming environment is one of the major successes and benefits of RAS technology. Implementing different environmental parameters in geographical locations where those parameters would not be a natural occurrence (for example, cold climate fish species in warm climates locations, e.g., rainbow trout produced in Malta) as

can be done with RAS, expanding the potential of aquaculture for different species production in areas where would not be possible otherwise (Ahmed and Turchini, 2021). This technology offers several advantages, including increased control over water quality, disease management, reduced water usage, and higher productivity. RAS can operate year-round without being affected by external environmental factors. Furthermore, RAS systems can be established near urban centres, reducing the costs of transportation and providing the consumers with fresher seafood products (Helfrich and Libey, 1991). However, to maintain a high productivity it is necessary to maintain a higher stocking density, which can promote different aggression displays. To mitigate aggression in high-density aquaculture systems these displays must be observed and adequate monitoring protocols implemented.

Future research on this theme should be focused on the relation of aggression with feed intake and stomach fullness, as a continuation to the study presented by Panasiak (2021). Such research could augment the efficiency of automatic feeding, where improved feed management would possibly reduce feed waste, and, therefore, optimize fish growth. Additionally, it would be interesting to quantify which behaviours are considered to be normal in rainbow trout, when integrated in a RAS aquaculture facility. Afterwards, such results should be compiled and aggregated with the aggressive behaviours, to obtain a behaviour dataset to develop a rainbow trout aggressive behaviour index. An aggression index would be a way to quantitatively measure the degree of aggression displayed by a population of farmed fish (with varying levels of low aggression behaviours to high aggression behaviours), benefiting the aquaculture sector by optimized fish health and welfare, increased production efficiency, reduced cannibalism and injuries, allowing for effective allocation of resources, improved decision-making, and adoption of sustainable production methods. By effectively managing aggression, aquaculture operators can optimize productivity while ensuring the well-being of the fish populations.

## **5. CONCLUSIONS**

This project was funded by the EU Horizon 2020 iFishIENCi project in order to help meet the consortium's objectives to improve feeding and fish farming efficiency via an AI to automate and identify behaviours in rainbow trout. By investigating the feeding

patterns and responses of rainbow trout in relation to different feeding ratios, it was possible to identify important insights into the impact of feed availability in the aggressive behaviour of rainbow trout in RAS:

- ✓ The number of individuals inside the tank influences behaviour visibility, therefore, the relation between total biomass and visibility must be adequate to allow reliable data retrieval.
- ✓ The lower the percentage of FR, the higher the probability of having a high frequency of aggressive behaviours. Therefore, FR has an influence in the frequency of behaviours.
- ✓ Daily feeding time did not influence behavioural frequency.
- ✓ The Maximum FR was demonstrated to be the feed percentage most efficient in terms of bringing fish to satiation, thus reducing aggression.
- ✓ Frequency of aggressive behaviours generated by fish could be used to develop an aggression index, which would help farmers to assess fish welfare and feeding efficiency in RAS.

The results of this study show that rainbow trout aggressiveness in RAS is significantly influenced by the availability of feed, with the highest FR proven to be the best for lowering antagonism in these fish after comprehensive testing. The present thesis offers important information, with potential to improve aquaculture methods based on the assumptions that good feed management may successfully curb aggressive tendencies in farmed rainbow trout populations, leading to healthier fish welfare in RAS settings. Adequate feed management and availability plays a vital role in the aquaculture industry, by promoting optimal growth and nutrition, reducing costs, ensuring sustainability, improving feed conversion efficiency, and assuring feed quality. By implementing effective feed management practices, the industry can enhance the productivity, profitability, and sustainability.

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## **Appendix I**

### Rainbow trout feeding times behavioural frequency

Frequency values were registered throughout the entire project, providing the totality of behaviours observed in the different feeding times, feeding rates and comparison of frequency between the three tanks in the study. Two final tables with the mean frequencies of behaviours were obtained (Tables 5 6), as well as mean frequency graphs per feeding period, having a total of six frequencies graphs and twenty-four tables of individual behaviours.

All behaviours were registered between April and May, with the final week of April being the first week of the project where the first sampling was conducted, giving total biomass data and overall weight of the stocking density, referred on Table 4.

## 1.1 Prefeed Mean Behaviours

Table 5: Prefeed Mean Behavioural Frequencies from each Behaviour per Feeding Rate.

Feeding Period	9A M	PURSUING PREFEED			CIRCLING PREFEED			LATERAL PREFEED			FRONTAL PREFEED					
		12PM	3PM	5PM	9AM	12PM	3PM	5PM	9AM	12PM	3PM	5PM	9AM	12PM	3PM	5PM
<b>Recommended Feed</b>	56,00	41,08	51,42	63,00	26,58	26,00	30,75	38,50	3,75	2,17	4,00	4,08	0,00	0,17	0,42	0,50
<b>Maximum Feed</b>	31,75	36,25	40,75	28,67	15,17	17,83	22,08	17,25	2,08	2,67	2,92	1,42	0,00	0,25	0,08	0,00
<b>Minimum Feed</b>	42,42	40,92	44,58	48,08	42,42	23,33	26,67	28,33	3,75	2,17	3,67	4,58	0,17	0,08	0,17	0,25



Figure 13: Prefeed Average Behavioural Frequencies for the different feeding rates and per each feeding period

## 1.2 Postfeed Mean Behaviours

Table 6: Postfeed Mean Behavioural Frequencies from each Behaviour per Feeding Rate.

Feeding Period	9A M	PURSUING POSTFEED			CIRCLING POSTFEED			LATERAL POSTFEED			FRONTAL POSTFEED					
		12PM	3PM	5PM	9AM	12PM	3PM	5PM	9AM	12PM	3PM	5PM	9AM	12PM	3PM	5PM
<b>Recommended Feed</b>	32,00	30,75	33,58	30,17	16,00	15,17	18,58	16,08	1,08	2,00	1,42	0,83	0,08	0,33	0,17	0,17
<b>Maximum Feed</b>	24,83	22,17	25,42	12,67	15,08	11,92	14,00	6,67	0,83	1,33	1,08	0,42	0,08	0,00	0,00	0,00
<b>Minimum Feed</b>	32,83	33,58	33,50	28,42	22,08	23,58	20,58	18,67	2,08	2,08	2,00	1,75	0,00	0,00	0,00	0,00

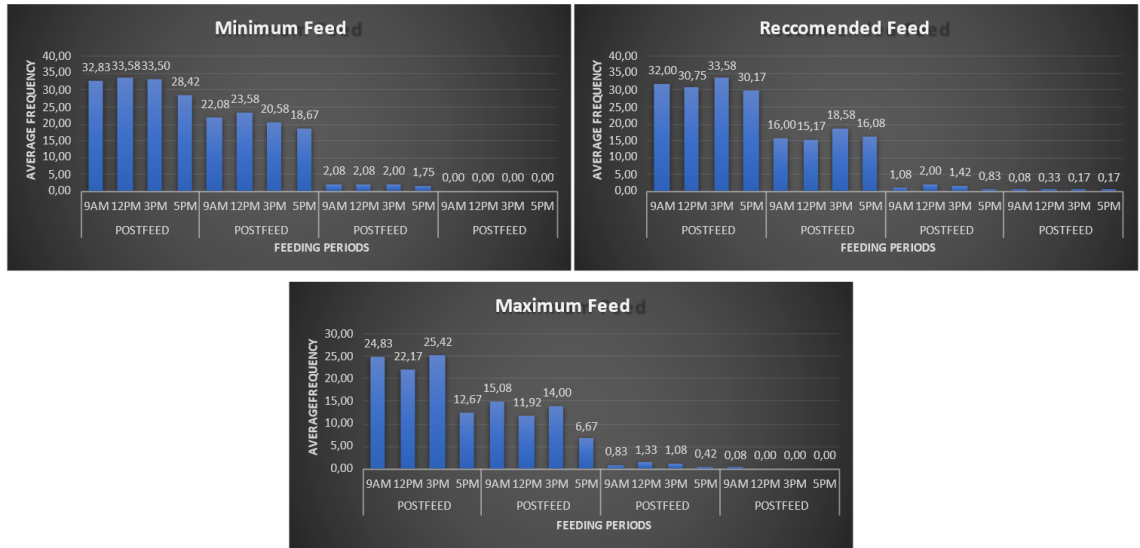


Figure 14: Postfeed Average Behavioural Frequencies for the different feeding rates and per each feeding period

## **Appendix II**

## Statistical data

A number of different statistical tests were conducted to obtain an accurate analysis regarding the influence of feed availability. An initial Levene's test was conducted to verify the assumptions, and it presented the relationship between feeding period and feed ratio, in the four different aggressive behaviours in both prefeeding and postfeeding events. Giving that to have a statistical significance, the probability value (p-value) has to be lower than 5% ( $p < 0.05$ ), in this case it was considered a 95% Confidence Interval (CI). Therefore, if the primary ANOVA test is greater than the 0.05, the behaviour frequency is not influenced by feed ratio.

Both a Kruskal-Wallis and a Dunn test were conducted *a posteriori* to verify the relation between FR and the behaviours and to verify which FRs had statistical differences, respectively. The following tables (7 - 18) presented the retrieved data from the referred tests, with significance codes (\*) statistical values, such as degrees of freedom (Df), variation of factors (Sum sq), significance factor (Mean sq), ratio of variance (F-value), probability value of statistics (Pr(>F)) and data sample (n).

### 1.1 Pursuing

#### 1.1.1 ANOVA Postfeed

Table 7: Primary ANOVA test pursuing postfeed

	<b>Df</b>	<b>Sum sq</b>	<b>Mean sq</b>	<b>F-value</b>	<b>Pr(&gt;F)</b>	<b>n</b>
<b>Day period</b>	3	4420	1473	1.445	0.248	9
<b>Feeding period</b>	2	14278	7139	10.34	0.000326***	12

#### 1.1.2 Post-hoc Dunn test prefeed

Table 8: Post-hoc Dunn test pursuing prefeed

	<b>Mean.rank.diff</b>	<b>p-value</b>
<b>Minimum-Maximum</b>	10.583(3)	0.415
<b>Recommended-Maximum</b>	7.16(6)	0.1912
<b>Recommended-Minimum</b>	-3.41(6)	0.4269

#### 1.1.3 Post-hoc Dunn test postfeed

Table 9: Post-hoc Dunn test pursuing postfeed

	<b>Mean.rank.diff</b>	<b>p-value</b>
<b>Minimum-Maximum</b>	15.08(3)	0.0014**
<b>Recommended-Maximum</b>	13.16(6)	0.0044**
<b>Recommended-Minimum</b>	-1.91(6)	0.6558

## 1.2 Circling

### 1.2.1 ANOVA Postfeed

Table 10: Primary ANOVA test circling postfeed

	<b>Df</b>	<b>Sum sq</b>	<b>Mean sq</b>	<b>F-value</b>	<b>Pr(&gt;F)</b>	<b>n</b>
<b>Day period</b>	3	1497	498	0.794	0.506	9
<b>Feeding period</b>	2	8327	4164	10.35	0.000325***	12

### 1.2.2 Post-hoc Dunn test prefeed

Table 11: Post-hoc Dunn test circling prefeed

	<b>Mean.rank.diff</b>	<b>p-value</b>
<b>Minimum-Maximum</b>	7.541(6)	0.1589
<b>Recommended-Maximum</b>	11.33(3)	0.0252*
<b>Recommended-Minimum</b>	3.791(6)	0.3779

### 1.2.3 Post-hoc Dunn test postfeed

Table 12: Post-hoc Dunn test circling postfeed

	<b>Mean.rank.diff</b>	<b>p-value</b>
<b>Minimum-Maximum</b>	7.541(6)	0.1589
<b>Recommended-Maximum</b>	11.33(3)	0.0252*
<b>Recommended-Minimum</b>	3.791(6)	0.3779

## 1.3 Lateral

### 1.3.1 ANOVA Postfeed

Table 13: Primary ANOVA test lateral postfeed

	<b>Df</b>	<b>Sum sq</b>	<b>Mean sq</b>	<b>F-value</b>	<b>Pr(&gt;F)</b>	<b>n</b>
<b>Day period</b>	3	48.7	16.25	1.162	0.34	9
<b>Feeding period</b>	2	110.1	55.03	4.701	0.016*	12

### 1.3.2 Post-hoc Dunn test prefeed

Table 14: Post-hoc Dunn test lateral prefeed

	<b>Mean.rank.diff</b>	<b>p-value</b>
<b>Minimum-Maximum</b>	9.83(3)	0.0658*
<b>Recommended-Maximum</b>	1.791(6)	0.6763
<b>Recommended-Minimum</b>	-8.041(6)	0.1218

### 1.3.3 Post-hoc Dunn test postfeed

Table 15: Post-hoc Dunn test lateral postfeed

	<b>Mean.rank.diff</b>	<b>p-value</b>
<b>Minimum-Maximum</b>	11.041(6)	0.0297*
<b>Recommended-Maximum</b>	4.708(3)	0.2781
<b>Recommended-Minimum</b>	-6.33(3)	0.2781

## 1.4 Frontal

### 1.4.1 ANOVA Postfeed

Table 16: Primary ANOVA test frontal postfeed

	<b>Df</b>	<b>Sum sq</b>	<b>Mean sq</b>	<b>F-value</b>	<b>Pr(&gt;F)</b>	<b>n</b>
<b>Day period</b>	3	0.333	0.1111	0.239	0.869	9
<b>Feeding period</b>	2	4.056	2.0278	5.993	0.00602**	12

### 1.4.2 Post-hoc Dunn test prefeed

Table 17: Post-hoc Dunn test frontal prefeed

	<b>Mean.rank.diff</b>	<b>p-value</b>
<b>Minimum-Maximum</b>	5.33(3)	0.4572
<b>Recommended-Maximum</b>	2.16(6)	0.7910
<b>Recommended-Minimum</b>	-3.16(6)	0.7910

### 1.4.3 Post-hoc Dunn test postfeed

Table 18: Post-hoc Dunn test frontal postfeed

	<b>Mean.rank.diff</b>	<b>p-value</b>
<b>Minimum-Maximum</b>	-1.41(6)	0.6327
<b>Recommended-Maximum</b>	7.66(6)	0.0194*
<b>Recommended-Minimum</b>	9.083(3)	0.0065**