SÓNIA MARINA ANTÓNIO SOARES

STUNNING AND SLAUGHTERING EFFECTS ON WELFARE OF GILTHEAD SEABREAM

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Master in Aquaculture and Fisheries

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Work authorship statement:

I declare to be the author of this work, which is unique and unprecedented. Authors and works consulted are properly cited in the text and are included in the listing of references included.

Sónia Marina António Soares

2023

ACKNOWLEDGMENTS AND DEDICATION

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My family. My dad João that always thought me to love animals and allowed me to try and save as many as possible. My brothers Ricardo and Hugo, my brother in law André and my sister in law Dayana, for always check in on me and supporting me since the beginning. My sister Joana, for all that and for convincing me to pursue Marine Biology.

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I would like to dedicate this thesis to my late mother, Isabel. I like to imagine that you would be proud of the path I chose.

I. RESUMO

Embora os peixes sejam vastamente utilizados pelo Homem, seja como animal de estimação, alimento, ou indivíduos de interesse cientifico, a sua sentiência mantém-se um assunto polémico entre a comunidade cientifica. Nós consideramos a existência de provas cientificas suficientes para concluir que os peixes são, de facto, seres sentientes capazes de sentir dor. Desta forma, práticas de abate humano devem ser aplicadas de acordo com as características de cada espécie. A Dourada (*Sparus aurata*, Linnaeus 1758) é uma espécie popular de produção europeia, contudo, o método de abate mais praticado para a mesma ainda é asfixia em gelo, considerado um método desumano de abate. Nós avaliámos o uso do método de atordoamento elétrico, de um ponto de vista de bem-estar, na dourada, utilizando o atordoamento com 2-fenoxietanol (um anestésico comprovado) como grupo de controlo positivo, assim como abate sem atordoamento prévio (o método mais praticado para esta espécie) como grupo de controlo negativo. As medições de bem-estar foram separadas em fisiológicas (frequência cardíaca e temperatura interna, medidas através de bio-loggers DST milli HRT) e comportamentais, baseados na perda da capacidade de natação livre, equilíbrio, reflexo vestíbulo-ocular (VOR), movimento opercular, bem como frequência de movimentos repentinos. O atordoamento não afetou a duração media de abate. Contudo a maioria dos indivíduos atordoados morreu mais depressa do que indivíduos sem atordoamento. A fibrilação arterial durante o abate em solução de gelo foi observada previamente em tratamentos sem atordoamento(~ 15 minutos de abate), com maior variabilidade (picos próximos de 80 bpm, em comparação com menos de 60 bpm nos outros tratamentos). Indivíduos sem atordoamento estavam conscientes durante o abate e o ikejime foi comprometido pelo mesmo, demorando mais tempo (média de duração maior do que 2 minutos, em comparação com menos de 1 minuto para indivíduos atordoados). O nosso estudo demonstra que atordoamento por choque electrico aumenta o bem-estar dos peixes, tanto por possibilitar um abate com os individous inconscientes em solução de gelo como por possibilitar uma técnica de ikejime mais rápida, quando comparado com o método mais praticado para esta espécie em aquacultura, que é abate por solução de gelo sem atordoamento prévio.

PALAVRAS-CHAVE: Dourada; atordoamento e abate humano humano; *bio-loggers*; bem-estar de peixes; aquacultura

I. ABSTRACT

Although fish are widely used by people, their sentience is still a polemic subject around the scientific community. We consider there is abundant scientific evidence to conclude fish are sentient beings capable of felling pain. Therefore, humane slaughter practices must be studied and applied according with the characteristics of each species. Although Gilthead seabream (*Sparus aurata*, Linnaeus 1758) is a popular farmed species in Europe, the most practiced slaughtering method for this species is still asphyxiation in ice, which is considered an inhumane form of slaughter. We evaluated the use of electrical stunning, from a welfare point of view, on gilthead seabream, with stunning with 2-phenoxyethanol (a proven anaesthetic) as a positive control group, and slaughter with no previous stunning (the most practiced method for this species) as a negative control group. Welfare was measured by physiological parameters (heart rate and internal temperature measured through bio-loggers DST milli HRT) and behavioural parameters, based on loss of free swimming, equilibrium, vestibulo-ocular reflex (VOR) and opercular movement (OM), as well as frequency of abrupt movements. Stunning did not reduce overall slaughter time in our study. However, the majority of the individuals of the stunning trials died faster. Heart fibrillation during ice-slurry was observed earlier on the treatment with no stunning (~ 15 minutes of slaughter), with higher variability (spikes reached almost 80 bpm, in comparation to less than 60 bpm for the other treatments). Individuals of the no stunning treatments were conscious during slaughter, contrary to the stunning treatments and ikejime was impaired by conscious fish, taking longer (average higher than 2 minutes for no stunning and less than 1 minute for the stunning treatments). Our results show that stunning by electrical shock increases the welfare of fish, both for a more rested slaughter during ice-slurry and faster ikejime, in comparation with the most commonly used method for this species in the industry, which is slaughter by ice-slurry without previous stunning.

KEYWORDS: Seabream; humane stunning and slaughtering; HR-loggers; fish welfare; aquaculture

II. ABBREVIATIONS AND SYMBOLS

Bpm: Beats per Minute

CCMAR: Centro de Ciências do Mar

cm: Centimetre(s)

CO_2: Carbon Dioxide

DG SANTE: Directorate-General for Health and Food Safety

e.g.: Exempli gratia (for example)

ECG: Electrocardiogram

EIC: Electrical Stunning & Ice-slurry slaughter

EIK: Electrical Stunning & Ikejime slaughter

EPPO: Estação Piloto de Piscicultura de Olhão

FEAP: Federation of European Aquaculture Producers

g: Gram(s)

HPI: Hypothalamic-pituitary-interrenal

HSA: Human Slaughter Association

IPMA: Instituto Português do Mar e da Atmosfera

L: Liter(s)

m: Meter(s)

mg: Milligram(s)

min: Minute(s)

mm: Millimetre(s)

N_2: Nitrogen

NIC: No Stunning & Ice-slurry slaughter

NIK: No Stunning & Ikejime slaughter

O_2: Oxygen

OM: Opercular Movement

pH: Potential of hydrogen

PIC: 2-phenoxyethanol Stunning & Ice-slurry slaughter

PIK: 2-phenoxyethanol Stunning & Ikejime slaughter

QI: Quality Index

QRS: Combination of the three deflections typically seen on a ECG

VOR: Vestibulo-ocular reflex

sec: Second(s)

SEM: standard Error of Mean

f_H: Heart rate

°C: Degrees Celsius

%: Percentage

~: Approximately

<: Less than

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Chapter 1. INTRODUCTION

"Are fish sentient beings?", "Do fish feel pain?" are some of the questions that arose during the fish sentience controversy that divided the scientific community. On one hand, there were scientists defending that behavioural responses to noxious stimuli differ from psychological experience of pain, which requires the function of a specific region of the cerebral cortex that fish lack, meaning fish are anatomically incapable of feeling pain (Rose, 2002; Key, 2016). On the other hand, an overwhelming amount of evidence suggests that fish experience pain like the rest of the vertebrates (*e.g.* Brown, 2014; Sneddon *et al.*, 2018; Brown & Dorey, 2019; Sneddon & Brown, 2020), with previous studies exhibiting responses to pain by some species (Reilly *et al.,* 2008), as well as response to analgesics (Sneddon, 2003).

Independently of the defended point of view, we know that fish respond to positive and negative stimuli differently, and since fish are widely used by humans (either harvested from the wild, farmed, studied by the scientific community, or kept as pets), the matter of their handling must not be taken lightly (Brown, 2014).

In the aquaculture industry, fish welfare is becoming recognized as a factor of product quality, which has led to the conduction of more studies to identify humane slaughtering methods applicable to fish farm (DG SANTE, 2017). Nowadays, some fish species such as Atlantic salmon (*Salmo salar*, Linnaeus, 1758) or rainbow trout (*Oncorhynchus mykiss*, Walbaum, 1792) are already commonly stunned and slaughter by percussion or electrical shock, which are considered humane methods of slaughter. However, for other species such as gilthead seabream (*Sparus aurata*, Linnaeus 1758), asphyxiation in ice is the most practiced slaughtering method, with other methods (*e.g.*, electrical stunning) still under experimental studies ((Poli *et al.,* 2005; DG SANTE, 2017).

The production of seabream is showing a growth trend, with an increase of production from 132,497.053 tons in 2014 to 180,961.583 tons in 2019, just in Europe (FEAP, 2020), which represents 264,994,106 and 361,923,166 individuals of 500 grams, respectively. As such, the study of humane slaughtering methods applicable to the aquaculture industry, for this species, is of great importance, since fish are sentient beings suffering with the actual practiced methods.

In 2009, the Treaty of Lisbon was approved, which acknowledged animals as sentient beings and enforced that the state members take into consideration the animals welfare requirements (Treaty of Lisbon, 2007). Since then, steps have been taken to increase welfare of farmed fish and some have already been implemented to several species, as mentioned before. However, common farming procedures still present relevant challenges for cultured fish and welfare is an urgent matter to address (Arechavala-Lopez & Saraiva, 2019; Saraiva & Arechavala-Lopez, 2019; Saraiva *et al,* 2022). The study of new methods, as well as making the necessary adaptations between species and widely implementing them throughout the industry is a time-consuming work still in progress.

1.1 Objectives

The aim of this study was to evaluated the efficacy of the use of electrical stunning, from a welfare point of view, on gilthead seabream, with stunning with 2-phenoxyethanol, a proven anaesthesia, as a positive control group, and slaughter with no previous stunning, the most practiced method for this species, as a negative control group. As for slaughter, we combined the stunning methods with two slaughtering techniques: ice-slurry, the most commonly used method, and ikejime, considered a humane slaughtering technique.

Chapter 2. STATE OF THE ART

2.1 Humane Slaughter

The principles of humane slaughter dictate that the method or methods chosen should prioritize the minimization of stress or pain in the animal, through fast stunning and death, which is determined by loss of brain function without recovery (Davie & Kopf 2006; AMVA, 2016). The applied method should take into consideration the characteristics of the animal in question and its environment (Ross & Ross, 2001). In fish, the guidelines and implementation of humane slaughter are not as advanced as in terrestrial animals (Knowles *et al*., 2007), possibly due to the polemic surrounding fish sentience and the difficulty to empathize with such an evolutionary distant group.

In order to classify a slaughter as humane, the animal must be fully unconscious at the time of slaughter (Kestin *et al*., 2002). According with the Human Slaughter Association (HSA, 2016), humane slaughter of fish for food purposes is advised to be conducted through either percussive stunning (where the fish receives a heavy blow to the head leaving it immediately unconscious) or electrical stunning (where an electrical charge is administrated to the fish's body leaving it unconscious). Contrarily, the HSA does not accept currently used methods that may cause pain and suffering to the fish, such as death by ice-slurry (where fish die of asphyxia or hypothermia depending on the proportion of ice and water), live chilling (where fish are placed in low temperature water, between 2-6°C, until immobile and slaughtered while conscious), gill cut without stunning (where conscious fish are removed from the water and their gills are cut), or carbon dioxide narcosis (where fish are placed on carbon dioxide saturated water until immobile, which sometimes is due to exhaustion and not by unconsciousness). Concerning alternative methods such as chemical stunning, some have been determined as humane methods of slaughter applicable in laboratory studies (*e.g*., tricaine and 2-phenoxyethanol), as well as in the food industry (in the case of the anaesthetic iso-eugenol), making the fish insensible to pain. However, their use is not approved in all countries (Javaheri & Moradlu, 2012; HSA, 2016).

2.2 Stunning

Fish have mechanisms for pain perception and, when disturbed (*e.g.*, by noise increment, removal from their natural environment, or crowding), their emergency response is activated, usually by increasing their stress levels, which has an adverse effect on their welfare (HSA, 2016). As such, the use of anaesthetics or other techniques for a "rested harvest" (fish fully sedated) is both beneficial for the fish welfare and the aquaculture industry, since this method can increase flesh parameters (*e.g.*, colour, firmness, delay of *rigor mortis*) (HSA, 2016). To qualify as an effective anaesthetic, the used product must block the hypothalamic-pituitary-interrenal (HPI) axis, which reduces the release of plasma cortisol, a stress indicator in fish (Webb *et al.*, 2007). In addition, the anaesthetic must be specimen specific, taking into consideration the fish species, size, health status, sex, and maturation phase, as well as environment specific, considering the water temperature, salinity, and pH (Ross & Ross, 2001). Furthermore, it is advisable to always withheld feed 24 hours prior to anaesthesia, to prevent an increase of both the metabolic rate and the oxygen consumption (Noga, 2011).

2.2.1 Carbon Dioxide

Carbon dioxide is a gas which can be introduced in the water to reduce oxygen saturation, leading to loss of consciousness without leaving residues on the fish (Noga, 2011; HSA, 2016). However, carbon dioxide is slow-acting and is linked to strong aversive movements, due to the stress caused by lowering of pH and oxygen levels, as well as not always resulting in loss of consciousness (HSA, 2016; Schneider *et al.*, 2018). As such, to ensure an efficient stunning, high concentrations of carbon dioxide should be used (HSA, 2016).

2.2.2 Nitrogen

The nitrogen stunning method is based on the principle that this gas replaces oxygen in the water which leads to loss of consciousness. Although working in a similar manner than other gases, such as carbon dioxide, previous studies in rainbow-trout show that nitrogen promotes less muscle activity, possibly indicating less stress, which can be explained by the fact that water pH is lower with carbon dioxide, and that individuals stunned by nitrogen exhibit higher pH levels than the ones slaughtered by asphyxiation, which is considered a non-humane slaughtering method (Wills

et al., 2006). Although both N_2 and CO_2 can be used as stun methods separately, their mixture has already been studied in some species. A 2015 study conducted by Zampacavallo *et al*. with seabass (*Dicentrarchus labrax*, Linnaeus, 1758) showed that the use of a mixture of 70% N_2 and 30% CO_2 reduced the stunning/slaughtering time by 40%, when compared with only ice-water. Similarly, a study conducted in gilthead seabream under the same mixture (and proportion) showed a reduction of mean stunning time (characterized by the fish turning their belly up) from approximately 52 minutes in ice-slurry to less than 2 minutes with the gas mixture (Roque *et al*., 2021).

2.2.3 AQUI-S®

AQUI-S® is a chemical product developed for fish anaesthesia that suppresses the sensory system (Javaheri & Moradlu, 2012). Its active ingredient is a synthetic form of isoeugenol, derived from eugenol which is the main components of clove oil (Noga, 2011). Although its use has been recommended for the aquaculture industry of many countries (Australia, Chile, New Zealand, Korea, Norway, Honduras, and Costa Rica), at the moment of writing this report, its use has not been approved on food products in Europe, due to the lack of reports concerning its pathological effects for humans (Javaheri & Moradlu, 2012; de la Rosa *et al*., 2021). In addition, evidence shows that fish anesthetized with eugenol may still present involuntary movement and that its safety margin is narrow, leading to ventilatory failure when higher doses are administered (Sladky *et al*., 2001). Nonetheless, this chemical has been proven to have fast induction in red pacu (*Piaractus brachypomus*, Cuvier, 1818) and to be effective in the deep anaesthesia of gilthead seabream (Sladky *et al*. 2001; Jerez-Cepa *et al*., 2021).

2.2.4 2-Phenoxyethanol

2-phenoxyethanol is a soluble liquid anaesthetic agent whose active ingredient is ethylene glycol monophenyl ether (Burka *et al*., 1997; Weber *et al*., 2009). This agent is widely used in research and aquaculture due to its characteristics, such as rapid action and low price (Klimankova *et al*., 2008). Previous studies have reported positive feedback of the use of this anaesthetic on a diversity of species, including species from the *Sparidae* family (Tsantillas *et al*., 2006; Vaughan *et al*., 2008; Weber *et al*., 2009). However, its mode of action has yet not been reported and is not

approved for fish harvested for food (Burka *et al*., 1997; Tsantillas *et al.,* 2006; Klimankova *et al*., 2008).

2.2.5 Electric Stunning

In electric stunning, an electrical current introduced either on the water (wet stunning) or directly on the fish body (dry stunning) interrupts the normal electrical activity of the brain (Lambooij *et al.,* 2008; Lines & Spence, 2014). Previous studies indicate that, if the correct voltage is applied in wet stunning, it can lead to immediate loss of consciousness and is successful when used in batches (Robb & Roth, 2003; Knowles *et al.,* 2007; Lambooij *et al.,* 2008) without the need to remove the individuals from the water, which is both beneficial from a welfare and economic point of view (Lines & Spence, 2014). However, this method can lead to epileptic seizures, fractured vertebrae, and haemorrhages (Robb & Roth, 2003), as well as pre-stun shocks, due to inadequate voltage or duration of the electrical application (Lines & Spence, 2014). In addition, electrical shock may cause disruption of electrocardiograms (ECG) and other data collectors on the fish, making it challenging to study the welfare implications of using such method (Lambooij *et al.,* 2008). In order to achieve a fast stun with prolonged insensitivity, the voltage and stunning time must be adequate for the fish species, body orientation, and water conductivity (Lines & Spence, 2014). Previous studies with freshwater species show positive results and it is even a common method used in the aquaculture industry for these species (Sattari *et al.,* 2010). However, this method's efficiency is lower in saltwater species, due to the reduced conductivity of salt water (Lines & Kestin, 2004).

2.3 Slaughter

2.3.1 Ikejime

Ikejime or brain spiking is a slaughter method in which a spike is placed on the base of the animal's skull and inserted into the brain, followed by maceration until causing trauma to the nervous tissue (Davie & Kopf, 2006; Noga, 2011). This method has several variations, depending on the fish size: from the front of the head (for big sized fish), from the side of the head (generally in medium sized fish) or through the gill cover (for smaller specimens, usually with a knife instead of a spike)

(AMVA, 2016). It is considered by many as the most humane and fast euthanasia method when performed by trained professionals (Davie & Kopf, 2006; Diggles, 2015). However, locating the correct part of the brain can be arduous and time consuming, since fish brains are small and location vary between species, possibly prolonging euthanasia time and resulting on unnecessary suffering (Diggles, 2015). Additionally, since slaughter must be performed individually, this method is not adequate for the aquaculture industry, from an economic point of view.

2.3.2 Ice-Slurry

The ice-slurry method is based on the principle that thermal shock induced by extreme cold causes loss of brain function, avoiding stress, and it is considered by many as a humane way to slaughter small warm water species (Wilson *et al*., 2009). However, this method is not considered suitable to medium to large individuals, or cold- or temperate-water adapted ones, such as the Atlantic salmon (Robb & Kestin, 2002; Foss *et al.,* 2011), because instead of inducing a quick thermal shock, it induces hypothermia over a long period of time.

Although some studies defend this method as humane for small tropical fish (Wilson *et al*., 2009), others do not consider hypothermia a true anaesthetic, since it does not inhibit the pain receptors of the individuals, possibly promoting stress, since the animals can be immobilized without being sedated, feeling the whole slaughtering process without being able to respond and dying slowly of asphyxiation, present vigorous movements, escape attempts and tachycardia (Lambooij *et al.,* 2008; Noga, 2011; HSA, 2016). Nonetheless, this method is commonly used in the aquaculture industry both for stunning and slaughter, for many fish species including seabream and seabass (de la Rosa *et al*., 2021; Roque *et al*., 2021).

2.4 Loggers

Bio-loggers are capable of recording variables within a specimen, such as living body temperature, swimming speed, acceleration, angular velocity, or cardiac rhythm, as well as environmental variables, such as water temperature and depth, or terrestrial magnetism. They can be either inserted into the abdominal cavity of the individuals through surgical procedures or attached externally (Muramoto & Naito, 2000; Brijs *et al.*, 2018; Bjarnason *et al*., 2019). After recording,

the loggers are retrieved from the animal and information is downloaded to a computer to be analysed (Muramoto & Naito, 2000), or they transmit such information directly to a computer via Bluetooth. Previous studies show that animals respond well to internal bio-loggers, with fast recuperation time and that this recording method allows more accurate information than the one obtained through traditional methods (animal physically connected to equipment), since we remove any external influence (Brijs *et al*., 2018; Hvas *et al*., 2020). However, a study conducted by Hvas *et al.* (2020) revealed that the loggers can have an adverse effect on fish growth (weight and length) and condition factor, although they still presented growth during the trials.

Concerning welfare and, most specifically, our study, the heart rate (f_H) is an interesting measurement of relevant importance, since it can reflect the stress levels on the fish at a determined moment (Hvas *et al.,* 2020). Bio-loggers equipped with electrocardiograms (ECG) are capable to determine the circadian rhythm of the heart rate in fish or their daily average heart rate (*e.g*., ~59 beats per minute in gilthead seabream (Mignucci *et al.,* 2021) and the occurrence of fluctuations, which could indicate a stress event for the individual (Brijs *et al*., 2018). A practical example of the use of bio-loggers to assess welfare in seabream is the Mignucci *et al*. (2021) study that revealed that this specie is very sensitive to disturbances (*e.g*., human presence), leading to shifts in the f_H. As such, the use of bio-loggers equipped with ECG will provide valuable information concerning gilthead seabream welfare during the evaluation of different stunning and slaughter procedures.

2.5 Behavioural analysis

The visual assessment of self-initiated behaviours, reflexes and other responses to stimuli can provide some intel concerning the level of brain function on fish during anaesthesia (Kestin *et al.,* 2002; Bowman *et al.,* 2020). Some of the parameters used to assess anaesthesia in fish include: swimming behaviour (normal or erratic swimming, or abrupt change of swimming direction); partial and total loss of equilibrium (fish loses its vertical position); response to handling and stimuli; opercular activity (diminish or irregular gill movement) (Kestin *et al*., 2002; Weber *et al.,* 2009; Bowman *et al.,* 2020). However, it should be noted that fish may be rendered immobilized without anaesthesia, which may affect the results of this type of analysis. (Kestin *et al*., 2002). As such, this method should be used in addition to physiological analysis, such as heart rate, brain function and blood sampling.

Chapter 3. METHODOLOGY

3.1 Preliminary Trials

Since bio-logger malfunction has been previously reported for this method (Lambooij *et al.,* 2008), a preliminary test was executed to evaluate the effect of electric stunning on the DST milli HRT bio-loggers. In this test, a previously tagged subject was stunned by the electrical stunner Fishkill EG100 and the logger was removed and evaluated. If malfunction had occurred, this method would have been removed from the study. However, since the electrical current did not affect the normal activity of the logger, this method was included in the present study.

3.2 Fish And Rearing Conditions

Thirty-one adult gilthead seabream (930.82 ± 139.88 g) obtained from a previous welfare study conducted by the scientific team, were rearranged in three groups (N = 31, 10 to 11 individuals per stunning method). Specimens were reared in 3,000 L cylindrical tanks (2 m diameter; 1 m depth; 90 cm water level) at Estação Piloto de Piscicultura de Olhão (EPPO), an IPMA facility (Instituto Português do Mar e da Atmosfera) in Olhão, Portugal. Feed was provided twice a day with commercial sinking pellets (Aller Blue Ex Vitamax; 6 mm) until apparent satiation. Physical and chemical water parameters were measured daily through a portable multiparameter monitor (*e.g.*, water temperature, oxygen saturation, and salinity).

On the trial day, the individuals were distributed in six stunning/slaughter combinations: No Stunning and Slaughter by Ice-slurry (NIC), No Stunning and Slaughter by Ikejime (NIK), Electrical Stunning and Slaughter by Ice-slurry (EIC), Electrical Stunning and Slaughter by Ikejime (EIK), 2-phenoxyethanol Stunning and Slaughter by Ice-slurry (PIC), and 2-phenoxyethanol Stunning and Slaughter by Ikejime (PIK).

3.3 Bio-Logger Implantation

All the individuals used in this study were tagged with DST milli HRT bio-loggers (Star-Oddi, Iceland, www.star-oddi.com), an animal implantable heart rate and temperature logger, at EPPO/IPMA. The DST milli HRT bio-loggers (length: 39.5 mm; height: 13 mm; weight: 12 g) (**Figure 3.1 C)**) were implanted internally through surgery, following the procedures described by Mignucci *et al*. (2021). Surgical procedures started after a 24 hours starvation period and had a duration of approximately 10-15 minutes each. Gilthead seabreams were taken from the housing tanks and anaesthetized with 2-phenoxyethanol (0.5‰, Sigma–Aldrich) and maintained in that state through a gill bath of 0.25‰ 2-phenoxyethanol. Skin was disinfected with a 5% povidone-iodine cream (Betadine) and an abdominal incision (~2 cm) along the ventral midline was performed with a scalpel. The bio-loggers were introduced in the intraperitoneal space of the thoracic cavity, near the pericardium (**Figure 3.1 B)**), and attached to the ventral thorax (**Figure 3.1 A)**) by two stitches, one of non-absorbable monofilament nylon suture and another of silk suture, to prevent bio-logger movement inside the fish which could lead to the disruption of accurate data recording. After implantation, the abdominal incision was treated with a 50/50 mix of Blastoestimulina and Furacín ointments and sealed with an absorbable monofilament glyconate suture. At the end of surgery, the wound was treated with Aloclair PLUS Gel and the individuals were returned to the housing tanks and monitored until recovery.

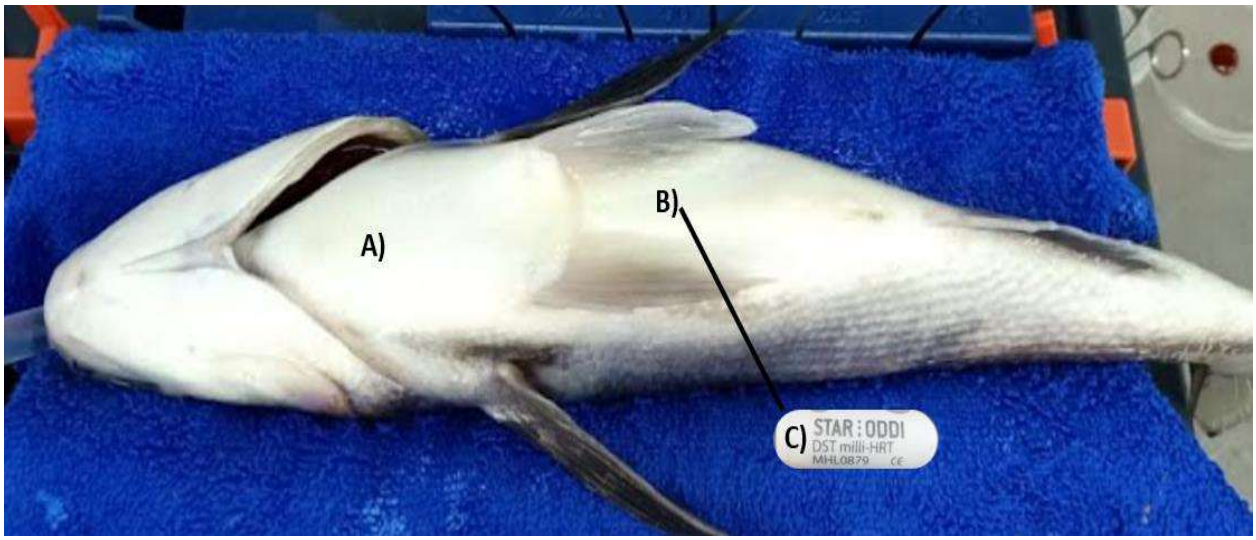

Figure 3.1: **Surgery for bio-logger placement**. **A)** Location where the bio-logger was attached to the ventral thorax, to prevent bio-logger displacement and bad heart rate reading. **B)** Location of the incision for the bio-logger implantation, in the intraperitoneal space of the thoracic cavity. **C)** DST milli HRT bio-logger produced by Star-Oddi (Iceland, www.star-oddi.com), not for scale.

3.4 Stunning

Mignucci *et al*. (2021) reported three days of surgery recovery in seabream. We left the fish undisturbed for a period of seven days after surgery to ensure that cardiac activity returned to basal levels (**Figure 3.2)**. Additionally, a starvation period of 24 hours was implemented to avoid unwanted increases in both metabolic rates and oxygen consumption. On the day of the trial, the housing tanks water was lowered, in order to help the catching of the individuals, and one to two gilthead seabreams were placed in harvesting buckets and immediately transported to the stunning area (~ 1 minute trip). A collaboration was made with a group belonging to Centro de Ciências do Mar (CCMAR), which collected blood samples of each individual immediately after stunning and slaughter, with exception of the individuals that were subjected to slaughter without previous stunning, in which case blood sample was collected only after the slaughtering event because blood collection was not possible in non-stunned individuals.

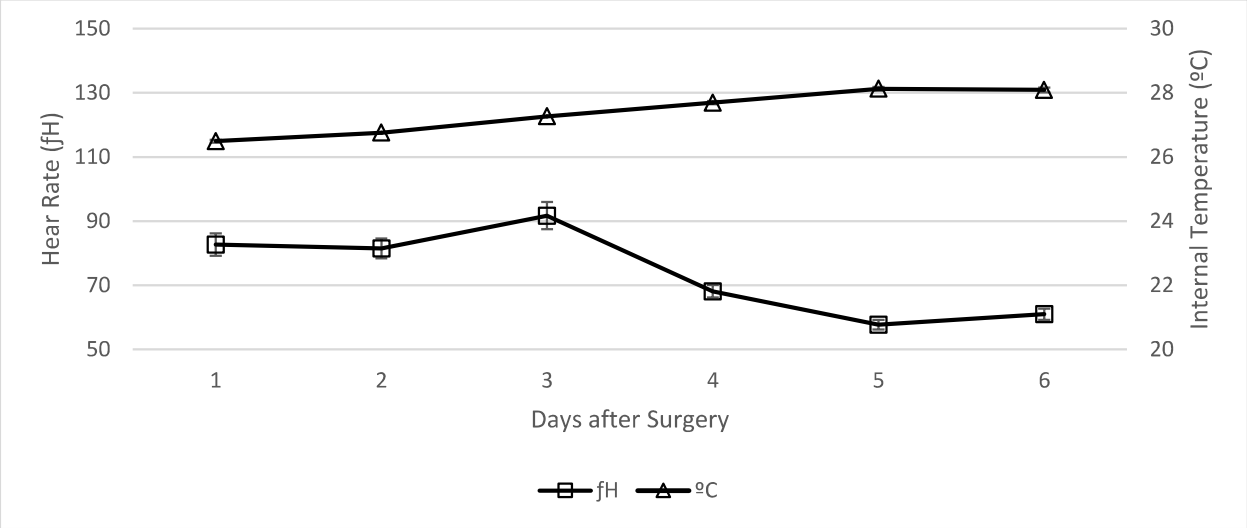

***Figure 3.2*. Heart Rate and Internal Temperature pre-trial**. Average heart rate (*f*H, black squares) and internal temperature (Celsius, black triangles) for all specimens (N= 31), during the seven days prior to the trial (day 1 represents the first day after surgery). Standard Error of Mean (SEM) is presented as the error bars.

Fish from the No Stunning and Ice-slurry (N= 5; mean body weight: 980.4 ± 116.4 g) and No Stunning and Ikejime trial (N= 4; 1013.75 ± 125.84 g) were immediately transferred to the slaughter station after capture.

Fish from the Electrical Stunning and Ice-slurry (N= 5; mean body weight: 994.8 ± 102.85 g) and from the Electrical Stunning and Ikejime trial (N= 5; mean body weight: 873.8 ± 84.64 g), were stunned using an individual non-commercial electrical stunner produced by Ace Aquatec Ltd. specifically for this study (**Figure 3.3)**. Fish were placed individually in the previously filled test tank (length: 500 mm; height: 400 mm) containing the electrodes. After the individual was placed on the tank, the tank lid was closed, and the voltage was applied to the tank (100 Volts; 6.32 ± 0.10 Amps) for a duration of 15 seconds.

Figure 3.3: **Electrical Stunner**. Electrical Stunner produced by Ace Aquatec Ltd. Stunner made of three parts: test tank (white tank), isolating transformer (blue box) connected to a multimeter (orange and black monitor) to monitor the stun voltage, and stun controller (light grey box) with voltage and time controllers.

Fish from the 2-phenoxyethanol and Ice-slurry (N= 5; mean body weight: 855.8 ± 65.10 g) and 2-phenoxyethanol and Ikejime trial (N= 5; mean body weight: 838.2 ± 176.02 g) were stunned using the water dispersible anaesthetic 2-phenoxyethanol. Fish were individually transferred to a 20 L plastic tank containing 2-phenoxyethanol at 1000 ppm concentration. Behavioural parameters were measured until the subject stopped showing VOR, which is usually the last sign of consciousness observed before anaesthesia (Kestin *et al.,* 2002). At that point the subject was moved to the slaughtering station.

3.5 Slaughter

Fish were slaughtered either by the ikejime or the ice-slurry methods.

Fish (N= 14, 4 for no stunning and 5 per stunning method, respectively) were slaughtered following the ikejime technique. The position of the brain was visually located using examples of the *Sparidae* family (*e.g.* yellow-finned bream, *Acanthopagrus australis*, Günther, 1859) available at Humane Killing of Fish (https://www.ikijime.com/). Fish were placed on their side on a table and covered by a wet cloth to reduce fish slippage during the procedure. The brain was spiked

rostrally between the eyes using an ikejime tool **(Figure 3.4)**, which was wiggled once inserted to ensure destruction of the brain.

Figure 3.4: **Ikejime Tool**. Ikejime tool used on the trial (© LumicaDirect.com). The shorter tool is used for skull penetration and the steel wire for destruction of the spinal cord, through the neural canal.

Fish (N= 15, 5 per stunning method/ treatment, respectively) were slaughtered by immersion in an ice-slurry (-0.62 ± 0.06 ºC), as described in Matos *et al*. (2010), with adjustment for individual stunning. Fish were individually placed in a 20 L Styrofoam tank containing the ice-saltwater slurry (4:1 proportion ice-saltwater). To allow fish monitoring, a pneumatic trough was placed over the gill and eye area **(Figure 3.5)**. Fish were kept on the ice-slurry until loss of consciousness signs were demonstrated.

Figure 3.5: **Ice-slurry set up.** Slaughter by Ice-slurry set up. Pneumatic trough placed over the gill and eye, to assess consciousness (VOR and OM).

3.6 Physiological Analysis

Before the trial, bio-loggers were programmed with the Mercury v 6.14 software, to record the beats per minute (BPM) once every hour for 4 seconds between the logger implantation and initiation of the trial, which was used to determine surgery recuperation time as well as heart rate baseline. On the trial day, the loggers recorded 10 consecutive seconds every 30 seconds from the moment the water in their housing tank was starting to drop.

After slaughter, the bio-loggers were retrieved and the data was transferred to a computer, where it was analysed with the help of the program Star-Oddi HRT Analyzer v 1.0.2, Star-Oddi Mercury v 6.14 software and Star-Oddi Pattern Finder v 1.24.

The Mercury v 6.14 software was programmed to automatically exclude heart rates with a quality index (QI) equal to 3 (considered by the program to be bad quality data), or a bpm limit inferior to 20 or superior to 200, since values below or above the selected ones are rare in gilthead seabream, based on the teams experience. Data whose QI was greater than 0 (considered great data) or that did not match the bpm established limits, was manually checked using Star-Oddi HRT Analyzer v 1.0.2 and/or the Star-Oddi Pattern Finder v 1.24. The latter was used in cases where the first was not sufficient to reach an agreeable value (the QRS waves characteristic of the cardiac cycle could not be measured accurately due to noise in the sample or value differed from the previous and/or

subsequent datapoint by a value equal or superior to 10). In addition, data was manually checked for heart variability and graded between 0 and 4 ("0" no variability and "4" total variability, meaning the data point was unreadable), as well as for wave amplitude (minimum, maximum and average, for each datapoint).

3.7 Behavioural Analysis

Behaviour data during the trial was collected with the help of an ethogram specially designed for each stunning/slaughter set. During the trials, we collected: Subject general information (housing tank identification, logger ID and fish weight); Stunner information for electrical stunning (volts, amps, shock time in seconds, water temperature and water salinity); Ice-slurry information (water temperature and salinity); Anaesthetic information for 2-phenoxyethanol (concentration, O_2 saturation, water pH, temperature and salinity); Duration of housing tank lowering water process, harvesting, stunning and slaughter; Latency for the signs of loss of awareness and sensibility (Loss of Free Swimming, Equilibrium, to Start Irregular Opercular Movement (OM), to Lose Opercular Movement and Vestibulo-Ocular Reflex (VOR)) **(Table 3.1)**; and finally blood sample beginning and end times (in hours, minutes and seconds).

Table 3.1: **Signs of loss of awareness and sensibility.** Description of the behavioural parameters analysed during this study.

Loss of Free Swimming	individual lost normal swimming activity
Loss of Equilibrium	individual lost its vertical position
Gain of Irregular Opercular Movement	individual demonstrates irregular gill movement
Loss Opercular Movement	individual ceases gill movement
Loss Vestibulo-Ocular Reflex	individual ceases vestibulo-ocular reflex/eye roll

3.8 Statistical Analysis

Statistical analysis of the data collected from both ethograms and bio-loggers was performed through the IBM SPSS Statistics v 28.0.1.0. P-values <0.05 were considered statistically significant. All values were reported as means and respective standard errors means (SEM), which were calculated through the following formula: STDEV.P(range)/SQRT(COUNT(range)).

Possible effect of time in water before harvesting for the trial (crowding event), in both f_H and slaughter time, was assessed through an ANCOVA test, to test the independence of the independent variable and covariate.

In accordance with Brijs *et al*. (2018), statistical analysis for *f*H and internal temperature, between treatments and individuals, were measured using a linear mixed model with first-order autoregressive repeated covariance matrix. This analysis was chosen based on the conception that data recorded close in time (loggers were recording every 30 seconds during the trial) is more dependent to each other than data temporally distant.

Levene statistic was used to test homogeneity of variances, in order to analyse the duration of the events on the trial. When this assumption was not meet, One-way ANOVA was substituted by the Independent-Samples Kruskal-Wallis Test with Pairwise Comparisons of Treatments.

Chapter 4. RESULTS

4.1 Bio-Logger Implantation/Surgery

The average time for bio-logger implantation was 25 minutes and 40 seconds (± 6 min. and 23 sec.) between beginning of anaesthesia administration and complete recuperation. The success rate was of 90.63%, with three individuals dying on the following days.

4.2 Trial Preparation And Harvesting

Trial preparations began with lowering of the housing tank water, to help harvest the fish. Since the time fish were kept in the lowered water (crowding event) and harvesting time differed between individuals **(Table 4.1)**, an ANCOVA test was conducted to determine if crowding could affect f_H or time for stunning and slaughter. Since the P value was >0.05 for both f_H (N= 4/5, Z= 1.837, P= 0.190) and duration (N= 4/5, Z= 0.000, P= 0.984), it was considered that the events pre-trial did not influence the final results, and therefore were not included in further analyses.

Table 4.1: **Duration of housing tank water lowering (crowding event) and harvest.** Data presented in average and SEM, per treatment (in hours, minutes and seconds). Electrical & Ice-slurry (EIC, N= 5), Electrical & Ikejime (EIK, N= 5), No stunning & Ice-slurry (NIC, N= 5), No stunning & Ikejime (NIK, N= 4), Anaesthetic & Ice-slurry (PIC, N= 5), Anaethetic & Ikejime (PIK, N= 5).

	Time (hh:mm:ss)	
	Crowding event	**Harvesting**
Electrical & Ice-slurry	01:22:42 ± 01:00:12	00:01:21 ± 00:01:55
Electrical & Ikejime	01:08:06 ± 00:49:48	00:00:37 ± 00:00:17
No stunning & Ice-slurry	01:36:12 ± 01:12:23	00:00:33 ± 00:00:16
No stunning & Ikejime	01:25:45 ± 01:06:16	00:00:29 ± 00:00:11
Anaesthetic & Ice-slurry	01:15:36 ± 00:54:10	00:00:32 ± 00:00:13
Anaesthetic & Ikejime	01:20:42 ± 00:54:35	00:00:35 ± 00:00:14

4.3 Duration

Stunning time did not meet the Levene test of homogeneity (P= 0.009). As such, we conducted the Kruskal-Wallis test, which determined that stunning by electrical shock was significantly faster than stunning by 2-phenoxyethanol (N= 5, P= 0.004 and 0.005) **(Table 4.2)**.

Slaughter time was defined from the moment the fish entered in contact with the slaughtering method until a few minutes after the indicators of activity ceased (for the behavioural analysis) and from the moment the fish entered in contact with the slaughtering method until heart rate stopped (for the physiological analysis). Slaughter time also did not meet the Levene test of homogeneity (P < 0.001). The Kruskal-Wallis test showed that the average slaughter time for Ikejime was significantly lower than for Ice-slurry (N= 4/5, P between <0.001 and 0.008 for stunning treatments and P= 0.034 for treatments with no previous stunning). The stunned method used appear to have no significance **(Table 4.2)**.

Table 4.2: **Average Stunning and Slaughter time according with the behavioural data.** In minutes and seconds. Data presented in average and SEM, per treatment. Electrical & Ice-slurry (EIC, N= 5), Electrical & Ikejime (EIK, N= 5), No stunning & Ice-slurry (NIC, N= 5), No stunning & Ikejime (NIK, N= 4), Anaesthetic & Ice-slurry (PIC, N= 5), Anaethetic & Ikejime (PIK, N= 5). Data with the same letter in the superscription within columns are not different from each other (P > 0.05). Stunning time NA (not applicable) for the trials with no previous stunning.

	Time (mm:ss)	
	Stunning	**Slaughter**
Electrical & Ice-slurry	00:15 ± 00:00 [a]	23:26 ± 03:42[a]
Electrical & Ikejime	00:15 ± 00:00 [a]	00:37 ± 00:09[b]
No stunning & Ice-slurry	NA	26:36 ± 01:15[a]
No stunning & Ikejime	NA	02:16 ± 01:47[b]
Anaesthetic & Ice-slurry	02:23 ± 00:43 [b]	21:28 ± 06:25[a]
Anaesthetic & Ikejime	02:10 ± 00:53 [b]	00:43 ± 00:13[b]

For slaughter by Ikejime, fish from the electrical stunning trial presented the fastest average time, followed by 2-phenoxyethanol stunning. No stunning presented the longest time for slaughter, as

well as time variability between individuals **(Figure 4.1)**, due to the fish being conscious and trying to move, increasing the difficulty to perform the Ikejime technique.

As for slaughter by Ice-slurry, the 2-phenoxyethanol stunning trial presented the fastest average time, followed by electrical stunning and no stunning. Similar with slaughter by Ikejime, no stunning presented the longest time for slaughter, however, stunning by 2-phenoxyethanol was the second fastest and presented the highest variation between individuals **(Figure 4.1)**.

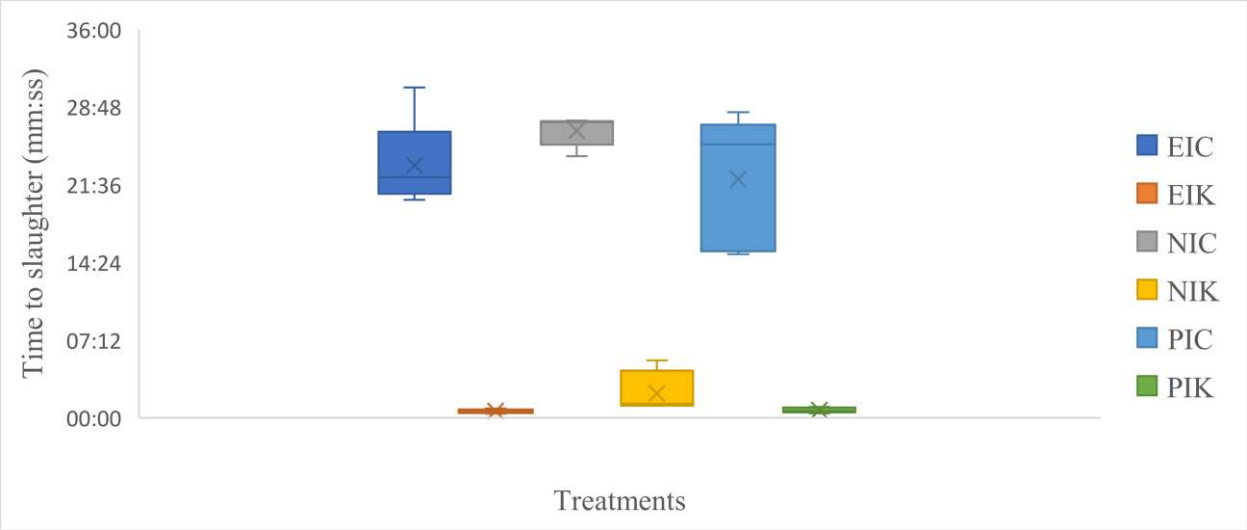

Figure 4.1: **Time to slaughter according with the behavioural data.** Box-and-whisker plot showing minimum (lower whisker) and maximum values (higher whisker), inner and outer quartile ranges (boxes), mean (X sign), and median value (horizontal bar inside the boxes) for the average time (in minutes and seconds), per treatment, for the time fish were in contact with the slaughtering method. EIC (N= 5), EIK (N= 5), NIC (N= 5), NIK (N= 4), PIC (N= 5), PIK (N= 5).

When analysing the survival trend for the Ice-slurry trial **(Figure 4.2)**, the following is observed: On the first 10 minutes of slaughter, all 5 individuals of each trial were considered alive. After 15 minutes, EIC had 5 individuals, PIC 4 and NIC 5. After 20 minutes, EIC and NIC remained the same, PIC had 3 individuals. After 25 minutes, EIC had 1 individual, PIC 2 and NIC still had 5 individuals alive. After 30 minutes, EIC was the only treatment with 1 individual alive and after 32 minutes and a half all individuals were considered deceased.

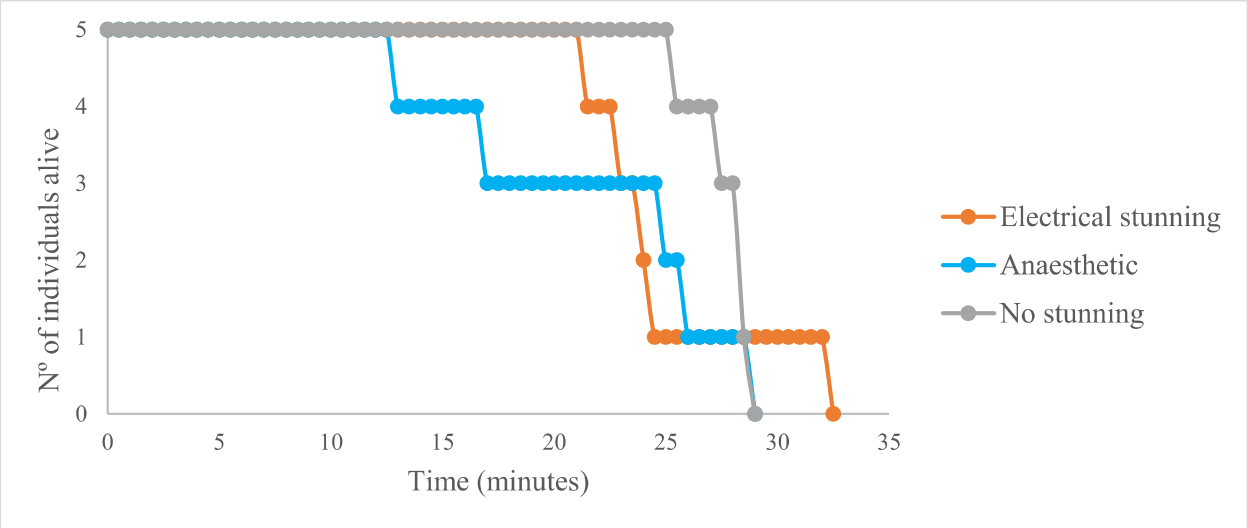

Figure 4.2: **Survival Curve for Ice-slurry Trial according with the physiological analysis**. Time, in minutes, it took for the five individuals on each trial to be considered dead (according with the ECG), once they were in contact with the ice-slurry (minute zero).

4.4 Physiological Analysis

4.4.1 No Stunning

On the day of the trials, the average heart rate and internal temperature of the individuals before starting was 97.5 bpm + 27.61ºC and 69.5 bpm + 27.60ºC, for slaughter by Ice-slurry and Ikejime, respectively. On both trials, it is possible to observe in **Figure 4.3** an increase in temperature after harvesting started (to 28.48 ºC for Ice-slurry and 28.34 ºC for Ikejime) due to the fish being moved to a bucket with probably slightly warmer water. This increase in temperature tendency was maintained for Ikejime for the duration of the trial and was most likely due to an adjustment to an increased temperature outside the water. For slaughter by Ice-slurry, the temperature presented the same increasing tendency until it dropped when the ice-slurry procedure started and continued to decrease for the duration of the trial (24.78 ºC after 5 min. and 18.08 ºC after 10 min.).

When the water of the tank stopped lowering, average f_H for Ice-slurry dropped to 79.75 bpm and increased 84 bpm for Ikejime. During the time the individuals were waiting to be fished, the f_H decreased to 78.55 bpm and 83.17 bpm, for Ice-slurry and Ikejime, respectively. For the Ice-slurry trial, f_H decreased when harvesting started (to 55 bpm), in contrast with Ikejime, where the f_H increased to 87.5 bpm.

For slaughter by Ice-slurry, the average f_H at the beginning of slaughtering was 88 bpm and decreased for the duration of the trial (27.25 bpm after 5 min. and 22.75 bpm after 10 min.). Seven minutes after the slaughter started, it is possible to see fluctuations in the f_H of the individuals, which was similar for all the ice-slurry trials (for electrical stunning after 20 minutes and for the anaesthetic trial after 22 minutes and 30 seconds). However, it is important to note that the fluctuations registered in this trial were more noticeable than the remaining trials.

Similar to Anaesthetic stunning **(Figure 4.4)**, the f_H spiked at the end of the trial, with a final registered f_H of 76 and 56 bpm, for no stunning and anaesthetic, respectively.

As for slaughter by Ikejime without previous stunning, the f_H spiked at the start of the slaughter process, which was similar to the electrical stunning trial and in contrast with the anaesthetic trial. The accurate performance of the ikejime technique might have had an effect on these results. For the anaesthetic trials, ikejime was reported as done properly for all the individuals, contrary with the other two methods , in which some incidents in the performance of Ikejime were registered, which might be the cause of the increase in the f_H of the individuals.

In this trial, one bio-logger from the NIC presented low data quality and was removed from the physiological analysis.

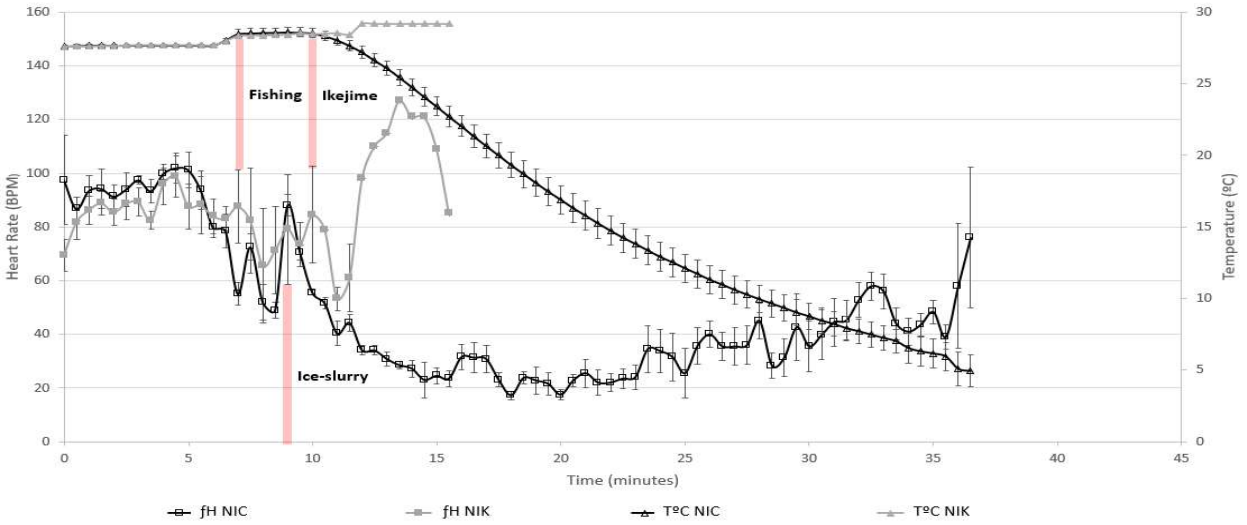

Figure 4.3: **Heart Rate and Temperature, No stunning trials.** Average (plus SEM) heart rate (*f*H) and temperature (ºC) for the NIC (black lines; N= 4) and NIK (grey lines; N= 4). Redish lines represent moment of main trial events.

4.4.2 2-phenoxyethanol Stunning

The initial average heart rate and internal temperature of the individuals before starting was 62.8 bpm + 27.92ºC and 71.4 bpm + 27.28ºC, for slaughter by Ice-slurry and Ikejime, respectively **(Figure 4.4)**. On both trials, the temperature followed the same pattern as the other trials, with an increase after harvesting started (28.50ºC for Ice-slurry and 27.80ºC for Ikejime) which was maintained for Ikejime and dropped for Ice-slurry when slaughter started and continued to decrease for the duration of the trial (24.09ºC after 5 minutes and 18.99ºC after 10 minutes).

When the water of the tank stopped lowering, average f_H increased to 94.2 bpm and 92 bpm, for Ice-slurry and Ikejime, respectively. For both trials, during the time the individuals were waiting to be fished, the f_H decreased to 87.54 bpm and 86.67 bpm, for Ice-slurry and Ikejime, respectively.

For Ice-slurry, the average f_H decreased when harvesting started, to 56.2 bpm, in contrast with Ikejime, where it increased to 92.25 bpm.

During stunning, f_H decreased from 79.2 to 58.3 bpm and from 100 to 63.6 bpm, in the first 30 seconds, for Ice-slurry and Ikejime, respectively.

For slaughter by Ice-slurry, the f_H presented a spike of 77.08 bpm between blood sampling and starting of the slaughter. After 5 minutes in the Ice-slurry, average f_H was 26.2 bpm, and 27.8 bpm after 10 minutes . After 22 minutes and 30 seconds of being in ice-slurry there was an increase in individual variability in the f_H. Similar to Ice-slurry without prior stunning, the f_H appears to have spiked at the end of the trial, with a final registered f_H of 76 and 56 bpm, for no stunning and anaesthetic, respectively.

As for slaughter by Ikejime, the f_H dropped after slaughter started (from 84.8 to 31 bpm), in contrast with the other Ikejime trials, possibly due to the fact that this trial was the only trial where there were no registered incidents during the performance of the Ikejime technique.

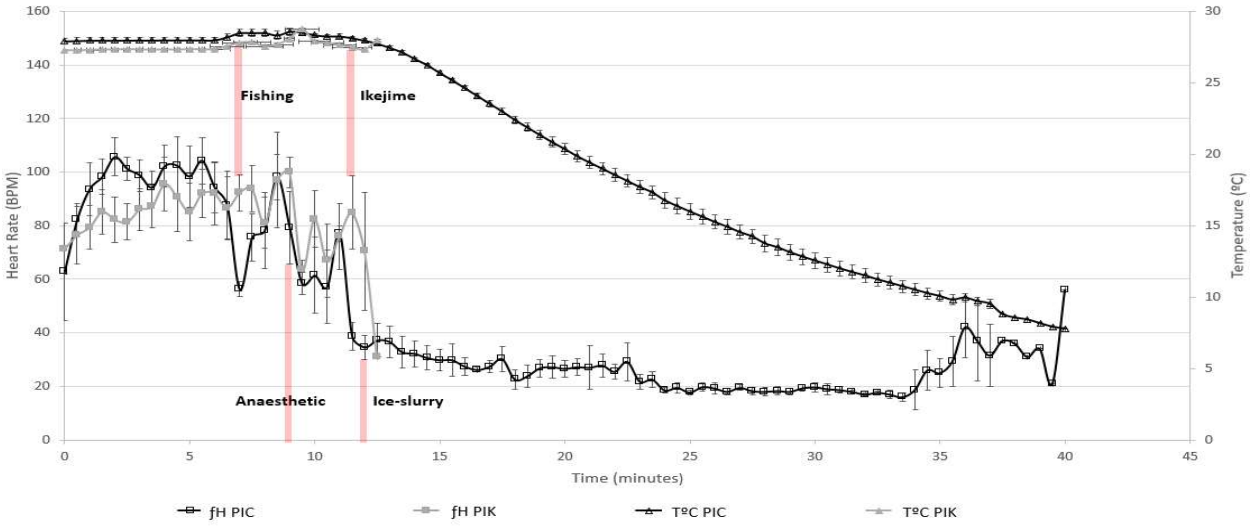

Figure 4.4: **Heart Rate and Temperature, Anaesthetic stunning trials.** Average (plus SEM) heart rate (fH) and temperature (ºC) for the PIC (black lines; N= 5) and PIK (grey lines; N= 5). Redish lines represent moment of main trial events.

4.4.3 Electrical Stunning

On the day of the trials, the average f_H and internal temperature before starting was 74.6 bpm + 27.91ºC and 67.4 bpm + 28.15ºC, for slaughter by Ice-slurry and Ikejime, respectively **(Figure 4.5)**. On both trials, we observed the increase in temperature after harvesting started (28.58ºC for Ice-slurry and 28.46ºC for Ikejime), which was maintained for Ikejime and dropped in Ice-slurry after slaughter started (25.07ºC after 5 minutes and 19.37ºC after 10 minutes), apart from a spike observed at minute 33 of the trial. This spike can be justified by the fact that only one fish was alive at the time, still presenting an internal temperature of 12.22ºC.

When the water of the tank stopped lowering, average f_H was 86.8 bpm and 103.8 bpm, for Ice-slurry and Ikejime, respectively. For Ice-slurry, during the time the individuals were waiting to be fished, the f_H increased to 98.11 bpm, in contrast with Ikejime, where it lowered to 102.17 bpm. For both trials, f_H started to decrease when harvesting started, to 96 bpm and 69.33 bpm, for Ice-slurry and Ikejime, respectively.

During electrical stunning, f_H decreased to 65.5 bpm for Ice-slurry and increased to 89.33 for Ikejime.

For slaughter by Ice-slurry, the f_H continued to decrease for the duration of the slaughter (31.2 bpm after 5 minutes and 18.4 bpm after 10 minutes). However, 20 minutes after slaughter began, it is possible to see variability in the f_H of the individuals, which was similar in all the ice-slurry trials.

As for slaughter by Ikejime, the f_H spiked after the slaughter started, which was similar to the Ikejiime without stunning trial and in contrast with the anaesthetic trial.

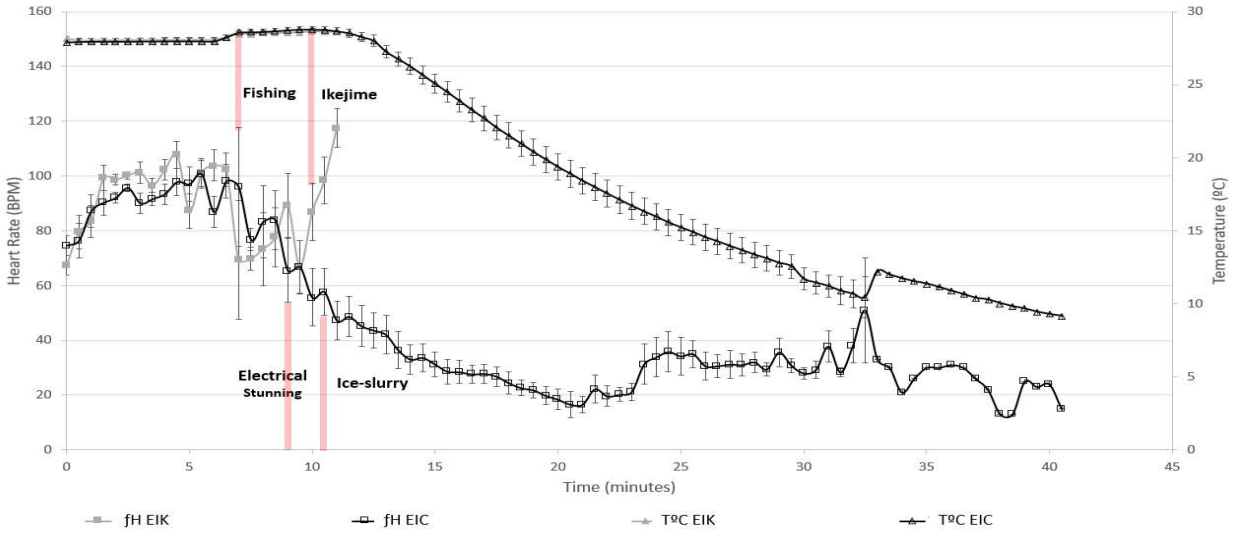

Figure 4.5: **Heart Rate and Temperature, Electric stunning trials**. Average (plus SEM) heart rate (fH) and temperature (ºC) for the EIC (black lines; N= 5) and EIK (grey lines; N= 5). Redish lines represent moment of main trial events.

4.5 Heart Rate during Slaughter

When comparing the slaughter methods, we observe that the lower temperature created during ice-slurry induced bradycardia (a reduction in the heart rate), having these fish lower f_H than the fish slaughtered by Ikejimef_H **(Figure 4.6)** . For slaughter by Ikejime, electrical stunning presented the highest f_H (94.13 bpm ± 18.74 bpm), followed by 2-phenoxyethanol stunning (79.37 bpm ± 39.17 bpm) and no stunning (64.19 bpm ± 25.68 bpm) **(Table 4.3)** . There was no significance between treatments (N= 4/5, F= 1.552, P= 0.229), however, there was significance between events (*i.e.,* day before the trial which represents the basal f_H level; stunning; slaughter) (N= 4/5, F= 5.247,

P= 0.012), with the basal f_H level being significantly different from the f_H during stunning (N= 4/5, t= 3.184, P= 0.004).

For slaughter by Ice-slurry, fish without stunning presented the highest f_H (35.02 bpm ± 5.10 bpm), followed by electrical stunning (31.98 bpm ± 7.63 bpm) and 2-phenoxyethanol stunning (26.72 bpm ± 8.40 bpm). Similar with slaughter by Ikejime, electrical stunning presented a highest f_H value when compared with 2-phenoxyethanol **(Table 4.3)**. However, there was no significance between treatments (N= 4/5, F= 2.330, P= 0.104), only on events (*i.e.,* day before the trial which represents the basal f_H level; stunning; slaughter divided in 5 minute intervals) (N= 4/5, F= 32.168, P <0.001), which was expected, since f_H goes down due to the drop in temperature caused by the ice-slurry (N= 4/5, t= between -4.113 and -5.776, P >0.001)

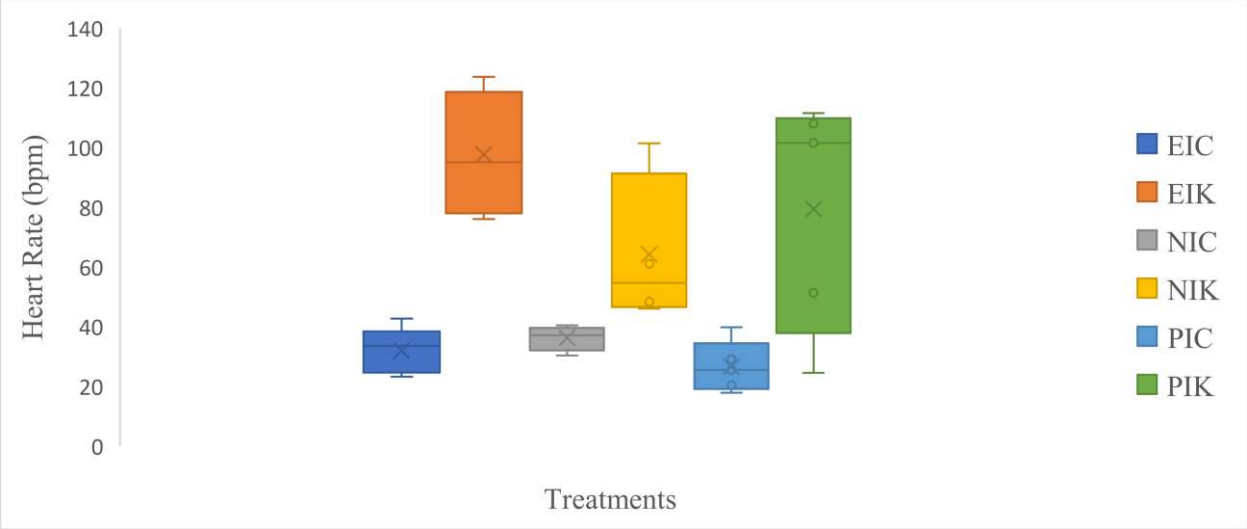

Figure 4.6: **Summary Average Heart Rate Slaughter**. Box-and-whisker plot showing minimum (lower whisker) and maximum values (higher whisker), inner and outer quartile ranges (boxes), mean (X sign), and median value (horizontal bar inside the boxes) for the average heart rate (in beats per minute), per treatment, for the time fish were in contact with the slaughtering method. EIC (N= 5), EIK (N= 5), NIC (N= 4), NIK (N= 4), PIC (N= 5), PIK (N= 5).

Table 2.3: **Summary of Stunning and Slaughter Heart Rate.** In beats per minute. Data presented in average and SEM, per treatment. Electrical & Ice-slurry (EIC, N= 5), Electrical & Ikejime (EIK, N= 5), No stunning & Ice-slurry (NIC, N= 4), No stunning & Ikejime (NIK, N= 4), Anaesthetic & Ice-slurry (PIC, N= 5), Anaethetic & Ikejime (PIK, N= 5). Stunning heart rate NA (not applicable) for the no stunning trials.

	Heart Rate	
	Stunning	**Slaughter**
Electrical & Ice-slurry	77.33 ± 26.31	31.98 ± 07.63
Electrical & Ikejime	89.33 ± 12.01	94.13 ± 18.74
No stunning & Ice-slurry	NA	35.02 ± 05.10
No stunning & Ikejime	NA	64.19 ± 25.68
Anaesthetic & Ice-slurry	69.27 ± 13.73	26.72 ± 08.40
Anaesthetic & Ikejime	82.60 ± 09.78	79.37 ± 39.17

4.6 Behavioural Analysis

4.6.1 Electrical Stunning

The duration of stunning (time of electrical current applied to the fish) was exactly 15 seconds for both trials. None of the individuals displayed either gasping or abrupt movements during stunning. **Loss of Free swimming**, **Loss of Equilibrium** and **Loss of OM** were immediate in all individuals after the subjects were subjected to electricity, with the exception of 1 individual that did not lose **VOR** in the Ice-slurry trial **(Table 4.4)**.

4.6.2 2-phenoxyethanol Stunning

The average time for stunning (time in 2-phenoxyethanol tank) was 2 minutes and 17 seconds. None of the individuals displayed either gasping or abrupt movement during stunning. **Loss of Free swimming:** observed in 4 of the 5 individuals of the Ice-slurry trial and in all of the Ikejime trial, and took on average 35 seconds; **Loss of Equilibrium:** took on average 1 minute and 31 seconds; **Irregular Opercular Movement**: detected in all individuals of the Ice-slurry trial and in 2 of the 5 individuals of the Ikejime trial after around 1 minute and 7 seconds; **Loss of OM**: after 1 minute and 30 seconds; **Loss of VOR:** after 1 minute and 27 seconds **(Table 4.4)**.

Table 4.4: **Latency behavioural parameters during stunning.** Average and SEM Latency (in minutes and seconds) of the main signs of loss of awareness and sensibility. Electrical & Ice-slurry (EIC, N= 5), Electrical & Ikejime (EIK, N= 5), No stunning & Ice-slurry (NIC, N= 5), No stunning & Ikejime (NIK, N= 4), Anaesthetic & Ice-slurry (PIC, N= 5), Anaethetic & Ikejime (PIK, N= 5). Stunning parameters NA (not applicable) for the no stunning trials.

	Stunning				
	Loss Equilib-rium	**Loss Free Swimming**	**Gain Irregu-lar OM**	**Loss VOR**	**Loss OM**
Electrical & Ice-slurry	00:00 ± 00:00	00:00 ± 00:00	00:00 ± 00:00	00:00 ± 00:00	00:00 ± 00:00
Electrical & Ikejime	00:00 ± 00:00	00:00 ± 00:00	00:00 ± 00:00	00:00 ± 00:00	00:00 ± 00:00
Anaesthetic & Ice-slurry	00:35 ± 00:13	00:36 ± 00:04	01:13 ± 00:23	01:34 ± 00:41	01:39 ± 00:26
Anaesthetic & Ikejime	00:28 ± 00:08	00:34 ± 00:10	01:00 ± 00:03	01:20 ± 00:34	01:20 ± 00:34
No stunning & Ice-slurry	NA	NA	NA	NA	NA
No stunning & Ikejime	NA	NA	NA	NA	NA

4.6.3 Slaughter by Ice-slurry

The average time for slaughter in the ice-slurry tank (-0.62 ± 0.06 ºC; 28.8 ± 1.17 psu) was 21 minutes and 28 seconds (± 6 minutes and 25 seconds) for the 2-phenoxyethanol trial, 23 minutes and 26 seconds (± 3 minutes and 42 seconds) for the Electrical stunning trial and 26 minutes and 36 seconds (± 1 minute and 15 seconds) for the No Stunning trial. Behavioural parameters may be observed in **Table 4.5**. Gasping was observed in all of the NIC individuals, on 3 individuals of the EIC and 2 of the PIC trial **(Table 4.6**). Abrupt movements presented a similar distribution, with the exception of the PIC trial, where only 1 individual presented abrupt movements (**Table 4.6**).

4.6.4 Slaughter by Ikejime

The average time for slaughter was 43 seconds (± 13 seconds) for the 2-phenoxyethanol trial, 37 seconds (± 9 seconds) for the Electrical Stunning trial and 2 minutes and 16 seconds (± 1 minute and 47 seconds) for the No Stunning trial. Behavioural parameters may be observed in **Table 4.5**.

Gasping was not observed in any of the trials (**Table 4.6**). Abrupt movements were observed in all of the NIK individuals, on 2 individuals of the EIK and 3 of the PIK trial **(Table 4.6**).

Table 4.5: **Latency behavioural parameters during slaughter.** Average and SEM Latency (time it takes for a certain behavioural to be noticed) in minutes and seconds for the main signs of loss of awareness and sensibility. Electrical & Ice-slurry (EIC, N= 5), Electrical & Ikejime (EIK, N= 5), No stunning & Ice-slurry (NIC, N= 5), No stunning & Ikejime (NIK, N= 4), Anaesthetic & Ice-slurry (PIC, N= 5), Anaethetic & Ikejime (PIK, N= 5). *: not an average, only one individual.

	Slaughter						
	Loss Free Swimming	**Loss Equilib-rium**	**Gain Ir-regular OM**	**Loss Ir-regular OM**	**Recov-ery VOR**	**Loss VOR**	**Loss OM**
Electrical & Ice-slurry	NA	NA	00:22 ± 00:14	13:14 ± 04:49	*00:33	02:30± 00:54	16:28 ± 06:33
Electrical & Ikejime	NA	NA	NA	NA	NA	NA	NA
Anaesthetic & Ice-slurry	NA	NA	01:42 ± 00:45	*15:00 ± 00:00	*18:21 ± 00:00	* 24:21 ± 00:00	18:23 ± 06:02
Anaesthetic & Ikejime	NA	NA	NA	NA	NA	NA	NA
No stunning & Ice-slurry	00:43 ± 00:41	00:57 ± 00:57	01:42 ± 00:45	06:16 ± 03:10	NA	15:00 ± 04:40	18:23 ± 06:02
No stunning & Ikejime	NA	NA	NA	NA	NA	NA	NA

Table 4.6: **Latency gasping and abrupt movements during slaughter.** Average and SEM Latency (time it takes for a certain behavioural to be noticed) in minutes and seconds for gasping and abrupt movements to occur. Electrical & Ice-slurry (EIC, N= 5), Electrical & Ikejime (EIK, N= 5), No stunning & Ice-slurry (NIC, N= 5), No stunning & Ikejime (NIK, N= 4), Anaesthetic & Ice-slurry (PIC, N= 5), Anaethetic & Ikejime (PIK, N= 5). *: not an average, only one individual.

	Slaughter	
	Gasping	**Abrupt movements**
Electrical & Ice-slurry	02:23 ± 01:09	03:56 ± 02:49
Electrical & Ikejime	NA	00:05 ± 00:03
No stunning & Ice-slurry	00:55 ± 00:35	01:14 ± 00:58
No stunning & Ikejime	NA	00:21 ± 00:21
Anaesthetic & Ice-slurry	06:15 ± 04:53	*00:45 ± 00:00
Anaesthetic & Ikejime	NA	00:03 ± 00:11

Chapter 5. DISCUSSION

Measuring suffering in fish can be demanding, since feelings are a subjective matter that can be difficult to quantify (Kestin *et al*., 2002; Duncan, 2005). In this study we combined physiological and behavioural parameters, to tackle this issue using quantifiable indicators. Although stunning did not significantly reduce slaughter time in our ice-slurry study, we observed that, after 25 minutes of slaughter, in the stunning treatments the majority of the individuals were deceased, contrary to the no stunning trial that, at the time, still had all individuals alive. In addition, heart fibrillation was observed earlier on the treatments with no previous stunning, with higher variability (spikes reaching almost 80 bpm, in comparation to less than 60 bpm for the other treatments). Concerning behavioural analysis, the individuals of the no stunning treatment were conscious during slaughter, contrary to the stunning treatments. Regarding slaughter by ikejime, slaughter time was again not significant, however, we observed that previous stunning led to a faster slaughter (average higher than 2 minutes for no stunning and less than 1 minute for the stunning treatments), since the ikejime technique is impaired by abrupt movements of the fish, which were more noticeable on the no stunning treatment. As such, our results show that stunning by electrical shock increases the welfare of fish, both for a more rested slaughter during ice-slurry and faster ikejime, in comparation with the most commonly used method for this species in the industry, which is slaughter by ice-slurry without previous stunning.

For the behavioural analysis, stunning and slaughter without previous stunning were considered completed when fish lost both VOR and OM, which are commonly the last behavioural parameters lost during stunning/anaesthesia (Weber *et al*., 2009; Bowman *et al.,* 2020). In humans, the presence/absence of VOR is considered a good indicator of recovery and survival in comatose patients, since VOR is linked to brain function (Meneses *et al.,* 2010; Wallace & Lifshitz, 2016). One interesting point that arose during this study, was: “when can we be certain that fish are fully unconscious, *i.e*., not feeling pain/suffering?”. In humans, coma patients are considered unconscious because they cannot be awaken by external stimuli (Laureys, 2005), however, evaluating pain can be arduous due to their physical condition (Demertzi *et al.,* 2009), as it was for our study. During slaughter by ice-slurry without previous stunning, for example, individuals lost VOR after around 15 minutes in the ice-slurry, which would indicate they had lost

consciousness. Nonetheless, the vestibular system is influenced by the bloodstream, and the metabolic rate lowers significantly during exposure to lower temperatures (Poli *et al.,* 2005; Wallace & Lifshitz, 2016), which in theory could influence VOR functioning.

The immediate loss of apparent consciousness during electrical stunning was in consensus with previous papers. Grans *et al*. (2016) described immediate loss of body movement, VOR and ventilation in African sharptooth catfish (*Clarias gariepinus*, Burchell, 1822) and Kestin *et al.* (2002) described, for gilthead seabream, immediate loss of equilibrium when fish were exposed to electric shock, as opposed to 5 minutes when exposed to ice-slurry, as well as immediate loss of reflex reactions such as breathing or VOR, as opposed to 10 minutes, respectively.

For the 2-phenoxyethanol anaesthesia, we found that the concentration of 1 000 ppm necessary for deep anaesthesia (loss of both VOR and OM) was not in consensus with other paper on seabreams. In 2006, Tsantillas *et al*. determined that, for white seabream (*Diplodus sargus L*., Valenciennes, 1830) and sharp snout seabream (*Diplodus puntazzo*, Walbaum, 1792), concentrations between 167 and 400 ppm were sufficient for anaesthesia under 3 minutes. However, the measured parameters in this study were loss of equilibrium and slow OM, with no accountability for loss of OM, which was the last parameter considered in our study. Although different species and individual characteristics may affect the anaesthetic effect, we believe that the discrepancy on the concentrations used may be associated to the different parameters considered, since Weber *et al.* (2009) also described concentrations higher than 1 000 ppm (600 000 ppm) for less than 3 minutes induction time, in senegalese sole (*Solea senegalensis*, Kaup 1858), considering the same parameters as our study.

As mentioned before, the presence or type of stunning did not influence significantly slaughter time for ice-slurry. This can be attributed to the fact that ice-slurry is a physiological process that lowers the temperature of the fish body, as well as its metabolic rate, diminishing the oxygen requirements of the individuals (Poli *et al.,* 2005) and takes a certain amount of time to achieve (either the individuals die by hypothermia or asphyxia). During hypothermia, in humans, there is a stable decrease of heart rate and possible fibrillation (Champion *et al.,* 2015), both observed during our trial, with a decrease of heart rate from the moment the slaughter started by ice-slurry started and an increase in heart variability after around 20 minutes of slaughter. It was also possible to observe bradycardia (lowering of the heart rate) in all treatments, which is a phenomenon

common when oxygen levels lower in water, called hypoxic bradycardia (Joyce *et al.,* 2015; Stecyk, 2017). Although the function of this phenomenon is still debated by the scientific community (Joyce *et al*., 2015), Farrel (2007) hypothesize this to be an adaptation to reduce the heart oxygen demand. Additionally, recovery of OM was registered in 2 cases in slaughter by ice-slurry: around 22 seconds after electric shock and 1 minute and 42 seconds after anaesthesia. These animals lost OM after 16 minutes and 28 seconds and 8 minutes and 23 seconds, respectively. This behaviour may be because fish are trying to maintain homeostasis, even unconscious. This phenomenon may be attributed to the autonomic nervous system, which regulates involuntary processes such as heart rate and respiration, and have evolved in fish to fight environmental hypoxia (Waxenbaum *et al.,* 1953; Sandblom & Axelsson, 2011). Finally, one interesting observation was that 2 individuals of the anaesthetic and ice-slurry trial were still considered alive during brain extraction (which was made in the end of the trial with a scientific researcher collaborating on this project), with one presenting minimum OM and mouth movements after it was taken out of the slaughter tank. Since we know that the metabolic rate lowers during ice-slurry (Poli *et al.,* 2005; Wallace & Lifshitz, 2016), it would be possible that the circulation of the anaesthetic in the fish body may be somehow disrupted, resulting in some gain of awareness. Further studies should be conducted to test this hypothesis.

Lastly, the average time (mean ± SEM) for bio-logger implantation was in consensus with previous studies (Brijs *et al*., 2019, Mignucci *et al.* 2021). Based on the experience of the team researchers and on results of a previous study with gilthead seabream (unpublished data), we expected a zero mortality rate during the bio-logger implantation and following recovery, instead of the 90,63% obtained. As such, we hypothesize that the mortality rate observed may be related to bacterial infection of the wound after surgery due to an increase in water temperature on the days following surgery, which might have facilitated a bacterial blooming. Mignucci *et al.* (2021) also reported that f_H levels returned to the basal level (around 60 bpm) on the third day after surgery. In our study, we reported similar levels on the fourth day (68.12 bpm).

Chapter 6. CONCLUSIONS AND RECOMMENDATIONS

These results indicate that stunning by electrical shock before slaughter is better, from a welfare point of view, than the most commonly used method for this species in the industry, which is slaughter by ice-slurry without previous stunning.

We recommend that future studies, if possible, use cameras to assist the behavioural analysis, in order to reduce human error, as described by Bowman *et al.* (2020). We found that measuring sudden behavioural parameters such as gasping events may be arduous by the naked eye, as well as faint self-initiated behaviours. Based on the duration results obtained in this study, it may be advantageous to install a camera on the stunning/slaughtering tanks and set an interval to record continuously, which would allow a better evaluation of the behavioural parameters. Furthermore, we think that the possible negative effect of ice-slurry in the 2-phenoxyethanol anaesthesia should be further studied in gilthead seabream.

In addition, since behavioural analysis may be unreliable in cases the fish are rendered immobilized but may have not lost consciousness (as is the case with ice-slurry), we think that it would be interesting to conduct a complete multiparametric study for this species, with the addition, for example, of the analysis of the brain activity through electro-encephalograms (Lambooij *et al*., 2008; Bowman *et al.,* 2020), as well as the analysis of the blood hormones (Brijs *et al*., 2018) and heart rate amplitudes.

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