UNIVERSIDADE DE SÃO PAULO
FACULDADE DE ZOOTECNIA E ENGENHARIA DE ALIMENTOS

DANIEL SANTIAGO RUCINQUE GONZALEZ

Unconsciousness assessment through electroencephalography (EEG), in the process of stunning for humane slaughter of Nile tilapia

Pirassununga
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Tese apresentada à Faculdade de Zootecnia e Engenharia de Alimentos da Universidade de São Paulo, como parte dos requisitos para a obtenção do título de Doutor em Ciências do programa de Pós-graduação em Zootecnia.

Área de Concentração: Qualidade e Produtividade Animal

Orientadora: Profa. Dra. Elisabete Maria Macedo Viegas

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ATA DE DEFESA

Ata de defesa de Tese do(a) Senhor(a) Daniel Santiago Rucinque Gonzalez no Programa: Zootecnia, do(a) Faculdade de Zootecnia e Engenharia de Alimentos da Universidade de São Paulo.

Aos 27 dias do mês de janeiro de 2021, no(a) Sala da Docente ZAZ (online) realizou-se a Defesa da Tese do(a) Senhor(a) Daniel Santiago Rucinque Gonzalez, apresentada para a obtenção do título de Doutor intitulada:

"Avaliação de inconsciência, por meio de eletroencefalografia (EEG), no processo de insensibilização para abate humanitário de tilápia do Nilo"

Após declarada aberta a sessão, o(a) Sr(a) Presidente passa a palavra ao candidato para exposição e a seguir aos examinadores para as devidas arguições que se desenvolvem nos termos regimentais. Em seguida, a Comissão Julgadora proclama o resultado:

Nome dos Participantes da Banca	Função	Sigla da CPG	Resultado
Elisabete Maria Macedo Viegas	Presidente	FZEA - USP	Não Votante
Judite das Graças Lapa Guimarães	Titular	FZEA - USP	APROVADO
Giuliana Parisi	Titular	UniFi(FZEA)	APROVADO
Dariane Beatriz Schoffen Enke	Titular	UNESP - Externo	APROVADO
Tatiana Emanuelli	Titular	UFSM - Externo	APROVADO
Juliana Antunes Galvão	Suplente	Especialista-ESALQ	APROVADO

Resultado Final: APROVADO

Parecer da Comissão Julgadora *

Eu, Erica Cristina Mello Ferraz _____, lavrei a presente ata, que assino juntamente com os(as) Senhores(as). Pirassununga, aos 27 dias do mês de janeiro de 2021.

Judite das Graças Lapa Guimarães

Giuliana Parisi

Dariane Beatriz Schoffen Enke

Tatiana Emanuelli

Juliana Antunes Galvão

Elisabete Maria Macedo Viegas
Presidente da Comissão Julgadora

* Obs: Se o candidato for reprovado por algum dos membros, o preenchimento do parecer é obrigatório.

A defesa foi homologada pela Comissão de Pós-Graduação em 01/02/2021 e, portanto, o(a) aluno(a) faz jus ao título de Doutor em Ciências obtido no Programa Zootecnia - Área de Concentração: Qualidade e Produtividade Animal.

Presidente da Comissão de Pós-Graduação
Vice-Presidente da Comissão de Pós-Graduação
- em exercício -
FZEA/USP

Dedico a minha família na Colômbia, José Santiago R, Diana R, Jenny R e Paula e no Brasil, Priscilla Tamioso, por serem minha fonte de força, amor e inspiração.

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“How it is possible that the most intellectual creature to ever walk the planet Earth is destroying its only home?”

Jane Goodall

RESUMO

RUCINQUE, D. S. **Avaliação de inconsciência, por meio de eletroencefalografia (EEG), no processo de insensibilização para abate humanitário de tilápia do Nilo**. 2021. 121 f. Tese (Doutorado) – Faculdade de Zootecnia e Engenharia de Alimentos, Universidade de São Paulo, Pirassununga, 2021.

No **Capítulo 1** apresenta-se uma breve introdução ao assunto, assim como os objetivos da tese. No **Capítulo 2** o objetivo foi avaliar os óleos essenciais de *Ocimum americanum* (OA) ou *Lippia alba* (LA) como anestésicos para tilápia do Nilo, assim como a influência dos mesmos no aroma e sabor dos filés. Para a avaliação anestésica, tilápias do Nilo foram divididas em 4 grupos: etanol (ET), 2-fenoxietanol (PE) e óleos essenciais de *Ocimum americanum* (OA) ou *Lippia alba* (LA). Óleos essenciais de OA ou LA a 500 µL/L foram efetivos para indução de anestesia profunda com uma duração da inconsciência que permitiu a sangria. Anestesia com óleos essenciais de *Ocimum americanum* ou *Lippia alba* podem ser indicados como uma alternativa à hipotermia no pré-abate de peixes no Brasil. No **Capítulo 3** o objetivo foi avaliar a percussão mecânica perfurante *Spiking* e a eletronarcose como métodos para indução de inconsciência, e seus efeitos sobre o *rigor mortis*. Tilápias do Nilo foram divididas em três grupos, dois para percussão mecânica e um grupo para eletronarcose. A percussão mecânica com acesso lateral foi efetiva para induzir inconsciência sem recuperação em tilápia do Nilo, e seu uso em sistema automatizado pode ser uma alternativa ao método tradicional de hipotermia no Brasil. No **Capítulo 4** foi avaliada a inconsciência por meio de eletroencefalografia (EEG) em tilápias do Nilo submetidas a diferentes métodos ao pré-abate. Tilápias do Nilo foram divididas em cinco diferentes grupos para avaliar métodos no período pré-abate, anestesia com 2-fenoxietanol 1 ml/L (PE), anestesia com óleos essências de *Ocimum americanum* (OA) ou *Lippia alba* a 500 µL/L, *spiking* (SP) e hipotermia (HP). A mediana de tempo (s) para perder a consciência representada pela fase suprimida foi PE (136 ± 61), OA (171 ± 164), LA (171.5 ±77.8) e HP (252 ± 752) ($P<0.01$). A análise espectral sugere inconsciência após 3 minutos de exposição aos anestésicos PE, OA, LA devido à diminuição de Ptot, F50 e F95 e incremento na contribuição de frequências *delta* e diminuição nas frequências de *beta*. Os valores de HP sugerem que tal método não induz inconsciência. Anestesia com *Ocimum americanum* ou *Lippia alba* induz inconsciência determinada por EEG após 180 s de imersão. O uso da hipotermia não induz inconsciência em tilápia do Nilo e não cumpre com critérios de abate humanitário, sendo urgente sua substituição por métodos que cumpram critérios de abate humanitário. No **Capítulo 5** o objetivo foi avaliar a influência de diferentes métodos pré-abate: hipotermia (HP), anestesia com os óleos essências de *Ocimum americanum* (OA) ou *Lippia alba* e *spiking* (SP) sobre parâmetros da qualidade da carne de tilápia sem evisceração e refrigerada durante 15 dias. O uso da percussão mecânica *spiking* (SP) e anestesia com óleos essenciais de *Ocimum americanum* (OA) ou *Lippia alba* (LA) em tilápia do Nilo sob refrigeração não alterou negativamente os seguintes parâmetros de qualidade da carne pH, rigor mortis, TVB-N e valor K quando comparado ao método tradicional de hipotermia.

Palavras-chave: anestesia, abate, bem-estar animal, bem-estar de peixes, hipotermia, indicadores de comportamento, insensibilização, óleos essenciais, qualidade da carne

ABSTRACT

RUCINQUE, D. S. **Unconsciousness assessment through electroencephalography (EEG), in the process of stunning for humane slaughter of Nile tilapia.** 2021. 121 p. Tese (Doutorado) – Faculdade de Zootecnia e Engenharia de Alimentos, Universidade de São Paulo, Pirassununga, 2021.

Chapter 1 presents a brief introduction to the subject and the aim of these. **Chapter 2** assessed *Ocimum americanum* (OA) or *Lippia alba* (LA) essential oils as anaesthetics in Nile tilapia and their influence on the aroma. For the anaesthetic evaluation, Nile tilapia were divided into four groups: ethanol (ET), 2-phenoxyethanol (PE), *Ocimum americanum* (OA) or *Lippia alba* (LA), all at 500 μL/L. OA or LA essential oils at 500 μL/L were effective in inducing deep anaesthesia with unconsciousness duration of 126 s, allowing time for bleeding. Anaesthesia with *Ocimum americanum* or *Lippia alba* essential oils may be indicated as an alternative to hypothermia at pre-slaughter handling of fish in Brazil. **Chapter 3** aimed to assess mechanical spiking and electrical stunning as methods to induce unconsciousness in Nile tilapia, and their effects on *rigor mortis*. Nile tilapia were divided into three groups, two groups for spiking and one group for electrical stunning. Mechanical spiking with lateral access is effective to induce unconsciousness without recovery, delaying the onset of rigor mortis in Nile tilapia, thus its use in an automatic system could be an alternative to traditional slaughter of hypothermia in Brazil. **Chapter 4** aimed to assess unconsciousness using electroencephalography EEG in Nile tilapia under different methods at pre-slaughter. Nile tilapia were divided in five different methods at pre-slaughter: anaesthesia with phenoxyethanol at 1 mL/L (PE), anaesthesia with *Ocimum americanum* (OA) or *Lippia alba* (LA) essential oils at 500 μL/L, spiking (SP) and hypothermia in ice/water (2:1). The median time (s) to lose consciousness as represented by the suppressed phase was PE (136 ± 61), OA (171 ± 164), LA (171.5 ±77.8) and HP (252 ± 752) ($P<0.01$). Spectral analysis suggested unconsciousness after 3 minutes of exposure to PE, OA or LA due to the decreases in Ptot, F50 and F95, and also the increase in contribution from the delta frequency and decrease in contribution from the beta frequency. The values obtained in the spectral analysis for fish submitted to HP suggest that this method does not induce unconsciousness. When used in Nile tilapia, phenoxyethanol-like anaesthetic showed both EEG traces and spectral analyses consistent with unconsciousness after 140 s of immersion. Anaesthesia with 500μL/L of the essential oils *Ocimum americanum* or *Lippia alba* induced unconsciousness after 180 s of immersion, as determined by EEG. The use of hypothermia at pre-slaughter of Nile tilapia does not induce unconsciousness and should be urgently substituted by methods that meet criteria of humane slaughter. **Chapter 5** assessed the influence of different pre-slaughter methods: hypothermia live chilling (HP); anaesthesia with the essential oils *Ocimum americanum* (OA) or *Lippia alba* (LA) and mechanical spiking (SP) on the meat quality parameters of Nile tilapia stored under refrigeration for 15 days. The use of mechanical spiking and anaesthesia with the *Ocimum americanum* (OA) or *Lippia alba* (LA) essential oils in Nile tilapia did not negatively change the following quality parameters of the meat: pH value, *rigor mortis*, TVB-N and K-value as compared to the traditional method of hypothermia.

Keywords: anaesthesia, animal welfare, behaviour indicators, essential oils, fish welfare, hypothermia, meat quality, slaughter, stunning, unconsciousness

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SUMMARY

1 Chapter 1 Introduction

According to the Brazilian Fisheries Association, fish production totalled 758,006 t in 2019, increasing 4.9% in relation to 2018. Nile tilapia represents 57% of all fish farming, with 432,149 t in 2019 (PEIXE BR, 2020). Such quantity involves a high number of individuals, therefore fish welfare becomes a relevant issue to reduce stress and suffering during the production process.

Animal welfare is a concept applied to sentient beings which are those who experience emotions associated with pleasure and suffering and who are motivated to promote their evolutionary fitness not as part of a well-planned, long-term strategy to ensure the wellbeing of future generations, but through the simpler, but no less intense, need to feel good about themselves (WEBSTER, 2006).

Scientific recognition of the ability to feel pain in fish is supported with publications from international bodies related to both food safety (EFSA, 2009) and fish welfare and sentience (BROWN, 2014; CHANDROO; DUNCAN; MOCCIA, 2004; SNEDDON, 2003, 2004, 2006; SNEDDON et al., 2014). Additionally, the World Organisation for Animal Health – OIE guarantees fish welfare during the transport, slaughter and killing of farmed fish for disease control purposes (OIE, 2015). The most important issue related to poor welfare is the slaughter process (HUNTINGFORD et al., 2006; LINES; SPENCE, 2014). In Brazil, humane slaughter regulations do not include fish (BRASIL, 2000) and hypothermia (live chilling in ice/water) is used in most fish slaughterhouses at pre-slaughter handling (OLIVEIRA FILHO et al., 2014). Such method is not considered humanitarian as it does not induce an immediate loss of consciousness in addition to aversive behaviour when fish are immersed in water and ice mixture (LAMBOOIJ et al., 2015; PEDRAZZANI et al., 2009; VAN DE VIS et al., 2003). In Norway stunning of farmed fish during at pre-slaughter is mandatory in fish destined to human consumption (NORWAY, 2009) and methods as electrical stunning and automat percussión are used. Anaesthetics to induce unconsciousness before slaughter are also used, for example, the isoeugenol (Aqui-s®, New Zeland) is used at slaughter of fish for human consumption in Australia, New Zeland, Chile, Korea, Costa Rica and Honduras. In 2011, the use of isoeugenol for fish slaughter in the European Union was approved (EU, 2011).

In order to consider slaughter humane, the animals must be rendered unconscious immediately or without pain prior to slaughter, and should remain so until dead. Stunning methods should induce an immediate loss of consciousness. If loss of consciousness is

not immediate (i.e. anaesthesia), the induction of unconsciousness should not cause avoidable pain and suffering in the animals (VELARDE; RAJ, 2016).

Behavioural indicators (swimming, equilibrium, response to handling, response to painful stimuli, vestibulo-ocular reflex and breathing) have been used to assess unconsciousness in fish (BALDI et al., 2018; KESTIN; VAN DE VIS; ROBB, 2002; RUCINQUE; WATANABE; MOLENTO, 2018; VEIT et al., 2017). However, measuring behaviour can be limited if fish is paralyzed by electro-immobilization or muscular contraction (VAN DE VIS et al., 2014). Hence, new methods for stunning/slaughtering fish and/or others animals should be validated through electroencephalography (EEG).

The EEG is the electrical activity record from electrodes placed at various locations on the scalp (human) or head (other species). It consists of the summated electrical activity of populations of neurones together with a contribution from the glial cells. Neurones are excitable cells with intrinsic electrical properties that result in the production of electrical and magnetic fields (MURRELL; JOHNSON, 2006). The recording of spontaneous cortical electrical activity (EEG) has been widely used as a means of assessing the state of sensibility of animals during slaughter (VELARDE et al., 2002).

EEG is one of the steps required by the European Food Safety Authority to register new methods for humane slaughter in animals. Knowledge regarding humane methods for stunning/slaughtering Nile tilapia is scarce (LAMBOOIJ et al., 2008; OLIVEIRA FILHO et al., 2014; PEDRAZZANI et al., 2009) and only electrical stunning was studied through EEG.

1.1 Aim

The aim of this thesis was to assess unconsciousness in fish through Electroencephalography (EEG) in the process of stunning by electrical stunning, percussion and anaesthesia for humane slaughter of Nile tilapia (*Oreochromis niloticus*).

The thesis was divided in Chapters. In Chapter 2 the effect of *Ocimum americanum* or *Lippia alba* essential oils like anaesthetics in Nile tilapia was studied. Part of the results was reported as oral presentation at the *Aquaciência 2018*, by Professor Elisabete Maria Macedo Viegas, on 17-21 September 2018, and part as a poster presentation, at the Latin American and Caribbean Aquaculture 2019 *Lacqua 2019*, São José, Costa Rica, 19-22 November 2019, by Professor Elisabete Maria Macedo Viegas. Results from Chapter 2 were published as a research paper (*Ocimum americanum* and *Lippia alba* essential oils

as anaesthetics for Nile tilapia: Induction, recovery of apparent unconsciousness and sensory analysis of fillets https://doi.org/10.1016/j.aquaculture.2020.735902) in the journal Aquaculture.

In Chapter 3 electrical parameters for stunning and mechanical percussion (*spiking)* in Nile tilapia were investigated. Part of the results was presented as a poster at the 53rd Congress of the ISAE International Society for Applied Ethology *ISAE 2019*, Bergen Norway, 5-9 August 2019, by Daniel Santiago Rucinque.

In Chapter 4, records of electroencephalography were applied for unconsciousness assessment during the process of pre-slaughter in Nile tilapia. The experiments were carried out with the participation of researchers from The Netherlands, Dr. Hans Van de Vis, Dr. Marien Gerritzen and Henny Reimert, with funding support from São Paulo Research Foundation (FAPESP) by way of the Regular Research Support (process number 2018/23317-3). The researchers supervised the student Daniel Santiago Rucinque during his stay of six months at the Wageningen Reserarch University in The Netherlands in 2018.

Chapter 5 we studied the effect of different methods at pre-slaughter, (*Ocimum americanum* or *Lippia alba* essential oils as anaesthetics, mechanical spiking and hypothermia) in Nile tilapia under refrigeration during 15 days, on meat quality indicators. The table 1.1 summarizes the experiments done in this thesis.

Table 1.1 Summary of the experiments done during the development of this thesis.

Chapter	Aim	N	Weight and length (mean ± SD)	Methods
2	This study aimed to assess the times for induction and recovery using *Ocimum americanum* or *Lippia alba* essential oils as anaesthetics in Nile tilapia, and their influence on the flavour and aroma of fillets.	n=40 fish/group	(224.05 ± 67.56 g, 22.10 ± 2.41 cm)	Behaviour for anaesthesia evaluation, glucose, lactate and cortisol. Sensory analysis (control comparison)
3	Our aim was to assess mechanical spiking and electrical stunning as methods to induce unconsciousness in Nile tilapia, and their effects on *rigor mortis*.	n=56 total	(574.0 ± 170.8 g; 30.9 ± 3.1 cm)	Behaviour for (un)consciousness evaluation and *rigor mortix* index
4	The aim of this study was to assess unconsciousness during pre-slaughter using	n=159 total	(484.0 ± 129.3 g, 29.0 ± 2.4 cm)	Electroencephalography (EEG): visual and spectral analysis

	electroencephalography EEG in Nile tilapia under anaesthesia with *Ocimum americanum* or *Lippia alba* essential oils, hypothermia and spiking.			Electrocardiography (ECG) heart rate/ min and Behaviour for (un)consciousness evaluation
5	The aim of this research was to evaluate the influence of different pre-slaughter methods: (hypothermia in ice/water; anaesthesia with the essential oils *Ocimum americanum* or *Lippia alba* and mechanical spiking), on the meat quality parameters of un-gutted Nile tilapia stored under refrigeration (4-6 °C) for 15 days.	n= 14 fish/group	(598.7 ± 112.0 g, 31.3 ± 2.3 cm)	pH, rigor mortis, total volatile basic nitrogen (TVB-N), instrumental colour, degradation of ATP and gills histology

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2 Chapter 2 *Ocimum americanum* or *Lippia alba* essential oils as anaesthetics for Nile tilapia: Induction, recovery of apparent unconsciousness and sensory analysis of fillets

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Abstract

In Brazil, humane slaughter regulations do not include fish, and hypothermia or immersion in ice water is used in most fish slaughterhouses for pre-slaughter handling. Such a method is not considered humanitarian as it does not induce an immediate loss of consciousness and fish show aversive behaviour when immersed in a water and ice mixture. This study aimed to assess the times for induction and recovery using *Ocimum americanum* or *Lippia alba* essential oils as anaesthetics in Nile tilapia, and their influence on the flavour and aroma of fillets. For the anaesthetic evaluation, Nile tilapia (224.05 ± 67.56 g, 22.10 ± 2.41 cm) were divided into four groups (40 fish/group): ethanol (ET), 2-phenoxyethanol (PE), *Ocimum americanum* (OA) or *Lippia alba* (LA), all at 500 μL/L. The times taken to reach total loss of equilibrium, anaesthesia and consciousness recovery were recorded using a stopwatch. Blood was collected from the caudal vein after 0, 1, 4, 8 and 24 h of anaesthesia for the analysis of glucose, lactate and cortisol. For the sensory analysis, fillets from fish exposed to essentials oils and hypothermia at pre-slaughter were evaluated by 122 untrained assessors to determine differences in the aroma and flavour attributes using a seven-point scale, ranging from 3 = substantially better than the control to − 3 = substantially worse than the control. The induction of deep anaesthesia (stage 4) was faster in the OA group (mean ± standard error of mean SEM) (126.8 ± 4.9 s), than the PE (152.3 ± 6.6 s) and LA groups (169.7 ± 10.9 s) ($p < .01$). A total of 91.7% (110/120) of the fish showed VOR (Vestibulo-Ocular Reflex) as the first behavioural response during consciousness recovery after anaesthesia. The levels of glucose (mmol/L) were similar amongst the groups at each time. The lactate levels (mmol/L) were higher at 0 h of sampling in LA (2.0 ± 0.2) than in ET (1.4 ± 0.8) ($p < .05$) and the cortisol levels (ng/mL) at 0 h were higher in ET (150.3 ± 55.3) than in OA (33.9 ± 9.7). Fillets prepared from Nile tilapia anesthetized using OA essential oil received higher sensory scores (1.5 ± 0.1) than fillets from both the *Lippia alba* (1.0 ± 0.1) and hypothermia control group (0.7 ± 0.1). OA and LA essential oils at 500 μL/L were effective in inducing deep anaesthesia with unconsciousness duration of 126 s, allowing time for bleeding.

Anaesthesia with *Ocimum americanum* or *Lippia alba* essential oils may be indicated as an alternative to hypothermia for the pre-slaughter handling of fish in Brazil.

Keywords: Fillets, Fish welfare, Fish anaesthesia, Recovery, Slaughter

2.1 Introduction

Recently, concern about fish welfare has been increasing in Western countries and a study with 2147 Norwegians showed that they are concerned about fish welfare (Ellingsen et al., 2015). A Latin American study showed that people recognize suffering in common practices using fish for hook and line fishing or in fish-and-pay ponds amongst others, and regarding slaughter, 76.0% and 72% of participants from Bogota, Colombia and Curitiba, Brazil, believed, respectively, that fish should be included in humane slaughter regulations (Rucinque et al., 2017). Public concern encourages new regulations that guarantee fish welfare in all steps of production, mainly at slaughter. For example, Norway and Sweden have specific regulations for the humane slaughter of fish destined for human consumption (Norway, 2009; Röcklinsberg 2015). Additionally, the World Organisation for Animal Health – OIE, by way of its aquatic animal health code, recommends practices to guarantee fish welfare during the transport, slaughter and killing of farmed fish for disease control purposes (OIE, 2015).

In Brazil, humane slaughter regulations do not include fish (Brasil, 2000) and hypothermia or immersion in ice water is used in most fish slaughterhouses for pre-slaughter handling (Oliveira Filho et al., 2014). Such a method is not considered humanitarian as it does not induce an immediate loss of consciousness and fish show aversive behaviour when immersed in a water and ice mixture (Lambooij et al., 2015; Pedrazzani et al., 2009; van de Vis et al., 2003). In Brazil, due to the absence of regulations for fish slaughter, to the authors' knowledge there is no official approval by the Ministry of Agriculture, Livestock and Supply to use oils as anaesthetics for fish slaughter. AQUI-S™ is used commercially in Australia and New Zealand as a pre-slaughter sedative during salmon killing (Robb and Kestin, 2002) but is not currently licenced for use in food animals in Norway and the European Union (Erikson, 2011). In order to consider slaughter humane, the animals must be rendered unconscious immediately or without pain prior to slaughter, and should remain so until dead. Stunning methods should induce an immediate loss of consciousness. If the loss of consciousness is not immediate (i.e. anaesthesia), the induction of unconsciousness should not cause avoidable pain and suffering in the animals (Velarde and Raj, 2016). Other methods such

as asphyxia, evisceration and bleeding are also used in Brazil, but do not meet the precepts of humane slaughter.

The use of anaesthetics may be an alternative to induce unconsciousness for the slaughter of fish. Anaesthetics are used in fish to reduce the consequences of stress and pain during handling procedures such as biometrics, identification, gamete extraction, vaccination and transport, and the most common route of administration is via inhalation. The anaesthetic agent is dispersed in the water and is absorbed across the gills. The effect is usually assessed via the induction and recovery times, reflex reactions to external stimuli and responsiveness to handling (Zahl et al., 2012). The principal agents used for anaesthesia in fish are benzocaine, tricaine methanesulphonate (MS-222), metomidate, isoeugenol, 2-phenoxyethanol and quinaldine. Nevertheless, all of these are considered aversive for zebrafish (Readman et al., 2013). Additionally, in some cases anaesthesia can, by itself, induce stress, as measured by the cortisol levels. MS-222 has been reported to induce high cortisol release rates in Atlantic salmon (*Salmo salar*), channel catfish (*Ictalurus punctatus*), gilthead sea bream (*Sparus aurata*), rainbow trout (*Oncorhynchus mykiss*) and South American catfish (*Rhamdia quelen*), following exposure (Priborsky and Velisek, 2018).

Hence, the search for new anaesthetics of natural origin has led to the use of plant-derived essential oils. Essential oils extracted from plants contain compounds produced during the secondary metabolism of plants. They constitute one of the most important groups of raw materials used in food, hygiene, cleaning products, pharmaceuticals, perfumery and others. Silva et al. (2015a) tested the sedative/anaesthetic effects of *Ocimum americanum* essential oil (EO) on *Rhamdia quelen* and observed that its use prevented the increase in cortisol and loss of sodium induced by aerial exposure. Kampke et al. (2018) studied the genotoxic effect of *Lippia alba* essential oil in Nile tilapia and mice (*Mus musculus*) and concluded that there was no DNA damage in fish or mice, and that it was safe for use in fish intended for human consumption. In addition, the effect of *Lippia alba* essential oil was observed in Nile tilapia, and a sensory analysis showed that the assessors detected no differences between exposed and non-exposed fish in a blind test for the taste and aroma of the fillets (Hohlenwerger et al., 2016). Therefore, the aim of this study was to assess *Ocimum americanum* or *Lippia alba* essential oils as anaesthetics in Nile tilapia and their influence on the aroma and flavour of the fillets.

2.1 Material and methods

2.1.1 Animals

One hundred sixty Nile tilapia (*Oreochromis niloticus*) were obtained from the Aquaculture Laboratory of The Faculty of Animal Science and Food Engineering, University of São Paulo, São Paulo, Brazil. The laboratory acquires juvenile fish from local farms and raises them in ponds. Two weeks before the experiment, the fish were transferred in buckets from the pond to four concrete tanks (4,500 L of capacity) (40/fish/tank) with constant water renovation for acclimation (28.3 °C, pH 6.91, 6.14 mg/L dissolved oxygen). The fish were fed with commercial feed containing 28% of crude protein according to the manufacturer (Laguna® Sport 28, State of São Paulo, Brazil). Twenty-four hours before the experiment the fish were transported from the concrete tanks to a recirculation system, where they were allocated to 100 L fiberglass tanks inside the system which contained a total of 25 tanks, each with three 500 L filters, totalling 4000 L (28.05 °C, pH 7.25, 5.85 mg/L dissolved oxygen, 0.004 mg/L NH3, 0.25 mg/L NO2). Trials took 3 days for each treatment. The fish were allocated in pairs, 2 fish/tank. The fiberglass material of the tanks prevented visual contact between fish in different tanks. Such a procedure simulated transportation conditions from farms to slaughterhouses. The fish were fasted 24 h prior to both transportation and experimentation. The experiment was approved by the Ethics Committee on Animal Use of the School of Animal Science and Food Engineering, University of São Paulo (CEUA/FZEA) under protocol number 4446150817 (Annex 3).

2.1.2 Essential oils and constituents

The *Ocimum americanum* (OA) and *Lippia alba* (LA) essential oils (EO) were purchased from a commercial store - Terra Flor Aromaterapia (Alto Paraíso de Goiás, Goiás, Brazil, https://terra-flor.com/terra-flor/). The compositions of both EOs were analysed by high resolution gas chromatography coupled to Mass Spectrometry (GC–MS) in the Chromatography Laboratory, Chemistry Department, Federal University of Minas Gerais, Brazil, using an Agilent-7820A gas chromatograph coupled to an Agilent mass selective detector, under the following conditions: HP-5 column (30 m × 0.32 mm × 0.25 μm); Temperature: 50 °C (0 min), 3 °C /min to 200 °C; Injector: 220 °C Split: 1/50; FID detector: 220 °C; Injection volume: 1 μL (concentration 1.0% in chloroform). The constituents of the EOs were identified by comparison of the mass spectra with a mass spectral library (Nist, 2002) and by comparison of the Kovats retention index with literature data (Adams, 2005).

2.1.3 Anaesthetic induction and recovery of apparent unconsciousness

The Nile tilapia (224.05 ± 67.56 g, 22.10 ± 2.41 cm) were divided into four groups (40 fish/group). The fish were netted from the tanks and placed in glass aquaria containing 10 L of water with constant aeration and 500 μL/L of EO previously diluted in ethanol (1:10) (98.8%) (Exodo®, São Paulo, Brazil). Such a concentration was reported for Nile tilapia anesthetized using *Lippia alba* EO (Hohlenwerger et al., 2016). The OA concentration was previously tested in a pilot study. To determine if ethanol (ET) had any anaesthetic effect, ET was used at a concentration that was equivalent to the dilution of 500 mL/L of essential oil. In addition, 2-phenoxyethanol (PE) (Sigma-Aldrich®, São Paulo, Brazil) at 1 mL/L was used as the positive control for anaesthesia due to its properties of inducing unconsciousness, as registered using an electroencephalogram (Lambooij et al., 2009).

The times taken to reach total loss of equilibrium - stage 3b, deep anaesthesia - stage 4 and consciousness recovery were recorded using a stopwatch, and the behaviours were assessed according to Kestin et al. (2002) and Schoettger and Julin (1967) (Table 2.1). The vestibulo-ocular reflex - VOR (Kestin et al., 2002) was included as an indicator of unconsciousness. In most vertebrates, vocalization, the presence of eyelids, and palpebral, corneal reflexes or changes in pupillary dilation or contraction are common indicators of unconsciousness. Such indicators cannot be extrapolated to fish and therefore swimming, equilibrium, response to handling, response to prick on lip and VOR were assessed. Furthermore, ten fish per group were filmed. Subsequently, the videos were reviewed to check the times for induction and anaesthetic recovery. The maximum time for the evaluation of induction was 10 min in the ET group and the anaesthetic solution was changed for every 10 fish. After deep anaesthesia, the fish were handled for the biometric measurements, which involved exposure to air for 1 min, and were then transferred to 15 L aquaria with water and constant aeration for recovery. Consciousness recovery was considered when any of the aforementioned indicators was observed, suggesting the recovery of consciousness and sensitivity to painful stimuli.

Table 2.1 - Behavioural assessment on different stages of fish anaesthesia (Adapted from Kestin et al., 2002; Schoettger and Julin, 1967).

Description	Stage	Behaviour
Sedation	1	Decreased reactivity to visual and vibrational stimuli; opercular and locomotor activity reduced slightly; darker in colour

Partial loss of equilibrium	2	Loss of equilibrium in water current; increased opercular rate; swimming ability disrupted
Total loss of equilibrium	3a	Usually turn over; swimming ability persists; opercular rate rapid; react to vibrational stimuli
Total loss of equilibrium	3b	Locomotion ceases; fin movement may continue; tactile response only to pressure on caudal fin or peduncle; opercular rate slowed
Loss of reflex activity	4	Failure to respond to external stimuli, particularly pressure on caudal fin or peduncle; opercular rate slow and erratic and loss of vestibulo-ocular reflex – VOR *
Medullary collapse	5	Opercular activity ceases
Recovery	-	Resumption of any behaviour: swimming, equilibrium, response to handling, response to prick on lip or VOR

* vestibulo-ocular reflex – VOR is an indicator of consciousness descripted by Kestin et al. (2002)

2.1.4 Blood sample and analyses

Blood was collected from the caudal vein with sterile heparinized syringes after 0, 1, 4, 8 and 24 h of anaesthesia (n = 8 for each group and collection time). Four tanks were chosen for analysis at each collection time. Each fish was sampled only once. Zero hour corresponds to the sample immediately after induction using 2-phenoxyethanol and essential oils or after 10 min of immersion in the ethanol group. A damp towel was used to cover the eyes of the fish to facilitate handling in a process that took no more than 1.5 min after removal. Hence, thirty seconds were spent for catching, weighing and measuring fish, and 60 s needed for blood sampling by a trained person. After recovery from anaesthesia or immersion for the ethanol group, all the fish returned to their respective tanks. One, four, eight and twenty-four hours after induction or immersion for the ethanol group, the fish were retaken from the tanks to collect the blood samples. Glucose was evaluated immediately after taking the sample using a glucometer (ACCU-CHEK®, City, Brazil). Subsequently, the blood was centrifuged at 6000 rpm for 9 min and the plasma transferred to cryotubes and stored at −18 °C prior to the lactate and cortisol analyses.

The lactate analysis was carried out using a commercial kit (Labtest, São Paulo, Brazil, Ref. 138–1, Calibra H Ref. 80–1, Qualitrol 1H Ref. 71–1), based on an enzymatic reaction and read at 550 nm (Mindray BS 120, China). The Cortisol analysis was carried out using the commercial kit Accu-Bind 3625-300B (Monobind, USA) following the manufacturer's instructions with analysis of the samples in duplicate and reading of the enzymatic reaction at 450 nm (Labsystems Multiskan MS, Finland).

2.1.5 Sensory analysis

The sensory analysis was carried out to determine whether there were differences in the aroma and flavour attributes between fillets prepared from Nile tilapia and exposed to 500 μL/L of OA or LA essential oils and the control group. For sensory analysis, the control fish were exposed to hypothermia (immersion in a 2:1 mixture of ice and water) for 30 min and killed by bleeding. After deep anaesthesia, the fish were killed by bleeding. The fillets were cooked in a microwave oven (portions of 10 g for 1 min) and evaluated by 122 untrained assessors. The differences in aroma and flavour from the control were measured using a seven-point scale, ranging from 3 = substantially better than the control; 2 = moderately better than the control; 1 = slightly better than the control; 0 = not different from the control; −1 = slightly worse than the control; −2 = moderately worse than the control; and − 3 = substantially worse than the control (Costell, 2002; Cunha et al., 2010). The samples were coded using random numbers and the sample presentation included a hidden control. The sensory scores were awarded to the OA and LA samples in comparison with the control sample. The sensory analysis was approved by the Human Research Ethics Committee of the School of Animal Science and Food Engineering, University of São Paulo, under protocol number 3.326.401 (Annex 4).

2.1.6 Statistical analyses

Prior to the data comparison between the groups, a normality test was carried out using the Shapiro-Wilk test. The variables of time to total loss of equilibrium, time for anaesthesia, time for recovery, levels of glucose, lactate, cortisol and scores for aroma and flavour did not follow a normality pattern. The Kruskal-Wallis test followed by the Dunn's test were used for comparisons amongst the groups when significant differences were found, and the significance level was set at $p < .05$. The data were analysed using the Minitab software, version 17.

2.2 Results

2.2.1 Constituents of the essential oils

Table 2.2 shows the chemical compositions of the EOs. The principal components of both OA and LA were linalool (74.3% and 74.1%, respectively) and β-caryophyllene (6.6% and 14.1%, respectively).

Table 2.2 - Chemical constituents of *Ocimum americanum* OA and *Lippia alba* LA essential oils used in the present study. KI means Kovats retention index.

Components	Relative %		KI calculated	
	OA	LA	OA	LA
α-pinene	1.1	-	982	-
Myrcene	0.5	0.1	1014	1013
p-menthadiene	0.1	-	1021	-
δ-carene	-	0.1	-	1022
Limonene	2.0		1037	
1,8-cineole	-	5.7	-	1040
Z-β-ocimene	0.4	-	1047	-
t-hydrate-sabinene	0.3	-	1073	-
Terpinolene	0.3	-	1086	-
linalool oxide	4.5	-	1089	-
Linalool	74.3	74.1	1103	1104
Isoborneol	0.5	0.1	1120	1118
Borneol	0.3	-	1129	-
Camphor	1.1	1.5	1132	1131
α-terpineol	0.7	0.3	1181	1182
Anethole	0.3	-	1189	-
Carvacrol	0.9	-	1277	-
Eugenol	0.3	-	1355	-
α-copaene	-	0.3	-	1366
β-elemene	-	0.1	-	1397
β-caryophyllene	6.6	14.1	1408	1410
α-bergamotene	-	0.3	-	1418
Humulene	1.3	1.2	1442	1443
Bicyclogermacrene	0.3	-	1486	-
γ-cadinene	0.3	-	1508	-
β-farnesene	-	0.2	-	1519
α-bisabolene	3.2	0.4	1543	1540
Nerolidol	-	0.6	-	1550
caryophyllene oxide	-	0.7	-	1570
Others	0.7	0.2		

2.2.2 Anaesthetic induction and recovery of apparent consciousness

Ethanol (ET) did not elicit any anaesthetic effect or sedation in the fish, for this reason induction and recovery times are not shown for ET in Figure. 2.1. Therefore, the anaesthetic effect observed in the EO fish can be attributed to the action of the EO. Induction of deep anaesthesia (stage 4) was faster in the OA fish (mean ± standard error of mean SEM) (126.8 ± 4.9 s), when compared to PE (152.3 ± 6.6 s) and LA fish (169.7 ± 10.9 s). Induction took longer in the PE and LA fish than in the OA fish ($p < .01$) (Fig. 2.1).

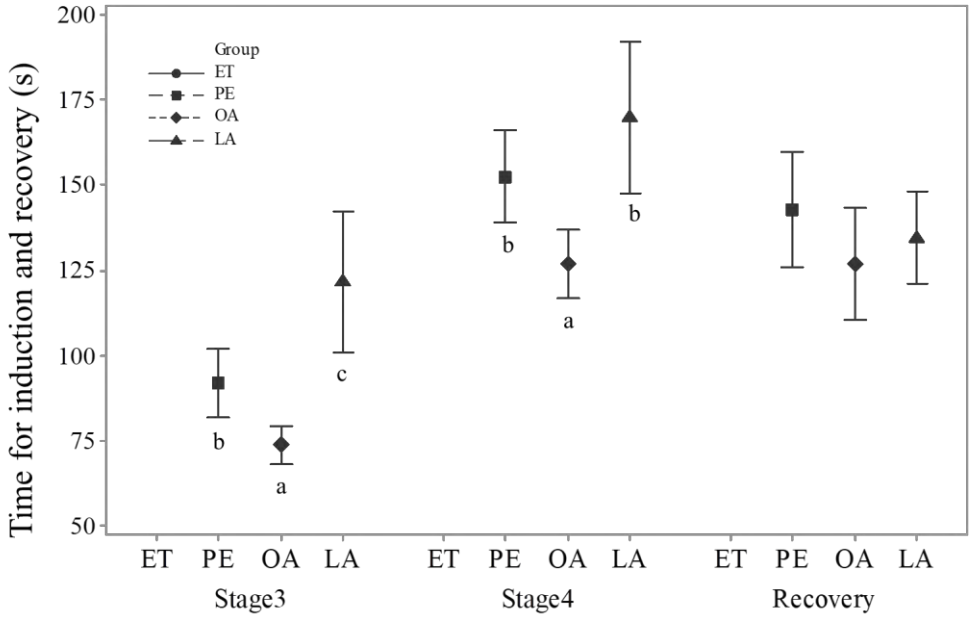

Figure 2.1 - Time (mean ± SEM) (s) required for induction and recovery from anaesthesia in Nile tilapia using ethanol (ET), 2-phenoxyethanol (PE), *Ocimum americanum* (OA) or *Lippia alba* (LA), 40 fish per group. The ET did not elicit any anaesthetic effect or sedation in the fish. The stages are shown according to Schoettger and Julin (1967) and the time to reach each stage is presented in seconds (s). Different letters indicate significant differences amongst groups at the same stage ($p < .05$, Kruskal-Wallis test followed by Dunn's test).

A total of 91.7% (110/120) of the fish showed VOR as the first behavioural response of consciousness recovery after anaesthesia. Other first behavioural responses of consciousness recovery were handling (6.7%) (8/120), response to a prick on the lip (0.8%) (1/120) and swimming (0.8%) (1/120). The times for consciousness recovery (s) were similar for all groups: 142.8 ± 8.2 s for PE, 126.9 ± 8.0 s for OA and 134.3 ± 6.7 s for LA fish ($p > .05$) (Fig. 2.1).

2.2.3 Blood analyses

The values for glucose (mmol/L) were similar between the groups at each time ($p > .05$) (Fig. 2.2). The lactate levels (mmol/L) were higher at 0 h for LA (2.0 ± 0.2) than for ET (1.4 ± 0.8) ($p < .05$) without differences in OA (1.2 ± 0.4) (Fig. 2.3), but after 1 h, 4 h, 8 h and 24 h the values for lactate were similar for all the groups ($p > .05$). At 0 h the cortisol levels (ng/mL) were higher in ET (150.3 ± 55.3) than in OA (33.9 ± 9.7) and were 44.5 ± 6.1 in LA fish and 75.2 ± 19.9 in PE fish without differences between LA and OA. The values found at the other sampling times levels were similar for all groups (Fig. 2.4).

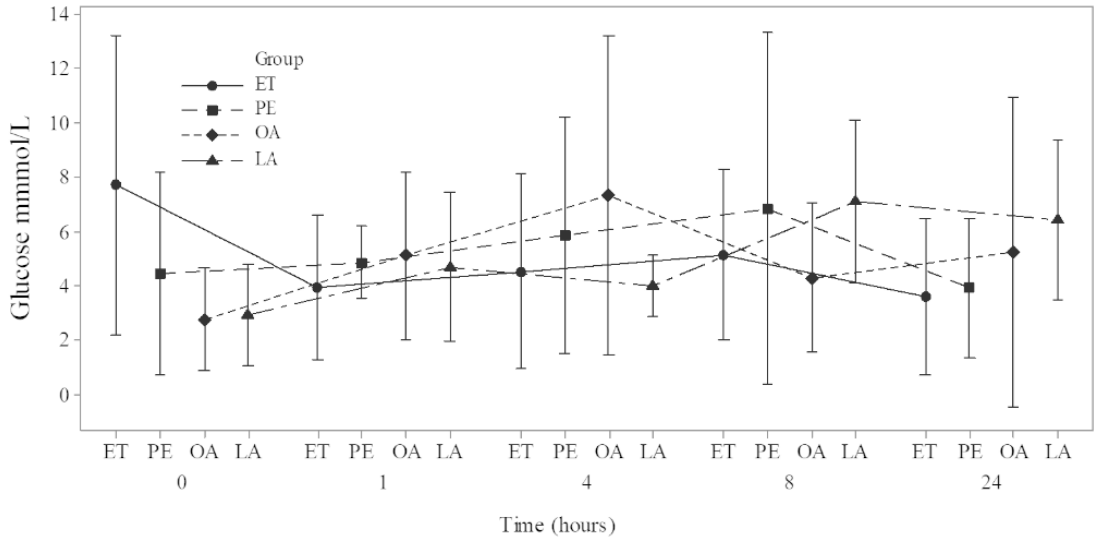

Figure 2.2 - The values for lactate (mmol/L) (mean ± SEM) after anaesthesia of Nile tilapia using ethanol (ET), 2-phenoxyethanol (PE), *Ocimum americanum* (OA) and *Lippia alba* (LA), 40 fish per group, 8 fish per time, .

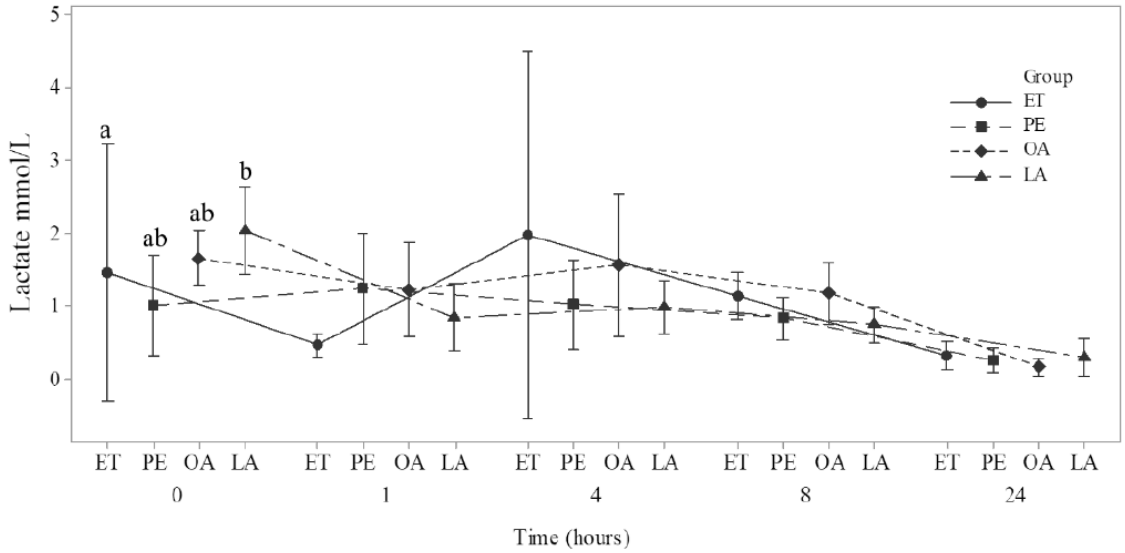

Figure 2.3 - The values for lactate (mmol/L) (mean ± SEM) after anaesthesia of Nile tilapia using ethanol (ET), 2-phenoxyethanol (PE), *Ocimum americanum* (OA) or *Lippia alba* (LA), 40 fish per group, 8 fish per time, different letters indicate significant differences amongst groups at the same sampling time ($p < .05$, Kruskal-Wallis test followed by Dunn's test).

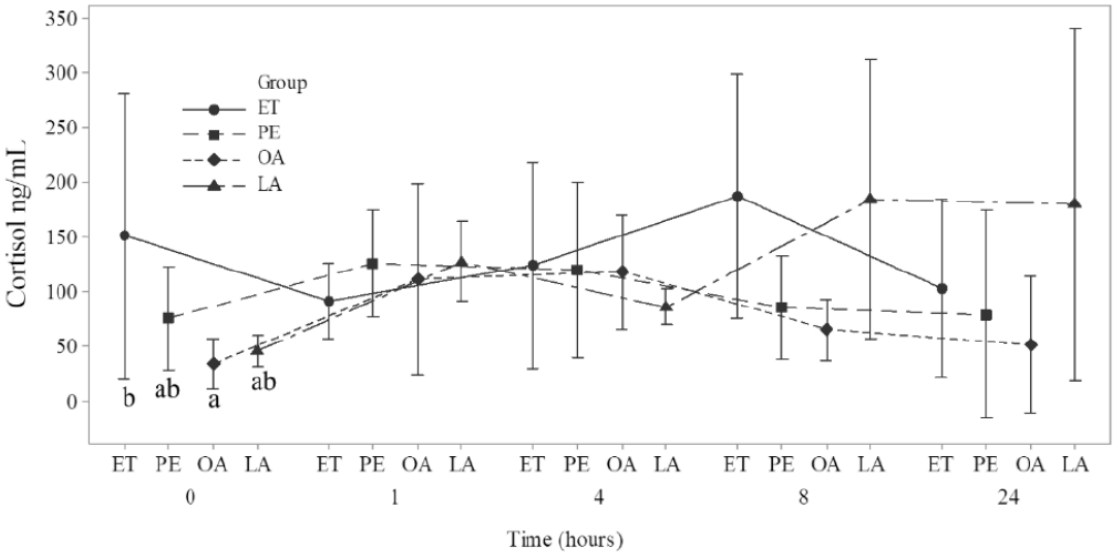

Figure 2.4 - The values for cortisol (ng/mL) (mean ± SEM) after anaesthesia of Nile tilapia using ethanol (ET), 2-phenoxyethanol (PE), *Ocimum americanum* (OA) or *Lippia alba* (LA), 40 fish per group, 8 fish per time, different letters indicate significant differences amongst groups at the same sampling time ($p < .05$, Kruskal-Wallis test followed by Dunn's test).

2.2.4 Sensory analysis

The fillets prepared from Nile tilapia anesthetized with *Ocimum americanum* received higher sensory scores (mean ± sem) for aroma (1.5 ± 0.1) than fillets from those anesthetized with *Lippia alba* (1.0 ± 0.1) and from the control group (0.7 ± 0.1) ($p < .01$) (Fig. 2.5A), but no significant differences were observed for the flavour scores ($p > .05$) (Fig. 2.5B).

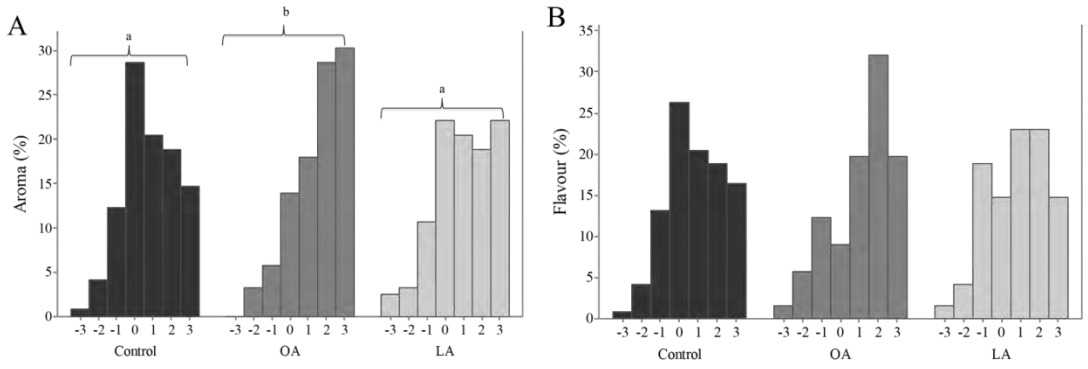

Figure 2.5 - Scores (%) awarded by one hundred and twenty two assessors for aroma (A) and flavour (B) of Nile tilapia fillets considering the following groups Control (hypothermia at pre-slaughter handling) and the *Ocimum americanum* OA or *Lippia alba* LA essential oils (anaesthesia before pre-slaughter handling). Different letters indicate significant differences amongst the groups ($p < .05$, Kruskal-Wallis test followed by Dunn's test).

2.3 Discussion

The essential oils *Ocimum americanum* or *Lippia alba* were effective for the induction of deep anaesthesia in Nile tilapia in less than 169 s. The mean time for the

recovery of consciousness was 127 s. Essential oils are highly aromatic natural chemicals, however in the sensory evaluation, fillets from fish anesthetized with LA received similar aroma and flavour scores as fillets from fish killed using the industrial slaughter method. Fillets from fish anesthetized with OA received higher aroma scores than those from the industrial slaughter method.

The mean time for the induction of anaesthesia was less than 3 min as reported in the literature (Ross and Ross, 2008), and the mean time for the recovery of consciousness provides sufficient time for the bleeding of the unconscious fish. Unconsciousness times greater than 127 s (mean) meet the requirements for humane slaughter. However, such a time does not conform with the 247 s of recovery reported by Hohlenwerger et al. (2016) for juveniles (weighing 4.25 ± 1.34 g and measuring 5.70 ± 1.00 cm) of Nile tilapia anesthetized with *L. alba* essential oil at 500 μL/L, probably due to different behaviours considered for consciousness recovery. Other authors only recognize consciousness recovery when the fish return to normal swimming and equilibrium, explored the aquarium and reacted to external stimuli (Toni et al., 2015). In the present study, consciousness recovery was considered when the fish partially returned to normal swimming and equilibrium, response to handling, response to prick on lip and VOR. During slaughter, the observation of any indicator suggested consciousness recovery and sensitivity to painful stimuli. Anaesthesia at slaughter represents a reduction in stress and pain in comparison with hypothermia, which is the current method used for the pre-slaughter handling of fish in Brazil. Oliveira Filho et al. (2014) reported a median time of 20 min for stunning Nile tilapia using hypothermia in ice and water.

The lack of anaesthetic induction in the ethanol group confirms that the effect of essential oils is due to their natural composition, in agreement with the literature data (Heldwein et al., 2014). The chemical compositions of the essentials oils are similar to those reported in other studies, where the main component of *L. alba* is linalool, 37.7% according to Cunha et al. (2010), 47.6% according to Hohlenwerger et al. (2016), 50.6% according to Souza et al. (2017), 59.6% according to Heldwein et al. (2014) and 87.6% according to Toni et al. (2015). Linalool (20.18% from the leaf; 46.6% from the inflorescence) and 1.8-cineole (21% from the leaf; 8.4% from the inflorescence) are the main components found in the *Ocimum americanum* EO (Silva et al., 2015a; Silva et al., 2015b).

Soil type, rainfall intensity, temperature, genetics, chemical components and extraction methods affect the composition of essential oils (Souza et al., 2019). Three

chemical components with anaesthetic effects in fish were identified in *L. alba*: lilanalool, citral (Souza et al., 2018) and carvone (Ventura et al., 2019). Although synthetic linalool compounds are available, the use of natural essential oils may be better accepted by the final consumer. The use of essential oils as anaesthetics for pre-slaughter should consider oils that have a standardized composition, to avoid changes in both the induction and recovery times.

Most anaesthetics exert their effects by regulating the gamma-aminobutyric acid (GABA) receptor complex, GABA type A being the main inhibitory neurotransmitter in the central nervous system. Most of the physiological actions of GABA are generated via GABAA receptors (DeFeudis, 1977). These receptors are chloride ion channels that can be opened by GABA and modulated by a variety of pharmacologically and clinically important drugs such as benzodiazepines, barbiturates, steroids, anaesthetics, and convulsants (Sieghart, 2006). Heldwein et al. (2014) showed the involvement of the GABAergic system in the anaesthetic effect of *Lippia alba* essential oil on the silver catfish *R. quelen.*

Regarding the glucose levels, no significant differences were observed between the groups at the same time of sampling. In contrast, Nile tilapia anesthetized with *Lippia alba* EO at 500 μL/L had significantly higher plasma glucose levels than the control group both 1 and 4 h after taking the biometric measurements (Hohlenwerger et al., 2016). The absence of differences in the present study may be due to data variability, which presents high coefficients of variation, higher than 60.6% for all groups. Souza et al. (2017) reported ideal time for blood sampling less than 30 s, hence time for sampling can be another important factor when observing both high values and variation. In addition, a new environment and aggressiveness between couples may cause such variability in the blood glucose levels. Furthermore, maintenance in a recirculation system and recapture after 1, 4, 8 and 24 h to take the blood samples were limiting factors with respect to observing significant differences between groups for the values of glucose, lactate and cortisol.

With respect to the findings concerning the lactate levels, Deriggi et al. (2006) also reported an increase in plasmatic lactate for Nile tilapia anesthetized with eugenol. Concentrations below 0.5 μmol/mL were observed in fish submitted to no handling in contrast to fish exposed to handling (1.2 μmol/mL), eugenol at 20 mg/L (0.9 μmol/mL) and eugenol at 80 mg/L (1.5 μmol/mL) (Deriggi et al., 2006). The process of fasting, crowding, air exposition, transport and holding in a new environment promoted the

activation of the hypothalamic-sympathetic-chromaffin axis, leading to the release of catecholamines (Bonga, 1997). Catecholamines reduce liver glycogen and increase plasma glucose, cardiac output and gill blood flow. Furthermore, such stressors also activate the hypothalamic-pituitary-inter-renal (HPI) axis that releases cortisol, thus leading to an increase in liver glycogen, muscle metabolism, free fatty acids, and a decrease in immune responses, growth and reproductive capacity (Bonga, 1997). The results suggest that the use of EOs does not reduce the glucose and lactate levels in Nile tilapia, due to the fact that the handling before anaesthesia was similar to that of pre-slaughter handling.

The mean values for cortisol in fish anesthetized with EOs were similar to those reported for Nile tilapia submitted to chronic stress (Barcellos et al., 1999). A handling procedure simulating the pre-slaughter conditions probably had a negative impact on the metabolic stress responses in Nile tilapia. The *Ocimum americanum* and *Lippia alba* EOs seemed to acutely decrease the activation of the HPI axis (< 60 min) in Nile tilapia, since the plasma cortisol levels were lower in fish anesthetized with such EOs as compared to ethanol. The values at 0 h of sampling suggest a reduction in the activation of the HPI axis in Nile tilapia anesthetized with the EOs. Such a reduction is of short duration, due to the absence of differences in the cortisol values between groups 0 h after sampling. The lack of a clear stress response for cortisol in fish has been previously reported. The common carp (*Cyprinus carpio L.*) stunned by percussion, showed the lowest average glucose concentration in the blood plasma (87 ng/mL; 16–665) (median: Q1-Q3) as compared to fish stunned by electricity (800; 14–800) and asphyxia (800; 800–800) (Daskalova et al., 2016). Daskalova et al. (2016) reported serious difficulties in statistical processing and the inability to calculate averages due to the cortisol values, and the authors attributed such differences to individual neuroendocrine and behavioural differences. In several cases a high biological variability in cortisol values was reported amongst individuals submitted to the same experimental conditions, suggesting that cortisol measurements should be complemented by other metabolic indicators to validate its results (Magalhães et al., 2018).

The assessors attributed higher aroma scores to fillets from fish anesthetized with *Ocimum americanum* EO. The results of the sensory analysis suggest that the use of *Ocimum americanum* EO as a pre-slaughter anaesthetic for Nile tilapia can improve the fillet aroma without changing its flavour, in comparison with fillets from fish submitted to hypothermia. Furthermore, fillets from fish anesthetized with *Lippia alba* EO showed

no significant differences in the aroma or flavour of the fillets in comparison with the control group. Hohlenwerger et al. (2016) found that the participants detected no differences in a blind test for fillet aroma and flavour after anaesthesia of the Nile tilapia with *Lippia alba* EO at the same concentration used in the present study, in comparison with fish exposed to ethanol and killed by decapitation. In addition, Cunha et al. (2010) found no changes in aroma and flavour after the use of *Lippia alba* EO at 300 mg/L in the South American catfish *Rhamdia quelen*. The knowledge obtained indicates that the use of *Ocimum americanum* essential oil at 500 μL/L for anaesthesia in Nile tilapia, reported here for the first time, can improve the aroma of the fillet with no negative alterations that could compromise acceptability of the fillets.

Therefore, the use of *Ocimum americanum* and *Lippia alba* essential oils as pre-slaughter anaesthesia causes no decrease in the sensory quality of the Nile tilapia fillets and may thus be used as an alternative to the use of hypothermia for the pre-slaughter handling of fish in Brazil. This and other studies from Brazilian research groups (Veit et al., 2018; Veit et al., 2016) could support changes in the regulations for the humane slaughter of fish in Brazil.

2.4 Conclusion

The essentials oils of *Ocimum americanum* or *Lippia alba* at 500 μL/L were effective in causing deep anaesthesia in Nile tilapia during a median time of 127 s. The use of *Ocimum americanum* essential oil for pre-slaughter handling improves the aroma of Nile tilapia fillets. Anaesthesia with *Ocimum americanum* or *Lippia alba* essentials oils may be an alternative to hypothermia for the pre-slaughter handling of fish in Brazil.

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3 Mechanical spiking and electrical stunning as a method for Nile tilapia (*Oreochromis niloticus*) at pre-slaughter

Abstract

Nile tilapia is the fish species most relevant regarding fish farming in Brazil, with a total of 758,006 t in 2019. Slaughter without stunning and the great number of individuals involved represent critical points for fish welfare in Brazil. This study aimed to assess mechanical spiking and electrical stunning as methods to induce unconsciousness in Nile tilapia, and their effects on *rigor mortis*. Nile tilapia (574.0 ± 170.8 g; 30.9 ± 3.1 cm) were used, divided into three groups, two groups for spiking and one group for electrical stunning. Fish in the Lateral group (L) (n=20) were placed on their left lateral side, and hence the captive bolt for spiking accessed the right side of the head. Fish in the Frontal group (F) (n=20) were placed in a V-shaped wooden structure such that the captive bolt accessed the front of the head. Electrical stunning (E) (n=13) was applied in a plastic tank containing fresh water, where two stainless-steel plate electrodes (area 5.25 dm²) were placed at a distance of 14 cm on the left and right side of fish. The electrical parameters applied were: alternating waveform current, 173 volts, 60 Hz and 9.7 A, 0.07-0.13 S/m water conductivity during 1 s of exposition, observing the behaviour indicators to determine loss of consciousness. Ten fish from each spiking group (L and F) were refrigerated to measure the *rigor mortis* index (RMI) at 0, 3, 6, 24, 48, 72 and 120 h after bleeding. All the L fish lost consciousness immediately after the shot, but only 95% of the F fish (p=1.0). One L fish (5%) showed signs of recovery, whereas five F fish (25%) showed signs of recovery (p=0.182), and the median time for recovery was 240 s for L (1/20) and 52 s for F (5/20). Fish exposed to electrical stunning showed no signs of loss of consciousness. The progress of the RMI was delayed in the L fish by 41.2 ± 34.8 % 6 h post-slaughter, in contrast to 59.1 ± 50.0% in the F fish after the same period of time (p=0.0445). Mechanical spiking with lateral access was effective to inducing unconsciousness without recovery, delaying the onset of *rigor mortis* in Nile tilapia, and hence its use in an automatic system could be an alternative to traditional slaughter by hypothermia in Brazil.

Keywords: Fish welfare, behaviour indicators, electrical stunning, *rigor mortis.*

3.1 Introduction

Hypothermia in ice and water is traditionally used pre-slaughter in most fish slaughterhouses in Brazil. This method is recognized as highly stressful before loss of consciousness in several fish species, and is neither considered humanitarian nor recommended by the OIE (OIE, 2015). Nile tilapia is the fish species most produced in Brazil, totalling 758,006 t in 2019, increasing 4.9% in relation to 2018 (PEIXE-BR, 2020), representing hundreds of millions of individuals. Slaughter without stunning and the great number of individuals involved represent critical points for fish welfare in Brazil.

Humane slaughter requires the induction of unconsciousness prior to bleeding, so stunning before bleeding must induce a state of general unconsciousness (TERLOUW; BOURGUET; DEISS, 2016). Percussive stunning is the application of a blow to the head manually or using a device. A cartridge with gunpowder, compressed air or a spring under tension are the methods used to drive bolts against or through the skull of farm animals, and percussion is used to induce unconsciousness in salmon, trout and carp (LAMBOOIJ et al., 2002). Spiking or coring are irreversible fish stunning and killing methods based on physical damage to the brain by inserting a spike or core into the brain and the unconsciousness following percussion or spiking is generally irreversible if correctly applied. It has been reported that percussed Atlantic salmon die of cerebral haemorrhage (LAMBOOIJ et al., 2010). In cases where the loss of consciousness is transient, the fish should be killed before they recover consciousness. Percussion cannot be applied to all fish species, for example, percussion is not effective for European eels (VELARDE; RAJ, 2016).

Electrical stunning is an alternative for the pre-slaughter stunning of farmed animals. To apply it correctly, a device must be built, where the parameters such as voltage, current, frequency and waveform can be changed according to the species. Electrical stunning can induce the immediate loss of consciousness and sensibility in fish (VAN DE VIS et al., 2003) if appropriately applied but data has been reported showing that fish cannot be killed by the use of electricity, since the fibrillation of the heart is not permanent, and hence electrical stunning should be followed by a killing method to avoid recovery of the stunned fish (LAMBOOIJ et al., 2008; VAN DE VIS et al., 2014). The fish should be exposed to the set of electrical parameters for a maximum of 1 s, assessing the behavioural indicators. When the current is strong enough, it induces unconsciousness after just 1 s of exposure (LAMBOOIJ et al., 2008; ROTH et al., 2003). After assessment of the behavioural indicators, the electrical parameters should be validated by

electroencephalography to confirm unconsciousness (EFSA (EUROPEAN FOOD SAFETY AUTHORITY), 2018).

This study aimed to assess mechanical spiking and electrical stunning as methods to induce unconsciousness in Nile tilapia, and their effects on *rigor mortis.*

3.2 Material and methods

All procedures were approved by the Ethics Committee on Animal Use of the School of Animal Science and Food Engineering of the University of São Paulo (protocol 4446150817).

A total of fifty-three Nile tilapia (*Oreochromis niloticus*) (mean ± SD) (574.0 ± 170.8 g; 30.9 ± 3.1 cm) was used. Fish used for spiking were divided into two groups. The Lateral group (L) (n=20) were placed on their left lateral side such that the captive bolt accessed the right hand side of the head. Fish in the Frontal group (F) (n=20) were placed in a V-shaped wooden structure such that the captive bolt accessed the front of the head (Figure 3.1). Three previously euthanized fish were dissected in order to practice both the method and determine the exact point of the shot. Mechanical spiking was applied using a commercial gun for fish – Ikigun® (New Zealand). The number of attempts for efficient penetration, immediate loss of consciousness, number of fish showing recovery indicators and the recovery time were assessed. The loss of consciousness was determined according to behavioural assessments in a tank containing 200 L of freshwater (KESTIN; VAN DE VIS; ROBB, 2002). Fish showing any indicator of the recovery of consciousness were placed in a tank with water and 200 mg/ L of benzocaine before bleeding. The monitoring of the recovery of consciousness was carried out during 20 minutes after the shoot (table 3.1).

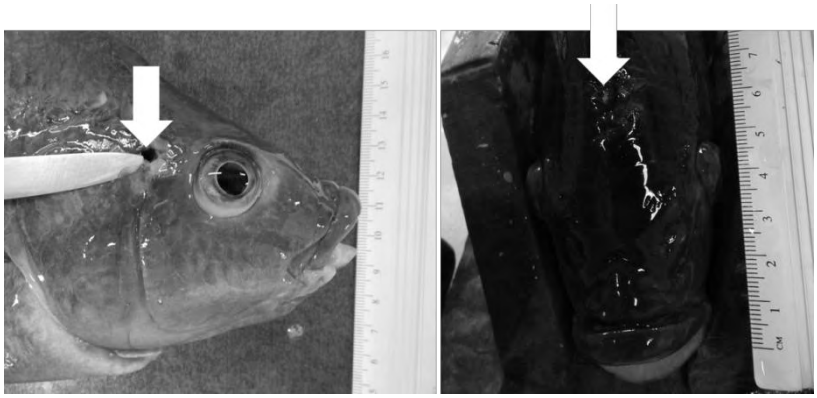

Figure 3.1 - The access points used for Lateral (A) and Frontal (B) access for mechanical spiking in Nile tilapia, where the white arrows represent the exact points of captive bolt access.

Table 3.1 – Protocol to determine the recovery of consciousness through behavioural evaluations after electrical stunning or mechanical spiking in Nile tilapia, modified from Kestin et al., 2000.

	Self-initiated behaviour		Response to stimuli		Clinical reflexes	
	Swimming	Equilibrium	Handling	Prick	Eye roll	Breathing
Behaviour / reflex	Swimming behaviour	Righting ability	Response to handling	Response to prick on lip	Vestibule-ocular reflex VOR	Rhythmic opercular activity
Procedure	Observe spontaneous swimming behaviour	Invert fish, observe righting response	Attempt to catch by tail and administer tail pinch, observe response	Prick lightly on lip with enough pressure to cause pricking sensation to human, observe response	Observe eye movement when fish is rolled from side to side through the perpendicular	Observe opercula for rhythmic movement
Recovery of consciousness after shot or shift off the current	Slow or abnormal swimming, eg, upside down or Normal swimming	Slow to right or quickly rights	Only slow or feeble response after tail pinch(s) or Immediate vigorous escape attempt on first touch/pinch	Slow and reduced response or head shake or escape attempt	Partial VOR or one eye shows VOR or eyes roll relative to the head while attempting to remain upright when fish is rolled	Slow or irregular movement or regular opercula movement

Electrical stunning was applied to ten fish (n=13) to assess the effectiveness in inducing unconsciousness. Electrical stunning was applied in a plastic tank (length x width x height): 68 cm x 50 cm x 39 cm containing fresh water, where two stainless-steel plate electrodes (length x width, 30 x 17.5 cm) (area 5.25 dm²) were placed on the left and right side of fish with a separation of 14 cm. Two pieces of expanded polystyrene were used to fix the electrodes. Fish were placed individually in the stunning tank with their bodies parallel to the electrode plates, so that the current would flow across them (Figure 3.2), and small amounts of salt were added to the water to increase the salinity to 0.07-0.13 S/m (LAMBOOIJ et al., 2008; LINES; KESTIN, 2004) as low water conductivity can affect negative both the electrical field and the passing of current through the fish. The electrodes were connected to a power supply delivering a voltage of 0-250 V, with a sinusoidal alternating current and frequency of 60 Hz (frequency of the electrical network), the current delivered being calculated by a sensor fixed to the

equipment. The equipment was constructed by an electrical engineer in 2010 in Pirassununga, São Paulo, Brazil.

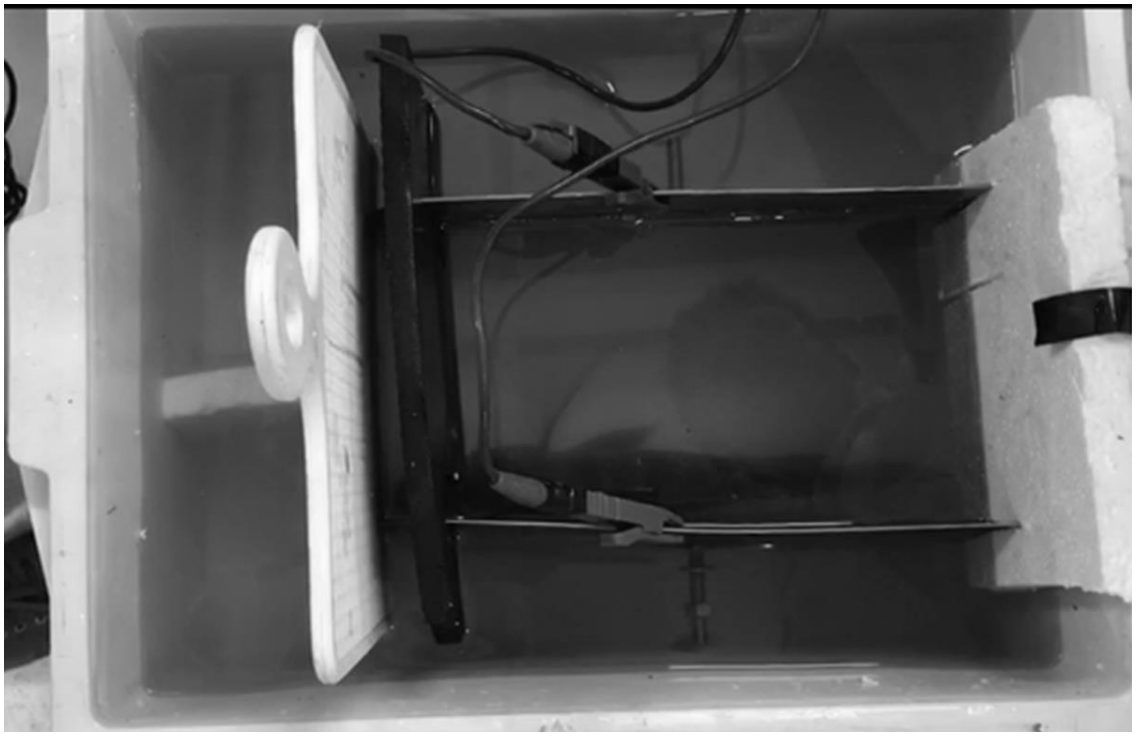

Figure 3.2 - Design of the tank and position of the electrodes for electrical stunning as applied to Nile tilapia (*Oreochromis niloticus*).

The electrical parameters applied were the alternating current waveform, 173 Volt (voltage), 60 Hz (frequency) and 9.7 A (amperes) for 1 s of exposition, observing the behaviour indicators to determine the loss of consciousness (KESTIN; VAN DE VIS; ROBB, 2002). Six fish were exposed to these parameters with a water conductivity of 0.07 S/m and seven with a water conductivity of 0.13 S/m. Due to lack of efficiency in the induction of unconsciousness in the E group (see Results), 13 fish were used and *rigor mortis* was not performed in such group. When the current is strong enough, it will induce unconsciousness after just 1 s of exposure (LAMBOOIJ et al., 2008; ROTH et al., 2003). The field strength and current densities were calculated using the following formulae (RETTER et al., 2018)

$$Field\ strength\ (\frac{V}{cm}) = \frac{\text{Voltage (Volt)}}{\text{distance between electrodes (cm)}} \quad (1)$$

$$Current\ density\ (A/dm^2) = \text{Conductivity of the water } (\frac{S}{m}) * \frac{\text{Voltage (Volt)}}{\text{distance between electrodes (dm)}} \quad (2)$$

Ten fish from each spiking group (L and F) were refrigerated in a cold chamber inside polystyrene boxes containing ice to measure the *rigor mortis* index (RMI) (BITO et al., 1983) at 0, 3, 6, 24, 48, 72, 120, 168, 240, 288, 336 and 384 h after bleeding.

The variables of the number of shot attempts and the RMI did not follow a pattern of normality, and hence the Mann-Whitney test was used at a significance level of $p<0.05$ to compare the treatments (L vs F). The Fisher's exact test was used to compare the proportions of fish showing specific behaviours amongst the treatments: loss of consciousness and recovery.

3.3 Results

Regarding spiking, all the L fish lost consciousness immediately after the shot, but only 95% of the F fish did so (P=1.0). All the fish that lost consciousness immediately after the shot stopped swimming, lost their equilibrium, did not respond to painful stimuli or handling, showed no VOR and ceased breathing. The number of attempts for an effective shot did not differ between the groups (median ± IQR) (1.0 ± 0 L; 1.0 ± 0 F) (p=0.5536). One L fish (5%) (420 g) showed indicators of recovery, but five F fish (25%) showed signs of recovery (p=0.182). The median weight of the F fish that recovered was higher (655 ± 182.5 g) than that of the F fish that did not recover (545.0 ± 185.0 g), but with no significant difference (p=0.0972). The median time for recovery was 240 s for L (1/20) and 52 s for F (5/20), but these times could not be statistically compared due to the absence of variance in L (Figure 3.3).

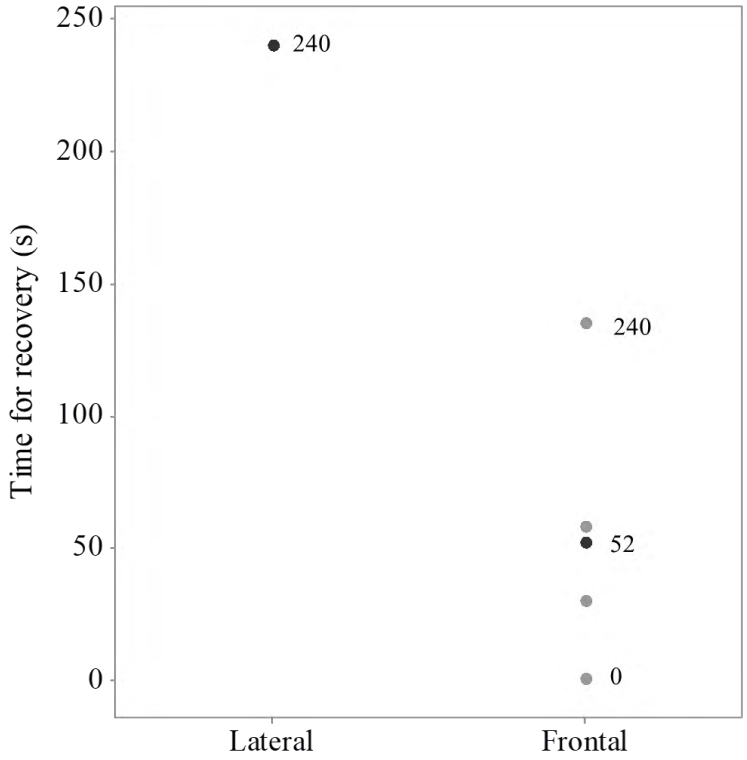

Figure 3.3 - Individual recovery times after spiking in Nile tilapia (*Oreochromis niloticus*) (n=20 fish/group). Black point indicates the median value.

The thirteen fish exposed to electrical stunning showed no behavioural indicators of a loss of consciousness. They did not lose their equilibrium, continued breathing and swam evidently. The field strength produced by the equipment was 12 (V/cm) and the current density was 0.87 (A/dm²) for water conductivity of 0.07 S/m and 1.6 (A/dm²) for water conductivity of 0.13 S/m. The field strength applied was the maximum capacity of the equipment, therefore electrical stunning was discarded for subsequent analyses of the *rigor mortis* index. Thus, side to side electrical stunning in fresh water using an alternating current of 173 volts, 60 Hz, 0.07-0.13 S/m water conductivity and 9.7 A for 1 s was not effective in inducing unconsciousness in Nile tilapia. Perhaps the use of other equipment with greater electrical current capacities, may allow the necessary pre-slaughter stunning in Nile tilapia

The progress of RMI was delayed in the L fish (median ± IQR) 41.2 ± 34.8 % at 6 h post-slaughter, in contrast with the F fish 59.1 ± 50.0% after the same period of time (p=0.0445) (Figure 3.4). Fish reached the full *rigor mortis* at 24 h after slaughter. The values at 0, 24, 48, 120 h post-slaughter can be not compared due to the lack of variance,

but previous studies carried out by our research team showed that all tilapia submitted to hypothermia reached full rigor mortis (100%) after 6 or 7 h.

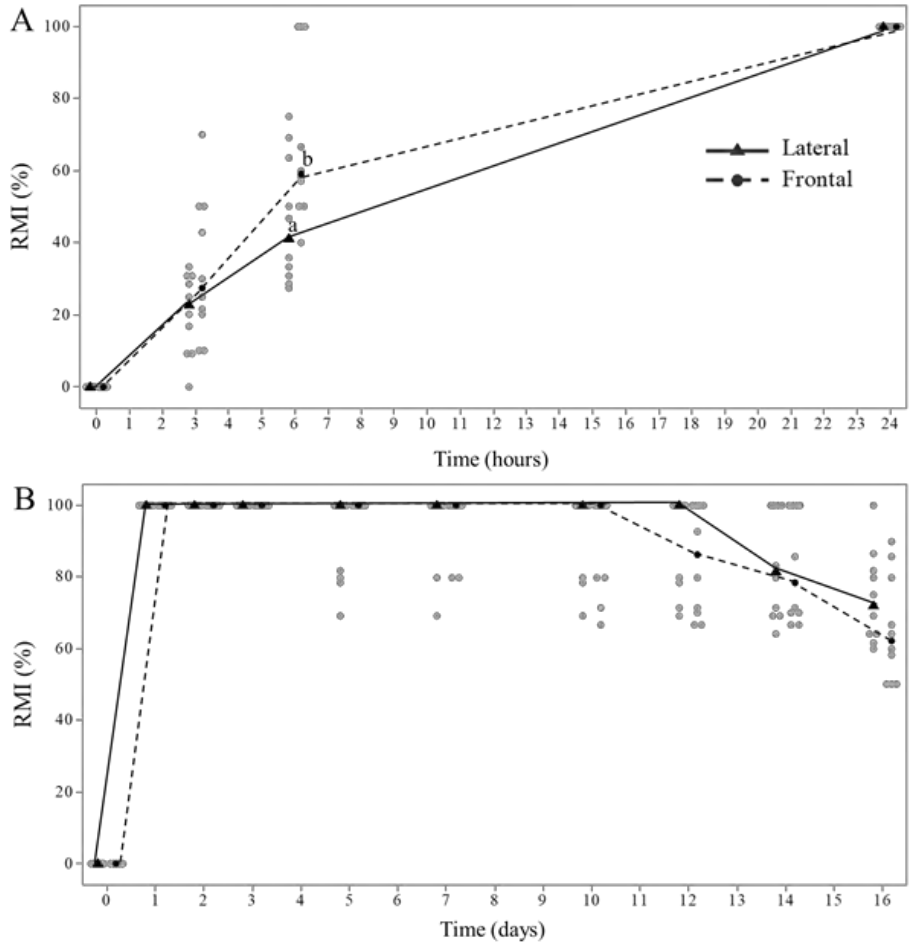

Figure 3.4 - Median values for the rigor mortis index RMI (%) in Nile tilapia (*Oreochromis niloticus*) (n=10 fish/group). (A) First 24 hours (B) Days. Letter indicates difference between groups at the same point in time ($p < 0.05$, Mann-Whitney test).

3.4 Discussion

The field strength (V/cm) of 12 and current density (A/dm²) of 0.87-1.6 used in this study were not strong enough to induce unconsciousness in Nile tilapia. LAMBOOIJ et al. (2008) reported a higher field strength of 13 (V/cm) and similar current density of 1.0 (A/dm²) with water conductivity of 0.07 S/m, 50 Hz in a sinusoidal waveform during 1 s for the effective induction of unconsciousness in Nile tilapia. Hence, technological and scientific development that allows for the construction of an equipment that produces more energy to form a higher field strength (>13 V/cm) could render the Nile tilapia unconscious and meet the criteria for humane slaughter. Having set the electrical

parameters according to the behavioural indicators after 1 s of exposure, the parameters must be validated by electroencephalography studies.

To the knowledge of the authors, this is the first time the use of spiking to stun/kill Nile tilapia has been reported, for this reason comparisons with previous papers using percussion was performed. Spiking is similar to the penetrating captive bolt stunning of farmed animals and is commonly used for tuna. For an effective application of the method, the spike should be rapidly inserted into the brain (EFSA, 2009). Regarding Nile tilapia, the process of spiking using lateral access was efficient for both the induction of unconsciousness and killing. Previous studies with salmon showed that, when correctly applied, percussive stunning not only renders the fish insensible, but also prevents any recovery (ROBB et al., 2000). Similar results were observed in the present study, since only one L fish (5%) showed indications of recovery, in contrast to 25% of the F fish. It is possible that the line that divides the operculum serves as an anatomical guide for a precise and accurate shot (Figure 3.1).

In addition, the only L fish that recovered took 240 s to recover after the shot. In Atlantic salmon (*Salmo salar*), percussion stunning using 7 to 8 bars produced the appearance of alpha, beta, theta, and delta waves on the EEG, and all the fish (n=8) except one, responded to noxious stimuli (LAMBOOIJ et al., 2010). Captive needle percussion was used to stun farmed eel (*Anguilla anguilla* L.) using a shooting pressure of 8 bar and an air injection of 3 bar for 1.5 s, and at least 93% of the eels were effectively stunned by a correctly positioned captive needle pistol (LAMBOOIJ et al., 2002). Spiking using a lateral access was efficient to induce immediate unconsciousness and to prevent the return of consciousness in 95% of the fish (1/20). New studies can measure the kinetic energy delivered by the commercial gun Ikigun®. Due to a lack of efficiency in Nile tilapia weighing more than 655 g, precaution is recommended for its use with heavier fish.

The application of spiking can be extended to other species such as pacu (*Piaractus mesopotamicus*) and tambaqui (*Colossoma macropomum*), due to their economic importance in Brazil. More research is necessary to develop an automated system for Nile tilapia and other species of interest in Brazil. The validation of spiking by the assessment of unconsciousness as determined by electroencephalography will allow for the development of new fish slaughter methods that meet fish welfare criteria.

The results of IRM suggested a delay in the onset of *rigor mortis*, since Nile tilapia submitted to hypothermia reach total *rigor mortis* (100%) 7 hours after slaughter (OLIVEIRA FILHO et al., 2014). We suggest studying *rigor mortis* in Nile tilapia

stunned with different methods at slaughter. Increased muscular activity, stress at slaughter and the endocrine response influence the post mortem biochemical processes of the fish, principally anaerobic glycolysis and the ATP degradation rate (HUSS, 1995). Such biochemical processes can influence the onset and release of *rigor mortis*, which, in turn, largely determines the spoilage rate of the fish (HUSS, 1995).

Atlantic salmon (*Salmo salar*) stunned by CO_2 showed an earlier onset and resolution of *rigor mortis*, in contrast with salmon stunned by percussion or electricity (ROTH et al., 2002). In addition, tambacu (*Colossoma macropomum* X *Piaractus brachypomus*) killed by bleeding, reached the maximum state of *rigor mortis* 2 h after slaughter (KODAIRA; TOMÉ; PÉREZ, 2001). Hence, humane methods at slaughter can reduce the energy expenditure and delay the onset of *rigor mortis*. Spiking in Nile tilapia seems to be an efficient method for stunning and killing.

3.5 Conclusion

Mechanical spiking with lateral access was effective in inducing unconsciousness without recovery, and its use in an automatic system could be an alternative to traditional slaughter by hypothermia in Brazil. Side to side electrical stunning in fresh water using an alternating current, 173 Volts, 60 Hz, 0.07-0.13 S/m water conductivity and 9.7 A for 1 s was not effective in inducing unconsciousness in Nile tilapia.

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4 Chapter 4 Electroencephalography (EEG) for the assessment of unconsciousness during the Nile tilapia stunning process

Abstract

Recently, new concerns about fish welfare are increasing in Western culture. A study with Norwegians showed they are concerned about fish welfare and a Latin American study showed that 76.0% and 72% of participants in Bogota, Colombia and Curitiba, Parana, Brazil, respectively, believed that fish should be included in humane slaughter regulations. In Brazil, the humane slaughter regulations do not include fish and hypothermia (live chilling in ice/water) is used in most fish slaughterhouses for pre-slaughter handling. The aim of this study was to assess unconsciousness during pre-slaughter using electroencephalography EEG in Nile tilapia under anaesthesia with *Ocimum americanum* or *Lippia alba* essential oils, hypothermia and spiking. Nile tilapia (484.0 ± 129.3 g, 29.0 ± 2.4 cm) (n=159) were divided in five different methods at pre-slaughter: anaesthesia with 2-phenoxyethanol at 1 mL/L (PE), anaesthesia with *Ocimum americanum* (OA) or *Lippia alba* (LAO) essential oils at 500 µL/L, lateral spiking (SP) and hypothermia (HP) in ice and water (2:1). Fish were restrained and lidocaine used in the place of insertion the EEG and electrocardiography ECG electrodes. The Labchart was used to carry out the visual and spectral analyses of the EEG. The EEG traces were divided into: normal (similar to that of the baseline), transitional (increase in amplitude and reduction in frequency or 1/3 reduction in amplitude as compared to the baseline), suppressed (1/2 reduction in amplitude as compared to the baseline) and isoelectric (minimal brain activity, characterized by < 0.5 µV of amplitude). For spectral analysis the values for total power (Ptot), median power frequency (F50), spectral edge frequency (F95), and the contribution of each frequency to the power spectrum: delta (0–4 Hz), theta (4–8 Hz), alpha (8–12 Hz) and beta (12–32 Hz) of each 2 s were calculated. The median time (s) to lose consciousness as represented by the suppressed phase was 136 ± 61 for PE, 171 ± 164 for OA, 171.5 ±77.8 for LA and 252 ± 752 for HP ($P<0.01$). Spectral analysis suggests unconsciousness after 3 minutes of exposure to PE, OA and LA due to the decreases in Ptot, F50 and F95, and also the increase in contribution from the delta frequency and decrease in contribution from the beta frequency. The values obtained in the spectral analysis for fish submitted to HP suggest that this method does not induce unconsciousness. The times to consider the fish unconsciousness were: 89.5 ± 27.2 s for OA; 138.0 ± 92.8 s for LA; 826.5 ± 319.3 s for HP; and 0 ± 0 s for SP. Only three of the

ten SP fish recovered 187.0 ± 243.0 s after shot, and only one of the ten soon after shot. When used in Nile tilapia, 2-phenoxyethanol-like anaesthetic showed both EEG traces and spectral analyses consistent with unconsciousness after 140 s of immersion. Anaesthesia with 500µL/L of the essential oils *Ocimum americanum* or *Lippia alba* induced unconsciousness after 180 s of immersion, as determined by EEG. The behavioural indicators suggest the recovery of consciousness 200 s after removal from the anaesthetic, which is sufficient time to bleed the fish while still unconscious, so these essential oils are an alternative for the humane slaughter of fish. The use of hypothermia for the pre-slaughter of Nile tilapia does not induce unconsciousness and should urgently be substituted by methods that meet criteria of humane slaughter.

Keywords: Anaesthesia, essential oils, fish welfare, hypothermia, slaughter, spectral analyses

4.1 Introduction

Recently, new concerns about fish welfare are increasing in Western culture. A study with 2,147 Norwegians showed they are concerned about fish welfare (ELLINGSEN et al., 2015) and a Latin American study showed that people recognized suffering in common practices with fish and that 76.0% and 72% of participants in Bogota, Colombia and Curitiba, Parana, Brazil, respectively, believed that fish should be included in humane slaughter regulations (RUCINQUE; SOUZA; MOLENTO, 2017). Public concern encourages the approval of new regulations that guarantee fish welfare in all steps of the production system, mainly at the moment of the slaughter. For example, Norway has specific regulations for the slaughter of fish destined for human consumption (NORWAY, 2009; RÖCKLINSBERG, 2015) and, in addition, the World Organization for Animal Health – OIE, by way of the aquatic animal health code, recommends practices that guarantee fish welfare during the transport and slaughter of farmed fish, for disease control purposes (OIE, 2017).

In Brazil, the humane slaughter regulations do not include fish (BRASIL, 2000) and hypothermia or immersion in iced water is used in most fish slaughterhouses for pre-slaughter handling (OLIVEIRA FILHO et al., 2014). Such a method is not considered humanitarian because it does not induce an immediate loss of consciousness and the fish show aversive behaviour when immersed in the mixture of ice and water (LAMBOOIJ et al., 2015; PEDRAZZANI et al., 2009; VAN DE VIS et al., 2003). To consider humane a

form of slaughter, the animal must undergo two processes: first, the animal must be rendered unconscious and, secondly, bled to induce death. Thus, stunning is applied to induce unconsciousness and insensitivity to pain, which should be of sufficient duration to ensure death by bleeding (LAMBOOIJ et al., 2008; OIE, 2015). Other methods, such as asphyxia, freezing, decapitation, medulla section, evisceration and bleeding are also used in Brazil, but do not meet the precepts of humane slaughter, since no method of effective stunning is used.

Studies on the use of natural anaesthetics prior to fish slaughter have increased in recent years, mainly the use of essential oils from the plants *Lippia alba*, *Ocimum americanum* and *Aloysia triphylla*, which do not show any negative effects on the aroma and flavour of the fish fillets (HOHLENWERGER et al., 2016; RUCINQUE et al., 2021; TEIXEIRA et al., 2016; VEIT et al., 2016, 2018) or cause aversion to the fish (BANDEIRA-JUNIOR et al., 2018), thus appearing as a humane alternative for fish slaughter.

To reduce the risks of pain and suffering during the slaughtering process, the new stunning methods must be validated by studies that evaluate unconsciousness by electroencephalography (EEG) according to recommendations of the European Food Safety Authority (EFSA, 2018). In a visual analysis of the EEG trace, one can identify patterns compatible with unconsciousness, such as, for example, a suppressed phase or isoelectric phase (MCKEEGAN et al., 2013; RAJ et al., 2008). The visual analysis is complemented by a spectral analysis, where, by way of the Fourier transformation, an EEG signal is dissected into its component spectra. In principle, such a method allows for the detection of underlying sinus waves that, on summing them up, result in the detection of a complex wave (TONNER; BEIN, 2006).

Furthermore, the spectral analysis of EEG traces is useful to identify different states of consciousness. Spectral variables can be calculated from the EEG such as the total power (Ptot), the median frequency (F50) and the spectral edge frequency (SEF or F95). Ptot is defined as the total area under the power spectrum curve; F50 represents the median frequency of the power spectrum curve and SEF represents the frequency where 95% of the power spectrum curve is located (TONNER; BEIN, 2006). F50 is more sensitive to changes in lower frequencies, whereas SEF is more sensitive to shifts towards higher frequencies (TONNER; BEIN, 2006). It is well recognized that increases in the EEG Ptot and associated decreases in both F50 and SEF, are correlated with clinical signs of the loss of consciousness and anaesthesia (MARTÍN-CANCHO et al., 2006;

MCKEEGAN et al., 2013; SANDERCOCK et al., 2014). The EEG frequencies can be divided into delta (0–4 Hz), theta (4–8 Hz), alpha (8–12 Hz) and beta (12–32 Hz) and increases in the delta and theta frequencies are related to the state of unconsciousness (GERRITZEN et al., 2006).

Thus the aim of this study was to assess unconsciousness during pre-slaughter using EEG in Nile tilapia under anaesthesia with *Ocimum americanum* or *Lippia alba* essential oils, hypothermia and mechanical spiking.

4.2 Material and methods

4.2.1 Animals

One hundred and seventy-six Nile tilapias of slaughter of weight 484.0 ± 129.3 g and length 29.0 ± 2.4 cm were acquired from a breeder close to Pirassununga town, Southwest Brazil, and transported to the Aquaculture Laboratory of FZEA/USP (State University of São Paulo) in Pirassununga. As part of the quarantine step, aimed at the fish resting after the stress of transportation, they were maintained in 4000L concrete tanks for two weeks before starting the experiment, maintaining the water parameters within the normal range for this species (pH = 7.41 ± 0.29, temperature = 23.1 ± 1.3 °C and dissolved oxygen of 5.92 ± 1.02). The fish were fed with commercial feed (28% protein, Laguna® Sport 28, São Paulo, Brazil) twice a day, and fasted for 48 h prior to the experiments. The procedures were approved by the Ethics Commission for the use of Animals (CEUA/FZEA) with the protocol nº of 4021010719 (Annex 5).

The water level in the concrete tanks was reduced on the day of the experiment to make it easier to capture the fish with a fish net. A maximum of ten fish were captured each time and transported to the slaughter room in up to 2 minutes, where they were placed in a maintenance tank (68 cm x 50 cm x 39 cm; 250 L) containing the same water present in the concrete tanks. Subsequently each fish was captured individually using a fish net and placed in a restriction tank (60 cm x 38 cm x 18 cm; 50 L) in up to 30 s, this tank also containing the same water present in the brick tanks. In the restriction tank, the fish was restricted by a net placed all round its body for the subsequent placement of the electroencephalogram (EEG) and electrocardiogram (ECG) electrodes.

4.2.2 EEG and ECG electrodes insertion

In order to determine the exact position of the brain and the location to insert the electrodes, two fish were slaughtered by euthanasia in 1 mL/L 2-phenoxyethanol, removed from the anaesthetic after 3 minutes, and bled for 3 minutes in iced water by

cutting the gills. After confirming death, a horizontal cut was made above the eyes of one fish to observe the cranium. The upper part of the cranium was exposed to determine the necessary position and length of the electrodes as recommended by Lambooij et al. (2003). Using the other fish, a cut was made along the line in the middle of the head to see the exact position of the brain, and the position of the heart was also determined. For the EEG, the correct position to place the two electrodes was determined as 2 cm caudal to the imaginary line at the end of the eyes and 0.7 mm to the left and 0.7 mm to the right of the sagittal axis of the head. The third electrode (earth) was placed 3 cm ventral to the dorsal fin. For the ECG, each electrode was placed on the midline of the left and right pectoral and lateral fins. The third electrode (earth) was placed in direct contact with the surface of the tail or inside the tank with water where the fish was immersed.

Prior to placing the electrodes, the fish were restricted inside the water in the restriction tank using a net fitted to the body with nylon clamps (Figure 4.1). The fish was then exposed to air by placing in a V-shaped wooden structure (Figure 4.1) and the eyes covered with a moist cloth. A 2 mg/kg (2%) dose of lidocaine diluted in 8.5% sodium bicarbonate (1:1) was then applied as a local anaesthetic to the insertion points of the 5 electrodes (3 for EEG and 2 for ECG) (McGILL, 2017), a procedure taking up to 60s. The fish was then placed back into the restriction tank for 90 s and then back into the V-shaped structure to fix the electrodes. The electrodes were in needle form, 30 mm long and 1.5 mm in diameter (55% silver, 21% copper and 24% zinc) and were placed percutaneously for both EEG and ECG. Surgical glue was used to fix the two electrodes to the head (LLONCH et al., 2012).

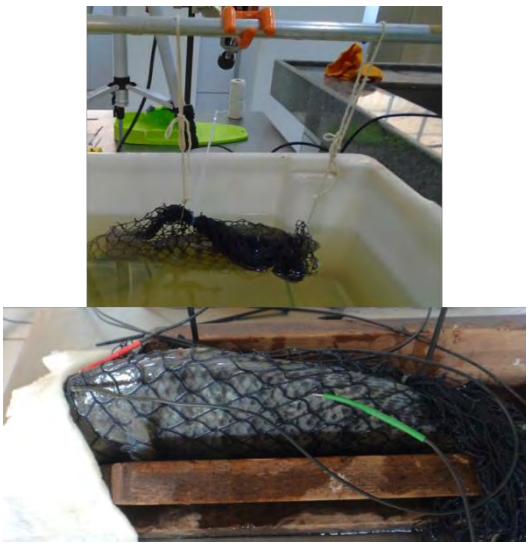

Figure 4.1 - (Upper part) - restriction in the water. (Lower part) –restriction in the V-shaped structure in order to place the EEG and ECG electrodes.

4.2.3 Description of the preparations used before applying the stunning methods

For the mechanical spiking, the data were collected for 90 s before the shot with the captive bolt pistol (SP) with the fish exposed to the air to record the baseline, continuing for 10 min after the shot. For anaesthetized fish or those submitted to hypothermia, the fish were put back into the restriction tank after fitting the electrodes in order to record the baseline during 90 s. After immersion in the anaesthetic, data were recorded for 10 min. In the case of hypothermia, the data were recorded for 20 min after immersion in the water/ ice mixture, due to the fact that median time to lose consciousness in fish submitted to hypothermia was 12 min, but some fish can react to stimuli even 18 min (PEDRAZZANI et al., 2009).

The EEG and ECG electrodes were connected to two Bio Amp (Model: FE231, ADInstruments Ltd, Australia) connected to a Power Lab 4/35 (Model: PL3504/P, ADInstruments). The EEG was amplified with the following configurations (Range: 500 uV, Low Pass Filter: 100 Hz, High Pass Filter: 0.3 Hz, Main Filter, Sample Rate: 1 K/s). The ECG was amplified with the following configurations (Range: 20 mV, low Pass Filter: 100 Hz, High Pass Filter: 0.3 Hz, Main Filter, Sample Rate: 1 K/s). The data were observed, saved and analyzed using the Labchart v 8.1.16 (ADInstruments). Since the fish were conscious while recording the baseline, a comparison was made with the baseline post-stunning. The fish were also filmed with an HD camera to observe and measure any behaviour that could interfere with the EEG and ECG signals.

4.2.4 Pre-slaughter stunning methods

The fish were randomly divided into 5 pre-slaughter stunning methods as follows: anaesthesia with 1 mL/L 2-phenoxyethanol (PE) (n=25), anaesthesia with 500 µL/L of the essential oil *Ocimum americanum* (OA) (n=26), anaesthesia with 500 µL/L of the essential oil *Lippia alba* (LA) (n=25), Hypothermia live chilling (HP) (n=15) in a mixture of water and ice (1:2) for 20 minutes and mechanical spiking with a captive bolt pistol (SP) (n=28). PE was chosen due to anaesthetics properties assessed through EEG in carp (LAMBOOIJ et al., 2009). The essential oils were purchased from Terra Flor (https://terra-flor.com), and the dose was tested in previously work (RUCINQUE et al., 2021). Prior to use, the oils were diluted in 98.8% ethanol (Exodo®, São Paulo, Brazil) in a 1:10 ratio. Anaesthesia was carried out in glass aquariums containing 15 L of water

and each anaesthetic was used for a maximum of 10 fish and the water and anaesthetic subsequently removed. After fixing the electrodes, the baseline was recorded in the restriction tank for 60 s before placing the fish in the glass aquarium. The beginning of immersion was the start of anaesthesia and recording continued for 10 min, when the state of consciousness of each fish was checked and stunned again with the gun if conscious, or bled from the cut in the gills if unconscious.

Mechanical spiking (SP) was carried out using a commercial gun (Ikigun®, New Zealand). After fixing the electrodes, the fish exposed to the air was placed on the percussion board where it was stunned and the data recorded for 10 minutes. After the shot, if any behaviour indicating consciousness was observed (VOR vestibule-ocular reflex, regular breathing, fin movements, response to painful stimulus), the fish was stunned again. After recording the data for 10 minutes, the state of consciousness of each fish was checked and stunned again if conscious, or bled from the cut in the gills if unconscious. After slaughter, the biometry of all fish was done.

4.2.5 Visual and spectral analyses

The Labchart was used to carry out the visual and spectral analyses of the EEG data traces and to calculate the heart rate (HR) from the ECG. For the visual analysis, the EEG data were filtered using a 5 to 45 Hz band pass filter, which eliminates interference from breathing and allows for comparison with the baseline. The EEG traces were divided into: normal (similar to that of the baseline), transitional (increase in amplitude and reduction in frequency or 1/3 reduction in amplitude as compared to the baseline), suppressed (1/2 reduction in amplitude as compared to the baseline) and isoelectric (minimal brain activity, characterized by < 0.5 µV of amplitude), similar to that described by MCKEEGAN et al. (2011, 2013). This subjective analysis was complemented by the spectral analysis. The swimming movements were recorded individually during the initial lag, including the number of movements, duration of the movements and end of the last movement.

For the spectral analysis, one fish from each group was selected and the EEG recordings were free of movements and interference from breathing or the ECG. The EEG recordings were filtered using a band pass filter (0.1 to 45 Hz), and the spectral analysis carried out using the following configurations (FFT size: 1K 1024; Data window: Hamming; Overlap: 50%, considering zero frequency) in 2 s epochs (MCKEEGAN et al., 2011, 2013). Using the Labchart, as from the power spectrum, one can calculate the

values for total power (Ptot), median power frequency (F50), spectral edge frequency (F95), and the contribution of each frequency to the power spectrum: delta (0–4 Hz), theta (4–8 Hz), alpha (8–12 Hz) and beta (12–32 Hz) of each 2 s (GERRITZEN et al., 2006; HERNANDEZ et al., 2019; SANDERCOCK et al., 2014). In all, 276 epochs were analyzed in FE, 276 OA, 272 LA, 563 HP and 267 SP. Seven criteria were considered for the spectral analysis in the minute by minute comparison of the recording with the baseline, in order to consider the fish unconscious:

-Ptot: increase or decrease

-F50: decrease

-F95: decrease

-Contribution of delta: increase

-Contribution of theta: increase

-Contribution of alpha: decrease

-Contribution of beta: decrease

Such criteria have been used in birds (MCKEEGAN; SANDERCOCK; GERRITZEN, 2013; SANDERCOCK et al., 2014), lambs (VELARDE et al., 2002), cattle (GIBSON et al., 2009) and fish (BOWMAN; HJELMSTEDT; GRÄNS, 2019; LAMBOOIJ et al., 2015). However, these studies did not evaluate all the criteria cited above as used in the present study.

The ECG was evaluated in relation to the heart rate per minute (HR). In the ECG channel, the 0.1 to 45 Hz band pass filter was used to eliminate noise, and the HR calculated by identifying the QRS peaks using the cyclic measurements tool of the Labchart.

4.2.6 Behavioural analyses

Nile tilapia were distributed at random for the behavioural tests using the essential oils OA (n=10), LA **(**n=10), immersion in water and ice for hypothermia HP (n=10) and the use of mechanical spiking SP (n=10).

In the case of anaesthesia with the oils, the fish were captured individually from the maintenance tank and placed in the anaesthesia aquarium. The time maintained in the

aquarium depended on the time the fish took to reach deep anaesthesia, with no response to a painful stimulus and the lack of a vestibule-ocular reflex (VOR) (Stage 4) (Table 4.1). The behaviour was verified every 60 s as from immersion in the tank.

Table 4.1 - Behavioural assessment at different stages of fish anaesthesia (adapted from Kestin et al., 2002; Schoettger and Julin, 1967).

Description	Stage	Behaviour
Sedation	1	Decreased reactivity to visual and vibrational stimuli; opercular and locomotor activity reduced slightly; darker in color
Partial loss of equilibrium	2	Loss of equilibrium in water current; increased opercular rate; swimming ability disrupted
Total loss of equilibrium	3a	Usually turns over; swimming ability persists; opercular rate rapid; reacts to vibrational stimuli
Total loss of equilibrium	3b	Locomotion ceases; fin movement may continue; tactile response only to pressure on caudal fin or peduncle; opercular rate slowed
Loss of reflex activity	4	Failure to respond to external stimuli, particularly pressure on caudal fin or peduncle; opercular rate slow and erratic and loss of vestibule-ocular reflex – VOR *
Medullary collapse	5	Opercular activity ceases

* vestibule-ocular reflex – VOR is an indicator of consciousness as described by KESTIN et al. (2002)

After reaching the deep anaesthesia stage, the fish was exposed to air for 60 s to simulate the process of exposure to air for bleeding. During this period the fish were measured, weighed and their response to painful stimuli recorded. After 60 s of air exposure, the fish from the OA and LA groups were placed in an aquarium free of anaesthetic to observe the recovery time of the different behaviours. Thus the times taken to lose swimming activity, equilibrium, response to handling, response to painful stimulus, VOR and respiration were recorded during anaesthesia. During recovery, the time taken for the fish to recover the first and second indicators was recorded, as also the time to recover VOR, respiration, equilibrium and swimming ability.

For the SP group, the pre-stunning handling time, considered as the time of exposure to air after capture up to shot, was measured using a stopwatch. After the shot the fish were put back into the water to observe their behaviour. Each fish was filmed individually in order to measure each recovery indicator. The post-shot observation was for 10 minutes or up to the moment when at least one recovery indicator was observed (KESTIN; VAN DE VIS; ROBB, 2002). On observing any recovery indicator, the fish

was stunned with the captive bolt gun for euthanasia. For the HP group the fish were placed individually in a tank with a water/ice (1:2) mixture at 2 ºC for 20 minutes, then exposed to air, the response to the painful stimulus recorded and immediately euthanized with the gun.

4.2.7 Statistical analyses

The Shapiro-Wilk normality test was applied to each variable. The variables of first movement lag, number of movements per fish, duration of the movements (s), end of last movement (s), lag for suppressed, lag for isoelectric, Ptot, F50, F95, delta (0-4 Hz), theta (4-8 Hz), alpha (8-12 Hz) and beta (12–32 Hz) did not follow a normal distribution. The times to lose equilibrium, swimming ability, response to a painful stimulus, VOR, breathing, anaesthesia (stage 4) and recovery of the first and second indicators also failed to follow a normal distribution. Only the times to recover from anaesthesia followed a normal distribution. The Kruskal-Wallis test followed by Dunn's test were used for comparisons amongst the groups when significant differences were found, and the significance level was set at $p < 0.05$. The Mann-Whitney test was used to compare the values obtained in the spectral analysis at each time versus the baseline value and the recovery from anaesthesia times. The data were shown as the median ± interquartile range (IQR).

4.3 Results

4.3.1 EEG visual analysis and movements

In all, the records of 98 fish were evaluated, as follows: PE (n=21), OA (n=23), LA (n=23), HP (n=13) and SP (n=18). Twenty-one fish were excluded from the analyses as follows: PE=4 no video; OA=3 no video; LA=2 no video; HP=2 problem with the electrodes; SP=10 recovery and euthanasia.

Four different patterns were identified on the EEG for the fish in the anaesthesia and hypothermia groups: normal, transitional, suppressed and isoelectric. Normal EEG represents activity with amplitude similar to that of the baseline or an increase as compared to the baseline after immersion or shot. Transitional represents a 1/3 reduction in amplitude as compared to the baseline or an increase in amplitude as compared to the baseline. Suppressed represents a >50% decrease in amplitude. Isoelectric: minimal brain activity with <0.5 µV using a 5 to 45 Hz band pass filter. Figure 4.2 shows the representative traces of each phase in a fish submitted to anaesthesia with phenoxyethanol (PE3).

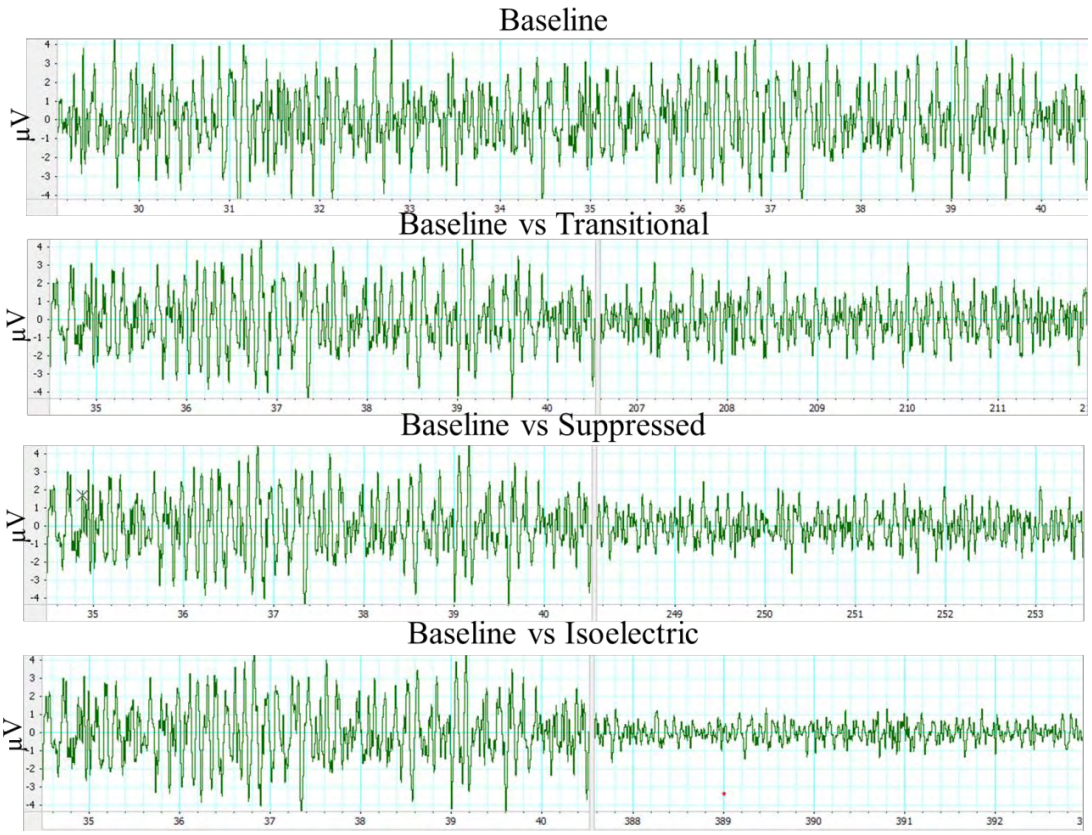

Figure 4.2 - A representative series of Electroencephalogram (EEG) traces illustrating the appearance of EEG in each phase identified (Baseline, Transitional, Suppressed and Isoelectric) in one fish (OA26). Neither movements nor artefacts were considered. The split view was used to compare each phase with the baseline.

Figures 4.3, 4.4, 4.5, 4.6 and 4.7 show the different events recorded for the first 5 minutes of evaluation for the PE, OA, LA, HP and SP fish, respectively. The transitional, suppressed and isoelectric phases were observed in the fish submitted to anaesthesia (PE, OA and LA). Only the fish submitted to HP presented the suppressed phase during the first 5 minutes of evaluation (Figure 4.5). The insult epileptiform phase, which is characterized by increases in the frequency and amplitude was identified in fish submitted to mechanical spiking, as shown in Figures 4.7 and 4.8.

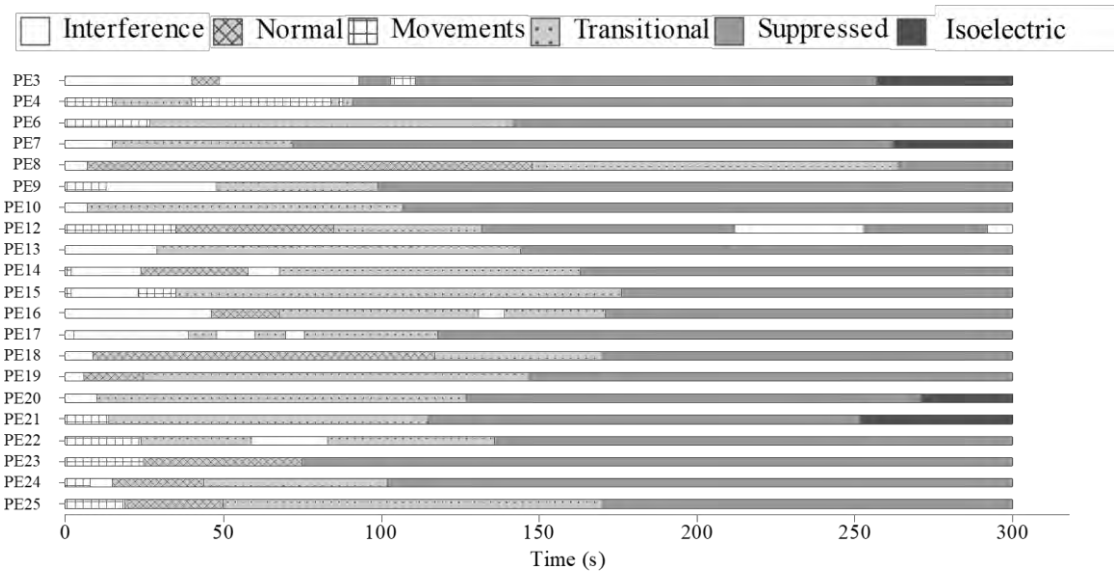

Figure 4.3 - Characteristics of the EEG trace in PE Nile tilapia (n=21) anesthetized with 1 mL/L 2-phenoxyethanol during the 300 s after immersion.

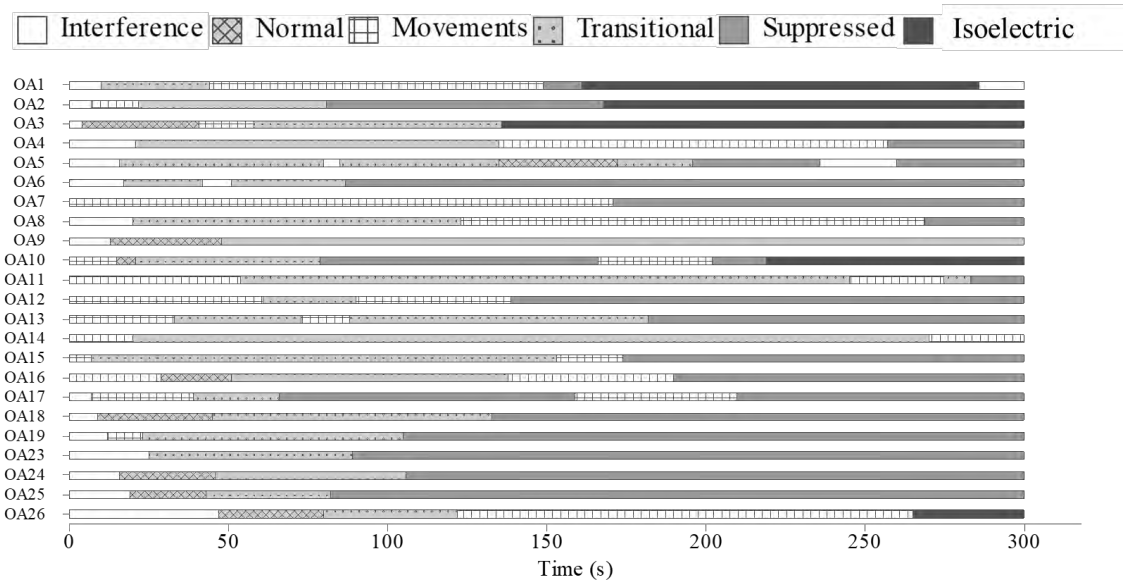

Figure 4.4 - Characteristics of the EEG trace in OA Nile tilapia (n=23) anesthetized with 500 µL/L *Ocimum americanum* essential oil during the 300 s after immersion.

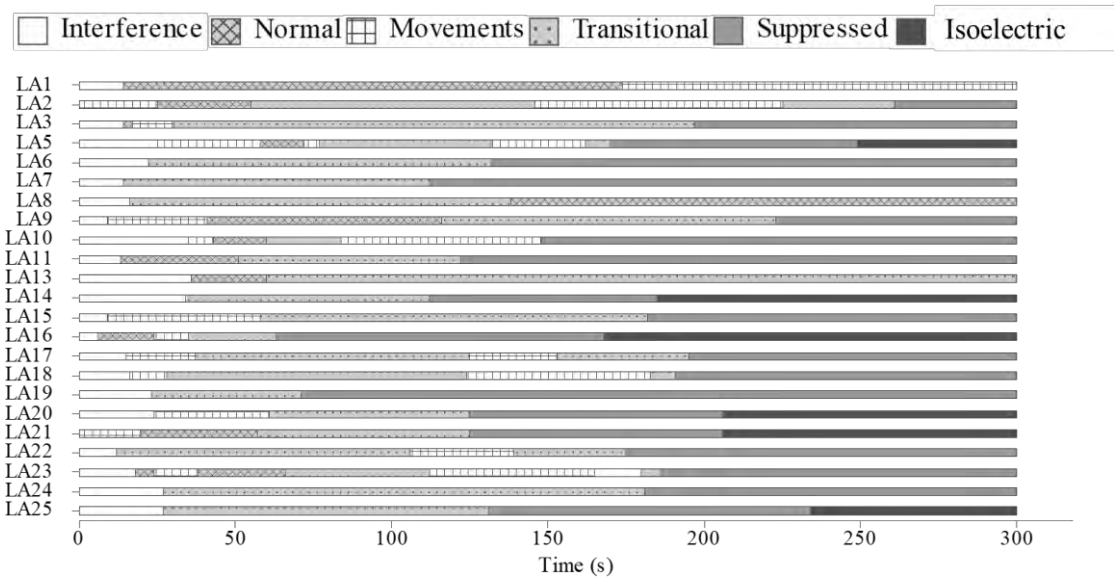

Figure 4.5 - Characteristics of the EEG trace in LA Nile tilapia (n=23) anesthetized with 500 µL/L *Lippia alba* essential oil during the 300 s after immersion.

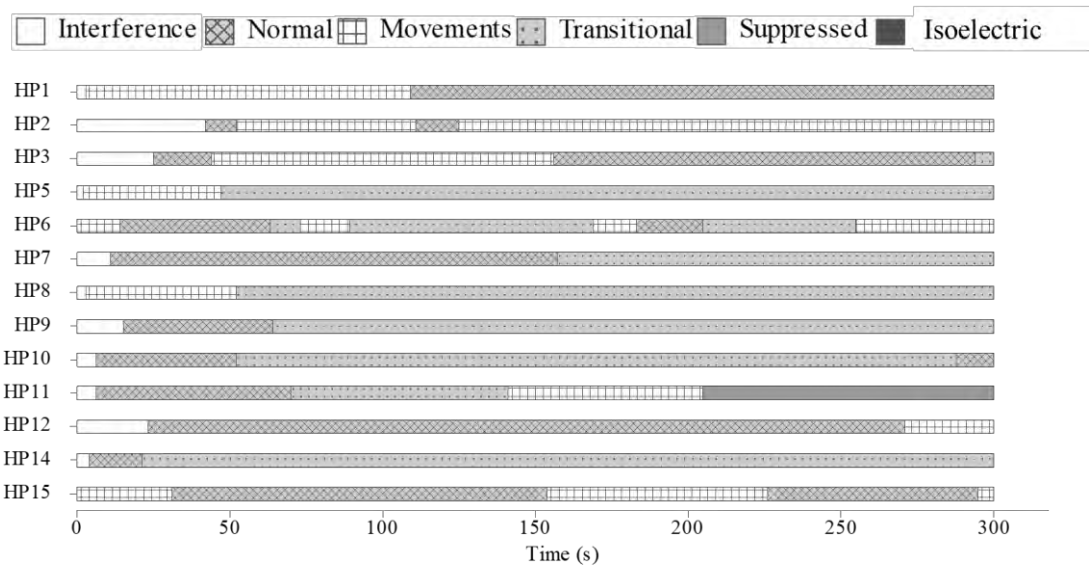

Figure 4.6 - Characteristics of the EEG trace in HP Nile tilapia (n=13) submitted to hypothermia during the 300 s after immersion.

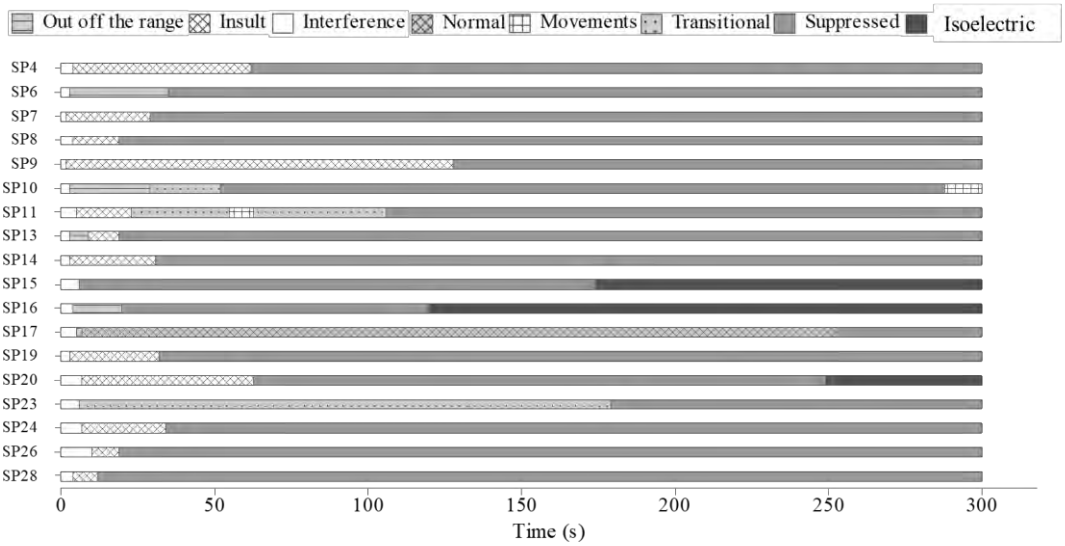

Figure 4.7 - Characteristics of the EEG trace in SP Nile tilapia (n=21) submitted to mechanical spiking during the 300 s after immersion.

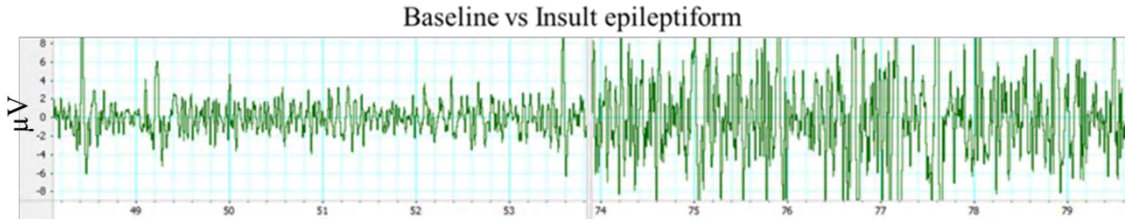

Figure 4.8 - A representative series of electroencephalogram (EEG) traces illustrating the appearance of EEG in one fish submitted to spiking (Baseline vs Insult epileptiform) (SP9).

The majority of the fish showed swimming movements after contact with the anaesthetic or with the ice/water mixture in hypothermia (PE 14/21: 66.7%, OA 18/23: 78.3%, LA 15/23: 65.2% and HP 12/13: 92.3%). For the SP group, only 11.1% (2/18) showed mouth or fin movements after the shot. Figure 4.9A shows the lag (median ± interquartile range) for the first movement after immersion or shot, which was significant for the HP (103 ± 544 s) and SP (225) ($P<0.01$) groups. The numbers of movements per fish were similar amongst the groups (Figure 4.9B), but the duration of the movements (s) was longer for OA (73.5 ± 78.5) and HP (115± 143.5) as compared to PE (18.5 ±17.2) ($P<0.01$) (Figure 4.9C). The end of the last movement took longer (s) for group HP (578 ± 722) ($P<0.05$) (Figure 4.9D).

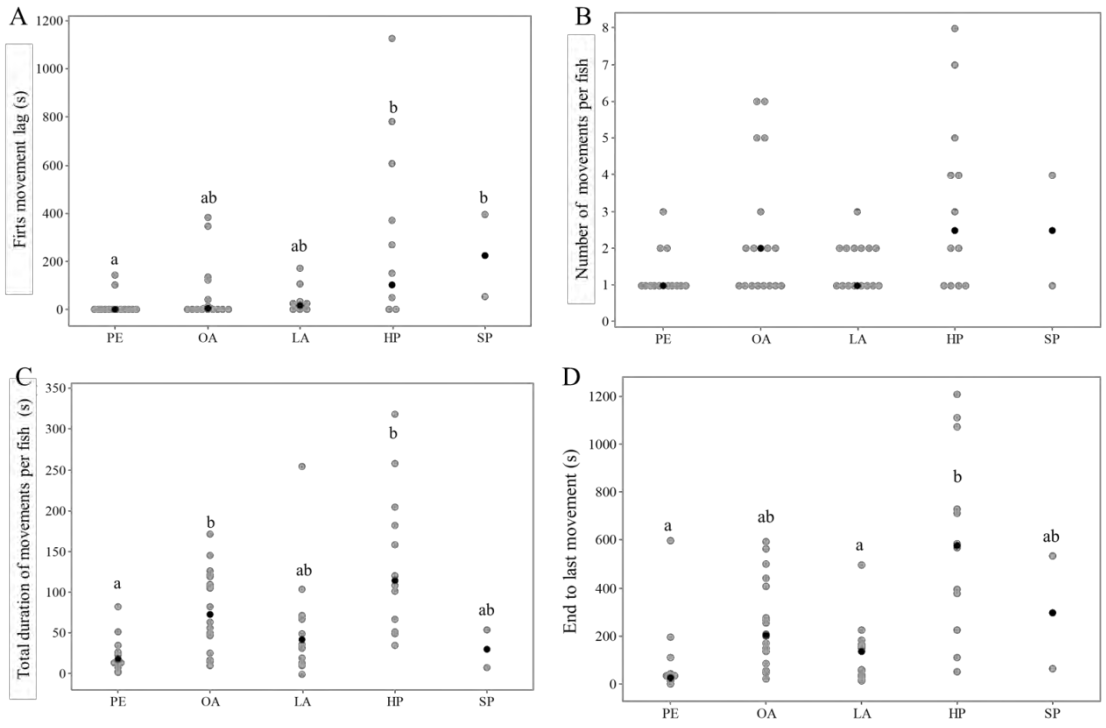

Figure 4.9 – Movements presented by the Nile tilapia during the recordings. Each grey dot represents an individual value. The black dots represent the median value of each group. (A) First movement lag (s), (B) Number of movements per fish, (C) Total duration of movements per fish (s), (D) End of last movement (s). Different letters indicate differences between the groups (Kruskall-Wallis test followed by Dunn's test ($P<0.05$).

Only 4.7% (1/21) of the LA fish showed a response to painful stimuli soon after anaesthesia, different from the HP fish (92.3% - 12/13). The fish in groups PE, OA and SP showed no response to pain soon after recording.

The median time (s) to lose consciousness as represented by the suppressed phase was PE: 136 ± 61, OA: 171 ± 164, LA: 171.5 ±77.8 and HP: 252 ± 752 (Figure 4.10 A) ($P<0.01$). In addition, 4/5 HP fish which became unconscious regained consciousness after 988 ± 580 s (Figure 4.11). Similarly, of the 29 fish submitted to mechanical spiking, only 17 (58.6%) lost consciousness, and 12/18 presented insult epileptiform traces starting (4 ± 4 s) after the shot. Thus the hypothermia method showed low efficiency to induce unconsciousness. The mechanical spiking method needs to be more accurate to guarantee unconsciousness in more than 95% of the fish.

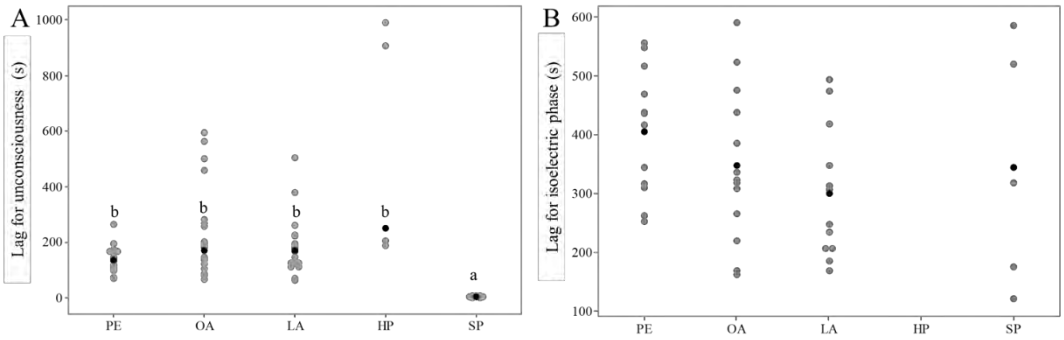

Figure 4.10 – (A) Lag for unconsciousness (s) after the stunning (immersion or shot). (B) Lag for isoelectric phase (s) after the stunning (immersion or shot). Each gray dot represents an individual value. The black dots represent the median value of each group (Kruskall-Wallis test followed by Dunn's test, $P<0.05$).

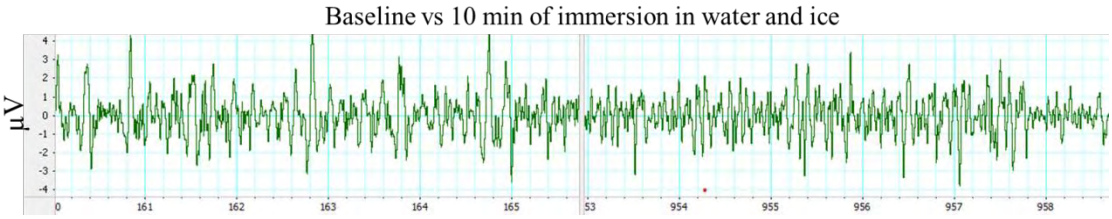

Figure 4.11 - A representative series of electroencephalogram (EEG) traces illustrating the appearance of EEG in one fish submitted to hypothermia (baseline vs 10 min immersion) (HP14).

The isoelectric phase was observed in 57.1% PE, 56.5% OA, 52.2% LA, 0% HP and 27.8% SP, and the lag (s) for it to appear was 426 ± 194 for PE, 322 ± 214.5 for OA, 276.5 ± 193.5 for LA, and 317.5 ± 405.5 for SP, being very similar amongst the groups ($P>0.05$) (Figure 4.10 B).

In addition, HP induced an irregular heart rate (HR) as shown in Figure 4.12, suggesting high stress levels for the use of this method. The fish submitted to anaesthesia showed an initial increase in the HR, but by the end of the anaesthesia, the values were similar to baseline (Figure 4.12)

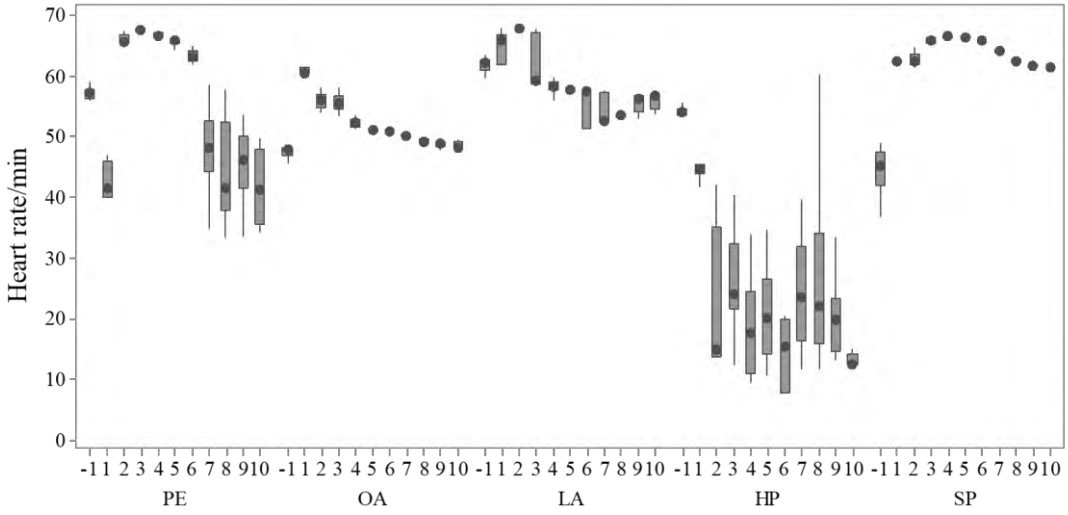

Figure 4.12 - Heart rate/min. The median is represented by each black dot for each minute. -1 signifies measurement on the baseline, and 1, 2, 3 … means minutes after stunning (immersion or shot).

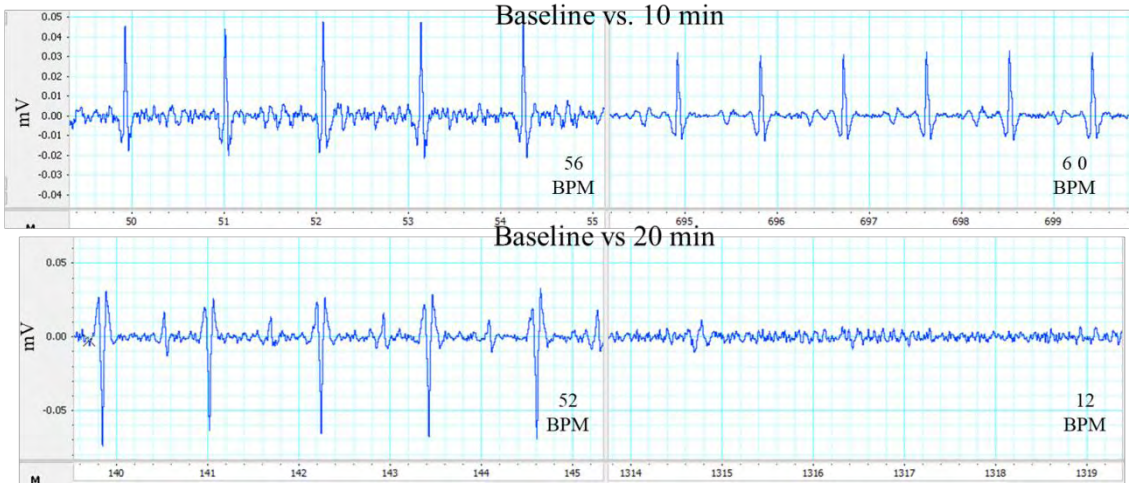

Figure 4.13 - Representation of the ECG and the value for the HR in fish submitted to OA (upper figure) and HP (lower figure). To the left the ECG at the baseline and to the right after 10 minutes of immersion in the anaesthetic or 20 min immersion in HP.

4.3.2 Spectral analyses

In each group, one fish with no interference from the ECG and/or respiration, was selected for the spectral analysis. Figure 4.14 shows the results of the variables for the spectral analysis (PE09) during the 10 min of recording.

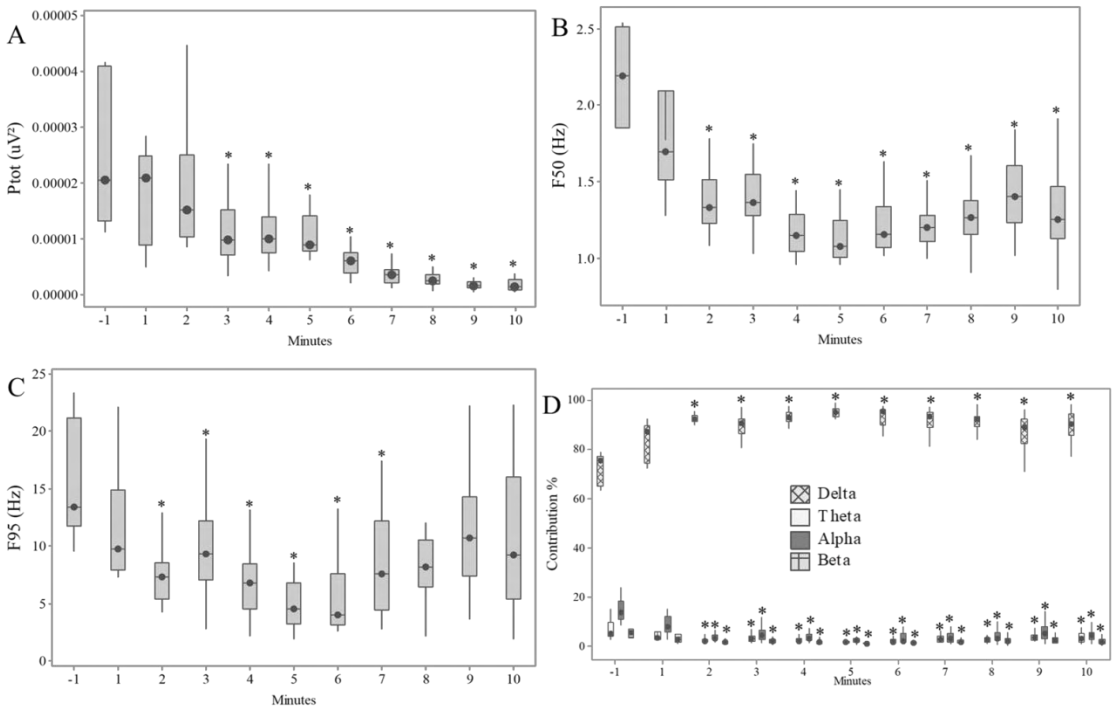

Figure 4.14 - Spectral variables in a PE fish (PE09). (A) Values obtained for Ptot (μV²), (B) F50 (Hz), (C) F95 (Hz) and (D) the power contribution (%) of the delta, theta, alpha and beta frequencies. -1 corresponds to baseline values. * indicates significant difference for each minute as compared to the baseline (Mann-Whitney test, $P < 0.05$).

The results of the spectral analysis suggest unconsciousness after 3 minutes of exposure to PE due to the decreases in Ptot, F50 and F95, and also the increase in contribution from the delta frequency and decrease in contribution from the beta frequency. PE met 4 out of the 7 criteria for the fish to be considered unconscious. This difference was observed throughout the time immersed in the anaesthetic and confirmed the results of the visual analysis.

In addition, the variables for the spectral analysis were similar for OA. With the exception of the values for Ptot and the theta frequency, after 3 min the other variables conformed with what one could expect for an unconscious fish, meeting 5 of the 7 criteria studied (Figure 4.15).

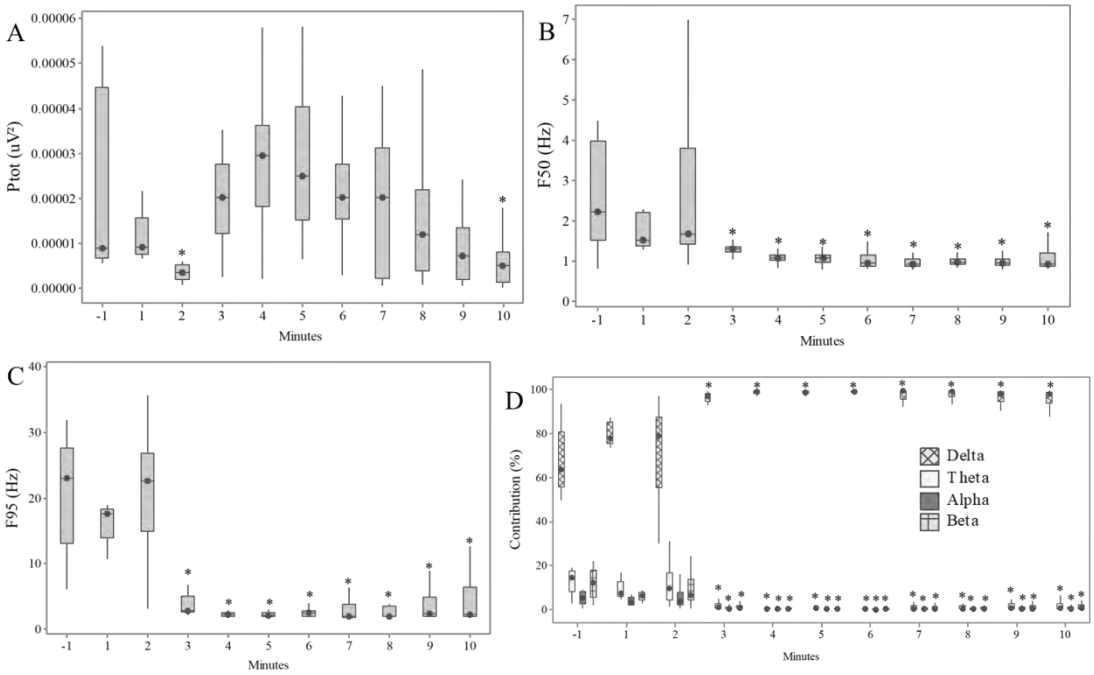

Figure 4.15 - Spectral variables in an OA fish (OA03). (A) Values obtained for Ptot (μV²), (B) F50 (Hz), (C) F95 (Hz) and (D) the power contributions (%) of the delta, theta, alpha and beta frequencies. -1 corresponds to baseline values. * indicates significant difference from each minute to the baseline (Mann-Whitney test, P <0.05).

Similarly, the values obtained for LA in the spectral analysis suggest unconsciousness after 3 min due to the decreases in Ptot, F50 and F95, increase in contribution of the delta frequencies and decreases in those from the alpha and beta frequencies, meeting 6 of the 7 criteria evaluated. This difference was observed throughout the time immersed in the anaesthetic and confirmed the results of the visual analysis (Figure 4.16).

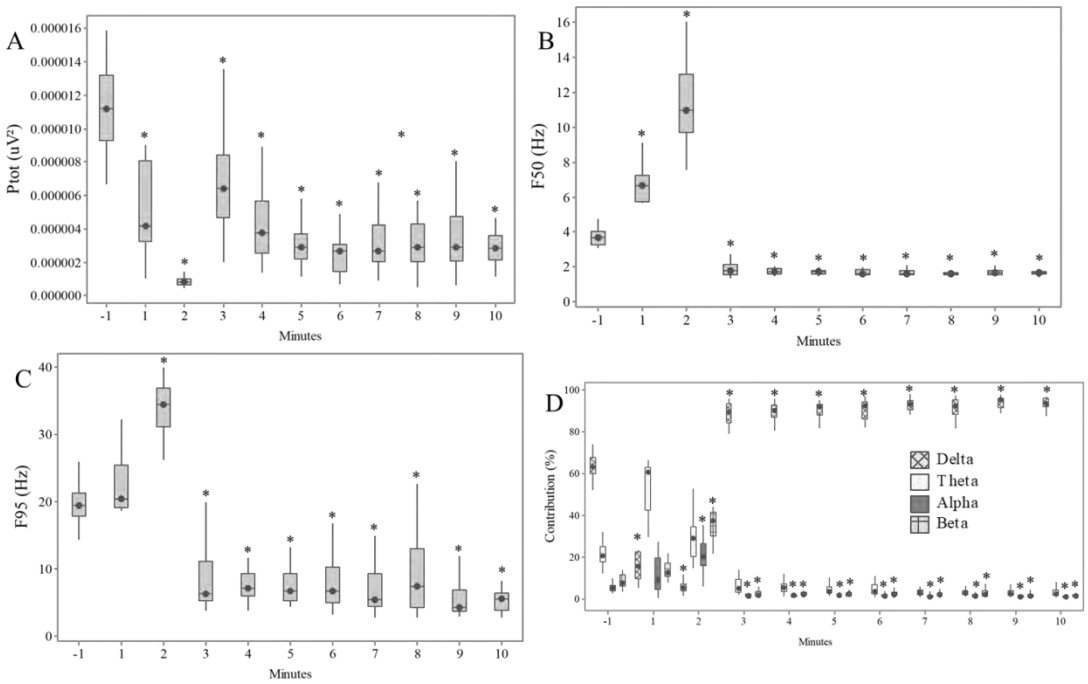

Figure 4.16 - Spectral variables in a LA fish (LA14). (A) Values obtained for Ptot (μV²), (B) F50 (Hz), (C) F95 (Hz) and (D) the power contributions (%) of the delta, theta, alpha and beta frequencies. -1 corresponds to baseline values. * indicates significant difference for each minute as compared to the baseline (Mann-Whitney test, P <0.05).

The values obtained in the spectral analysis for fish submitted to HP suggest that this method does not induce unconsciousness. Figure 4.17 shows that only the values for Ptot varied with time, whereas the values for F50, F95 and the contributions of the frequencies suggest the absence of unconsciousness, only meeting one of the 7 criteria studied.

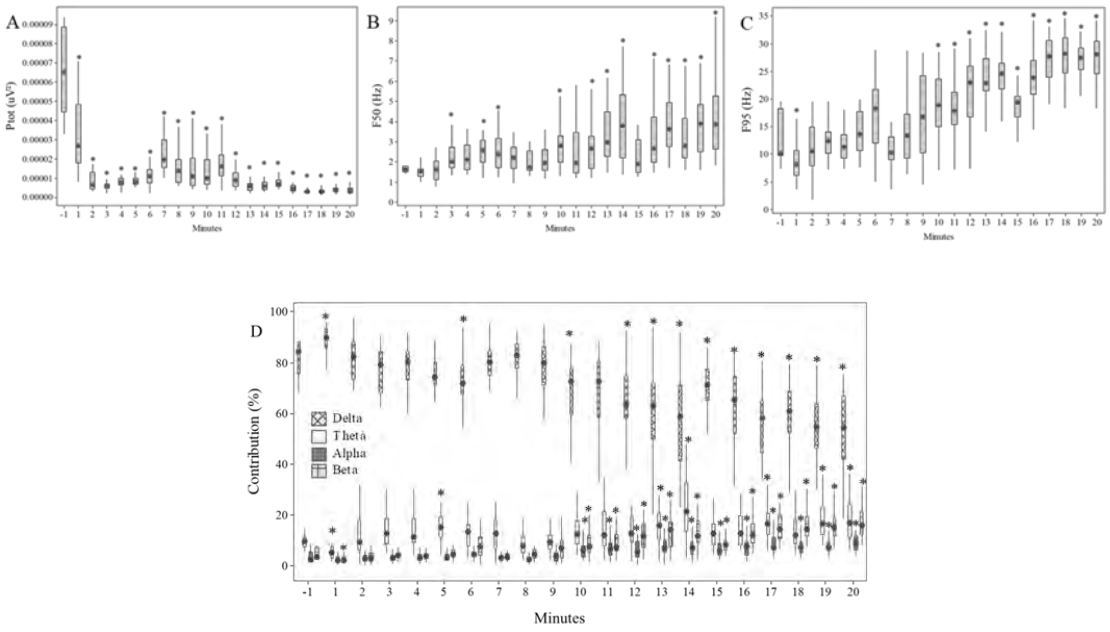

Figure 4.17 - Spectral variables in an HP fish (HP10). (A) Values obtained for Ptot (μV²), (B) F50 (Hz), (C) F95 (Hz) and (D) the power contributions (%) of the delta, theta, alpha and beta frequencies. -1 corresponds to baseline values. * indicates significant differences for each minute as compared to the baseline (Mann-Whitney test, P <0.05).

The values obtained in the spectral analysis of a fish submitted to SP can be seen in Figure 4.18. Only the values for F95 and the beta frequencies were compatible with an unconscious fish, only meeting 2 of the 7 criteria studied.

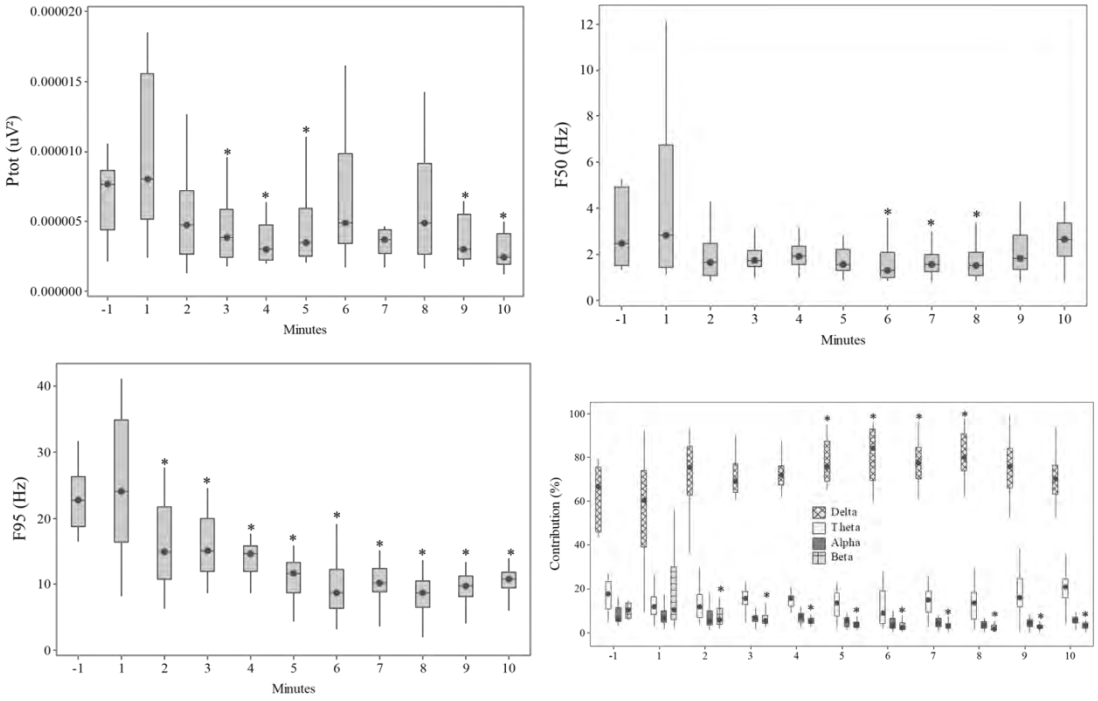

Figure 4.18 - Spectral variables in a SP fish (SP14). Values obtained for Ptot (μV²)(A), F50 (Hz)(B), F95 (Hz)(C) and the power contributions (%) of the delta, theta, alpha and beta frequencies (D).-1 corresponds to baseline values. * indicates significant differences for each minute as compared to the baseline (Mann-Whitney test, P <0.05).

4.3.3 Behavioural analyses

Figure 4.19 shows the lag for the loss of the indicators observed during each stunning process. The times taken to reach a level of deep anaesthesia (stage 4), where the fish no longer reacted to a painful stimulus or showed VOR were: 89.5 ± 27.2 s for OA; 138.0 ± 92.8 s for LA; 826.5 ± 319.3 s for HP; and 0 ± 0 s for SP (Figure 4.20). In addition, 4 of the 10 fish in the HP group reacted to a painful stimulus after 60 s of exposure to air. The HP group took significantly longer to lose all their behaviours, in contrast with the other groups (P<0.05). The pre-stunning time for the SP group was 27 ± 4 s. Since SP was a method inducing immediate unconsciousness, the time was much smaller than in the other groups. No differences were observed between OA and LA.

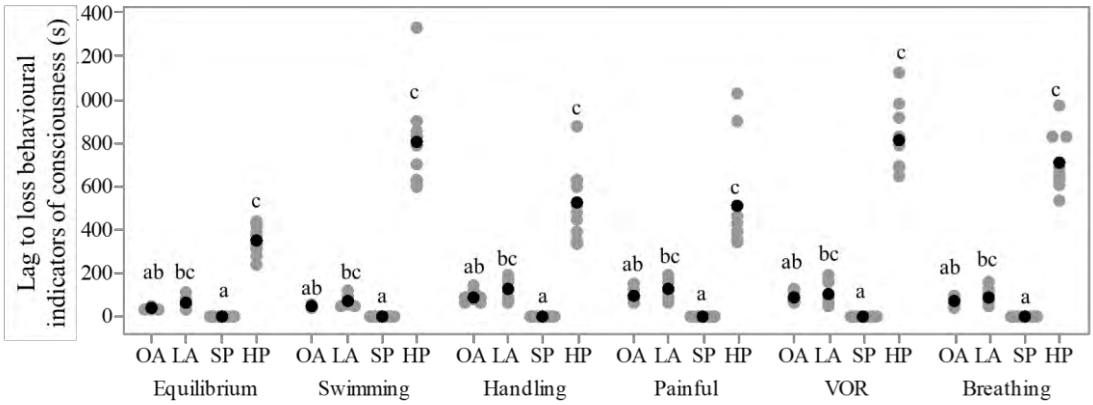

Figure 4.19 - Time taken to lose the indicators of consciousness in Nile tilapia submitted to anaesthesia with the essential oils *Ocimum americanum* (OA), *Lippia alba* (LA), spiking (SP) or hypothermia (HP). Each grey dot represents an individual value. The black dots represent the median value for each group. Different letters indicate differences between the groups for the same behaviour (Kruskall-Wallis test followed by Dunn's test) (P<0.05).

Only three of the ten SP fish recovered 187.0 ± 243.0 s after firing, and only one of the ten soon after firing. Breathing was the first indicator recovered by 9 of the 10 OA fish and 8 of the 10 LA fish. The first indicator presented by the SP fish that recovered was swimming ability. The second behaviour observed was VOR for OA (6/10) and LA (8/10). The time taken to recover the second indicator was longer for OA (485.0 ± 138.0 s) and different from LA (288.5 ± 169.8 s) (P<0.05).

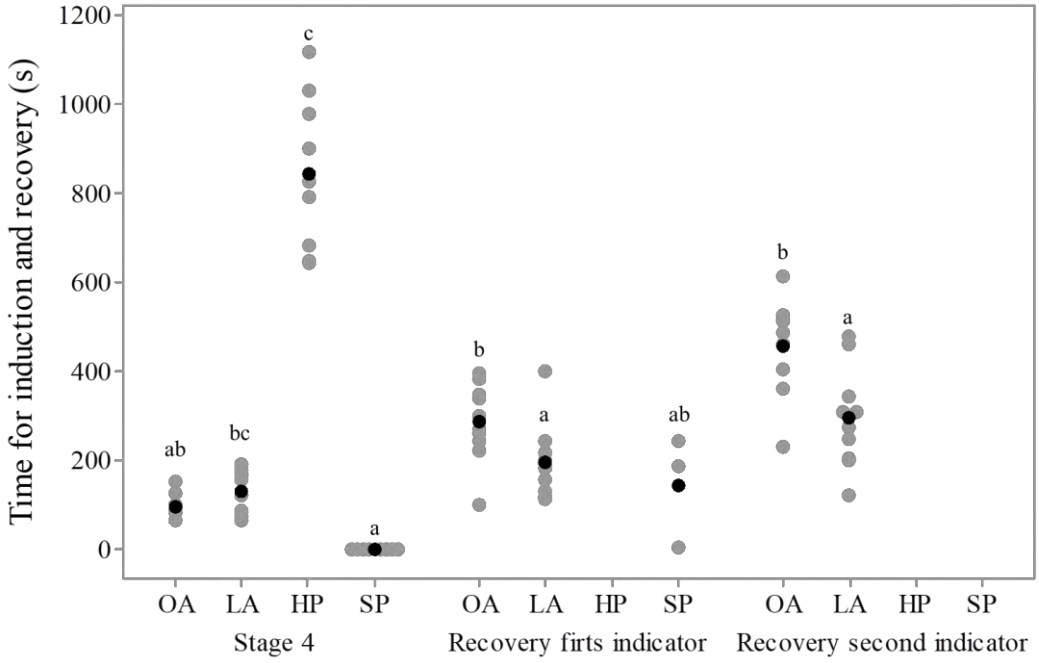

Figure 4.20 - Time taken for the induction of deep anaesthesia (stage 4) and recovery in Nile tilapia submitted to anaesthesia with the essential oils *Ocimum americanum* (OA), *Lippia alba*

(LA), and to spiking (SP) and hypothermia (HP). Each grey dot represents an individual value. The black dots represent the median value for each group. Fish from HP group were euthanized after 20 min of immersion, and fish from SP group were euthanized after observed any indicator of recovery after the shot. Different letters indicate differences between the groups for the same stage (Kruskall-Wallis test followed by Dunn's test) ($P<0.05$).

The duration of unconsciousness for the fish submitted to anaesthesia with the essential oils was determined as being up to the appearance of an indicator other than breathing. Hence the median value for the time (s) of the end of unconsciousness was longer for OA (485.0 ± 150.5) than for LA (288.5 ± 192.8) ($P<0.05$).

4.4 Discussion

The sounds emitted by Nile tilapia are at a frequency inaudible to humans. In addition, they do not present easily identifiable facial expressions, do not have a white conjunctiva, have no eyelashes, do not contract their pupils in response to light changes and the ocular globe has limited movement. Thus behaviours such as swimming, equilibrium, response to handling, response to a painful stimulus, VOR and breathing are useful indicators to determine unconsciousness (KESTIN; VAN DE VIS; ROBB, 2002). However, for methods that could induce paralysis such as electro-immobilization, muscle blockers, sedation or hypothermia, observing the behaviour shows limited results (LAMBOOIJ et al., 2009; VAN DE VIS et al., 2014), and hence the use of EEG is necessary to validate new methods of humane slaughter.

During a study involving sedation and anaesthesia in birds, SANDERCOCK et al. (2014) observed changes in the values of the spectral analysis, with increases in Ptot and decreases in F50 and F95 under inhalation anaesthesia (8% sevoflurane vaporized in 100% oxygen) as compared to the values obtained with conscious birds. Similar shifts in the values for Ptot and F50 were observed in broiler chickens submitted to low atmospheric pressure stunning (LAPS), with the Ptot values increasing in the poultry after LAPS (MCKEEGAN; SANDERCOCK; GERRITZEN, 2013). In addition the Ptot values were found to decrease in turkeys after intravenous anesthesia with 1 ml/4.5 kg sodium pentobarbital (HERNANDEZ et al., 2019). A decrease in the values for Ptot was also observed in turkeys stunned using a non-penetrative captive bolt (GIBSON et al., 2018). For these reasons, higher or lower Ptot values and decreases in the values for F50 and F95 were considered indicative of an unconscious fish.

The frequency bands were organized into delta (0 to 4 Hz), theta (4 to 8 Hz), alpha (8 to 12 Hz) and beta (12 to 32 Hz). Frequency shifts, more specifically, the suppression

of alpha and beta waves and the occurrence of delta and theta waves, are indicative of a loss of consciousness (GERRITZEN et al., 2006). Increases in the delta and theta patterns are correlated with states of insensibility, and an isoelectric EEG reflects no brain activity or brain death in poultry (GERRITZEN et al., 2006; HERNANDEZ et al., 2019). Thus frequency shifts are indicators of unconsciousness in the spectral analysis, completing the seven criteria evaluated (Ptot, F50, F95 and the contributions of the delta, theta, alpha and beta frequencies).

Fish submitted to PE, OA or LA met more than four of the criteria studied to consider the fish unconscious. A key point in the spectral analysis is to obtain good quality records with no interference from breathing or cardiac activity, but this issue was not previously reported in earlier studies (BOWMAN; HJELMSTEDT; GRÄNS, 2019; HERNANDEZ et al., 2019; and SANDERCOCK et al., 2014). BOWMAN et al. (2019) carried out a spectral analysis in rainbow trout (*Oncorhynchus mykiss*) submitted to anaesthesia with MS 222 and found no significant differences in the values for F50, low delta and theta frequencies and high alpha and beta frequencies during the evaluation time, as compared to non-anesthetized fish.

On applying EEG during the slaughter process, the transitional, suppressed and isoelectric phases were reported in hens, broilers, ducks and turkeys exposed to N_2- or CO_2- filled foam (MCKEEGAN et al., 2013) and in hens killed by CO_2 (MCKEEGAN et al., 2011). Such different phases were also described in experiments with pigs exposed to high CO_2 levels (VERHOEVEN et al., 2016). The transitional phase showed high amplitude, low-frequency activity or high frequency but a reduced amplitude signal; the suppressed phase showed a greatly suppressed EEG but containing some slow wave activity; and the isoelectric phase was residual low-level noise indicating a lack of EEG activity (MCKEEGAN et al., 2013). A conservative approach considers that the transitional phase is just a reduction in the state of vigilance, and thus the start of unconsciousness can be determined by the suppressed phase (MCKEEGAN et al., 2013). A characterization of the different patterns in the visual analysis of EEG traces for fish was not found in the literature consulted, making this study pioneer and original. The shifts associated with the spectral analysis values suggest a progressive induction of unconsciousness in fish submitted to anaesthesia with 2-phenoxyethanol, *Ocimum americanum* (OA) or *Lippia alba* (LA).

The use of 2-phenoxyethanol was previously reported as an efficient anaesthesia to induce unconsciousness in common carp (*Cyprinus carpio*) as verified by EEG

(LAMBOOIJ et al., 2009). Thus the results of the present study with a visual and spectral analysis of the EEG suggest the same effect in Nile tilapia due to the observation of phases compatible with unconsciousness, such as the suppressed and isoelectric phases: a reduction in movement after 180 s of exposure to the anaesthetic, the absence of response to a painful stimulus, reduction in the values for Ptot, F50 and F95, and an increase in the contributions of the delta and beta frequencies during the 10 min of anaesthesia. Thus the results suggest that the immersion of Nile tilapia in 1 mL/L 2-phenoxyethanol induces unconsciousness after approximately 171 s, so its use as a positive control for unconsciousness in Nile tilapia can be applied in future studies. The use of 2-phenoxyethanol is limited only for research proposes, and it cannot be used in fish destined to consumption.

With respect to anaesthesia with OA the results suggest efficiency in inducing unconsciousness since the suppressed and isoelectric phases were observed and the criteria of F50, F95 and the delta, alpha and beta frequencies were compatible with those of an unconscious animal. Similar to OA, the EEG of the LA group showed the suppressed and isoelectric phases and met 6 of the 7 criteria in the spectral analysis. During the behavioural analysis, the oils induced unconsciousness in approximately 3 minutes after immersion in the anaesthetic, a time favourable for their subsequent application in the field. Recent studies with the use of essential oils as anaesthetics for Nile tilapia were carried out based on behavioural indicators (HOHLENWERGER et al., 2016; RUCINQUE et al., 2021; TEIXEIRA et al., 2016). Thus the results of the EEG suggest the efficiency of the essential oils in the induction of deep anaesthesia. The anaesthetic effect of the essential oils may be mediated by activation of the inhibitory neurotransmitter γ-aminobutyric acid (GABA) in the central nervous system (CNS) of the fish (GARLET et al., 2019; HELDWEIN et al., 2012). The GABA mechanism regulates the entrance of Cl^- into the post-synaptic membrane, inhibiting the transmission of nerve stimulation with sedative, anaesthetic, anxiolytic and anticonvulsant effects. Drugs such as benzodiazepines and barbiturates also show GABA action and GABA receptors have been observed in the rainbow trout since 1983 (WILKINSON et al., 1983).

The stunning methods should induce immediate loss of consciousness, but if the loss of consciousness is not immediate the induction of unconsciousness should not cause avoidable pain and suffering in the animals. The main anaesthetics used (benzocaine, iso-eugenol, MS 222 and lidocaine) for the euthanasia of zebrafish (*Danio rerio*) induce aversion in the fish when placed in the anaesthetic solution (READMAN et al., 2013),

but according to BANDEIRA-JUNIOR et al. (2018), the use of the essential oils *Lippia alba* or *Aloysia triphylla* as pre-slaughter anaesthetics in zebrafish did not cause aversion in the fish. Thus the use of 500 µL/L of the essential oils *Ocimum americanum* (OA) or *Lippia alba* (LA) induces unconsciousness in Nile tilapia, as verified by EEG, after approximately 180 s of immersion, representing an alternative for the humane slaughter of fish.

The analysis of recovery from the anaesthesia according to the behavioural indicators was useful to determine the duration of unconsciousness, considering the return to consciousness as the observation of any of the indicators with the exception of breathing. The animals continued breathing while under the anaesthesia, but this did not signify being conscious or being sensitive to pain. In the case of very deep anaesthesia, a depression of the respiratory center can occur, leading to the absence of breathing or apnea, and in this case, support with respiratory ventilation is provided (MUIR et al., 2013). Having determined the recovery times for consciousness using the behavioural analysis, one can estimate a safe time to carry out the bleeding of the unconscious animal. At the slaughter scene, the fish should remain in the anaesthetic solution with the oil concentrations used in the present study for at least 3 min, and bleeding should be carried out immediately after removing the animal from the anaesthetic solution. In addition, the anaesthetic solution should be renewed at every 10 fish, thus we recommended a similar approach in future studies with essential oils as anaesthetics.

Hypothermia is a pre-slaughter method widely used for various fish species. The fish are immersed in a water and ice mixture at between 1:1 or 1:2, but with respect to the HP group in the present study, the visual and spectral EEG analyses suggested a low efficiency in inducing unconsciousness in the animals. During the first five minutes of evaluation only 1 in 13 fish presented the suppressed phase. In all, 5 of the 13 fish presented the suppressed phase during the 20 minutes recorded, and 4 of these 5 fish recovered. The spectral analysis confirmed the results of the visual analysis, and the fish only met one of the 7 criteria evaluated in the spectral analysis. To the authors' knowledge, this is the first time that unconsciousness was studied by way of EEG in Nile tilapia submitted to hypothermia. The hypothermia in water and ice at 2 ºC induced partial immobilization in the fish. The EEG results showed an irregular heart rate, suggesting that the thermal shock induced a high degree of stress.

Inefficiency in the induction of unconsciousness was also verified in turbot (*Scophthalmus maximus*) submitted to hypothermia as determined by an analysis of the

EEG frequencies (LAMBOOIJ et al., 2015). Hypothermia induces a loss of consciousness in eels (*Anguilla anguilla*, L.) after 12 ± 6 minutes, associated with an irregular heart rate (LAMBOOIJ et al., 2002). In addition, hypothermia is used in Mediterranean countries in the pre-slaughter of gilthead seabream (*Sparus aurata*) but only induces unconsciousness after 5-40 min of immersion, as verified by behaviour analyses (HUIDOBRO; MENDES; NUNES, 2001; VAN DE VIS et al., 2003). Similar results were observed for sea bass (*Dicentrarchus labrax*) immersed in iced water (2:1) at 1 ± 1 ºC, where the fish became motionless after 3 min, but when stimulated, presented responses after 9-11 min of immersion (SIMITZIS et al., 2014).

The transference of Nile tilapia from water at 25 ºC to immersion at 13 ºC (difference of 12 ºC) induces metabolic stress with negative consequences for the biochemistry and physiology of the fish (PANASE; SAENPHET; SAENPHET, 2018). A temperature difference of 21 ºC was used in the present study, but even so the heat shock was not efficient in inducing unconsciousness, since the behavioural analysis only observed an induction of unconsciousness after 826.5 ± 319.3 s of immersion in the water and ice mixture. Similar times have been reported for Nile tilapia submitted to hypothermia, where the fish lost consciousness after a median time of 750 s (PEDRAZZANI et al., 2009). Hypothermia is also not recommended as a method for slaughter or euthanasia in other animal groups, such as reptiles and amphibians (AVMA, 2016; SHINE et al., 2015). However, hypothermia is still used as a pre-slaughter method for fish in slaughterhouses in Brazil (OLIVEIRA FILHO et al., 2014; PEDRAZZANI et al., 2009), and so millions of individuals are being slaughtered totally conscious, causing high degree of suffering. Other methods not considered humanitarian are also used in the field in Brazil (asphyxiation, bleeding or evisceration) since the humane slaughter legislation does not include fish and has not been updated since 2000 (BRASIL, 2000). For the above mentioned reasons, there is an urgent need to substitute hypothermia for the pre-slaughter treatment of fish in Brazil in order to reduce the suffering of the individuals involved and obtain an ethically acceptable product.

Mechanical spiking was not effective in inducing unconsciousness in more than 60% of the fish used. In addition, the spectral analysis of the EEG only showed two indicators compatible with unconsciousness. In the behavioural analysis firing was effective in 90% of the fish, and 30% recovered consciousness. Thus the results suggest that the mechanical spiking technique should be refined before being used in the field for the stunning/slaughtering of Nile tilapia. Pre-stunning management requires refinement

since it took longer than the 15 seconds recommended by the Humane Slaughter Association guides (HAS, 2016). Pre-slaughter stunning methods should be effective in at least 95% of the fish (GRANDIN, 2010). Mechanical percussion produced the appearance of theta and delta waves and spikes, which were proceeded by no brain activity on the EEG, providing evidence of unconsciousness and insensibility in common carp (*Cyprinus carpio*) (LAMBOOIJ et al., 2007). The maximum speed of the captive bolt was 19.13 ± 0.76 m/s and only 2 of the 22 fish recovered consciousness 30 s and 180 s after percussion (LAMBOOIJ et al., 2007). The relationship between the force and the probability of recovery was described by (ROTH; SLINDE; ROBB, 2007) in Atlantic salmon. According to Lambooij et al. (2010), the use of a commercial percussion machine does not induce unconsciousness in Atlantic salmon (*Salmo salar*) when used with a force below 8.1 bar. Thus the development of pistols that deliver a greater force is necessary to guarantee fish welfare at slaughter.

The fish industry has the opportunity to be proactive and install humane fish slaughtering methods before their imposition by citizen pressure, and thus be in the vanguard of the welfare of fish destined for human consumption. The use of essential oils at slaughter in fish depends of regulations of each country.

4.5 Conclusions

When used in Nile tilapia, 2-phenoxyethanol anaesthetic showed both EEG traces and spectral analyses consistent with unconsciousness after 140 s of immersion. Anaesthesia with 500µL/L of the essential oils *Ocimum americanum* or *Lippia alba* induced unconsciousness after 180 s of immersion, as determined by EEG. The behavioural indicators suggest the recovery of consciousness 200 s after removal from the anaesthetic, which is sufficient time to bleed the fish while still unconscious, so these essential oils are an alternative for the humane slaughter of fish.

The development of national mechanical spiking equipment is necessary for research purposes for their subsequent application in the field. The use of hypothermia for the pre-slaughter of Nile tilapia does not induce unconsciousness and should urgently be substituted by methods that meet the criteria of humane slaughter.

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5 Chapter 5 Meat quality of Nile tilapia submitted to different pre-slaughter methods

Abstract

In Brazil, tilapia represented 57% of the total fish production with 432,149 t, representing a significant number of individuals. The stress induced by pre-slaughter procedures, such as fasting, fishing, transport and the method itself alter the physiological homeostasis, influencing the post mortem biochemical process, principally anaerobic glucose and ATP (adenosine triphosphate) degradation. Our aim was to evaluate the influence of different pre-slaughter methods: hypothermia in ice/water; anesthesia with the essential oils *Ocimum americanum* (OA) or *Lippia alba* (LA) and mechanical spiking (SP), on the meat quality parameters of Nile tilapia stored under refrigeration for 15 days. Nile tilapia (n=56) were divided into four (n=14/group) pre-slaughter stunning method groups: anesthesia with essential oils OA and LA (2 groups), SP and hypothermia (HP). For the methods using anesthesia with the essential oils *Ocimum americanum* (OA) or *Lippia alba* (LA), the fish were captured individually and placed in glass aquariums (15 L) with the essential oil at 500 µL/L until the fish reached deep anesthesia. Mechanical spiking (SP) was carried out using a commercial fish gun. For hypothermia (HP), the fish were harvested from the concrete tank and placed directly in a water and ice slurry (1:2) in a 250 L capacity tank. After anaesthesia (OA and LA), shot (SP) or 20 min of immersion in the tank (HP) fish were killed by bleeding. Un-gutted fish and fillets from tilapia were stored in a cold chamber inside Styrofoam boxes in alternate layers with ice during 15 days for analyses of pH, *rigor mortis* index (RMI), total volatile basic nitrogen (TVB-N), ATP (Adenosine triphosphate) and its metabolites, instrumental color at different times. Immediately after slaughter (0 h) the muscle pH values were similar for all groups (7.13 ± 0.43 SP, 6.86 ± 0.55 HP, 7.33 ± 0.57 OA, 7.23 ± 0.48 LA), but significant differences ($p<0.05$) were found 3, 6 and 24 hours after slaughter, being higher in group LA than in group SP. Regarding RMI, the values for the HP group indicate a quicker start to the *rigor mortis* process as compared to the OA and LA groups after 3 h (45 ± 59 % SP, 78 ± 44 % HP, 30 ± 14% OA, 33 ± 12 % LA). The values for ATP, ADP, INO, Hx and the *K-value* were similar for the different groups. After 8 days (192 h) of storage, the *K-values* were (46.2% SP; 50.0% HP; 52.5% OA; and 55.8 LA). The use of mechanical spiking and anesthesia with the essential oils *Ocimum americanum* (OA) or *Lippia alba* (LA) in Nile tilapia did not negatively change the following quality

parameters of the meat: pH value, rigor mortis, TVB-N and K-value as compared to the traditional method of hypothermia.

Keywords: animal welfare, essential oils, hypothermia, humane slaughter, meat quality

5.1 Introduction

Nile tilapia is one of the most relevant species farmed in the world. Data from FAO (Food and Agriculture Organization) in 2018, the production of tilapia was 4,555,4 thousands of tons, after of Grass carp (*Ctenopharyngodon idellus)* (5,704.0 thousands of tons) and Silver carp (4,788.5 thousands of tons) *(Hypophthalmichthys molitrix)* (FAO, 2020). According to the Brazilian Fisheries Association, in Brazil fish production totalled 758,006 t in 2019, representing an increase of 4.9% in relation to 2018 (PEIXE-BR, 2020). Tilapia represented 57% of the total fish production, totalling 432,149 t (PEIXE-BR, 2020), which is a significant number of individuals. In Brazil the main tilapia-producing states are Paraná, São Paulo and Santa Catarina. The weight of individuals can vary from 500 g to 1000g at slaughter, so the number of individuals is estimated at between 864,298,000 and 432,149,000, surpassing the numbers of individual cattle (17,650,718) and swine (46,356,359) slaughtered, although more than 5.6 billion individual birds are slaughtered per year in Brazil (BRASIL, 2019).

Tilapia is a highly important species in Brazilian aquaculture, due to its rusticity and resistance to diseases, handling, temperature changes and low oxygen levels, and it tolerates high density levels, utilizes an omnivorous diet and is easy to reproduce (VICENTE; ELIAS; FONSECA-ALVES, 2014). The greater part of tilapia is commercialized as refrigerated skinned fillets. The fillets are wide and have no bones, which attracts the consumer, but frozen fillets and slices are also commercialized in supermarket chains.

Due to the absence of specific norms, there is no standard process for slaughtering fish in Latin America and many other countries (BRASIL, 2000; VIEGAS et al., 2012). Hypothermia (live chilling in ice/water) is used as a pre-slaughter method in Brazilian slaughterhouses. Hypothermia is recognized as a method that does not follow the principles of humane slaughter and is not recommended by OIE (OIE, 2015). In the search for humane slaughter methods, the use of essential oils (HOHLENWERGER et al., 2016; RUCINQUE et al., 2021), electric stunning (LAMBOOIJ et al., 2008) and spiking have

been studied for Nile tilapia. In countries as Norway, Germany, UK, Netherlands there are specifically regulations for stunning farmed fish before slaughter (Norway, 2019).

Alternative slaughtering methods to hypothermia should not cause negative alterations in the final product. The stress induced by pre-slaughter procedures, such as fasting, fishing, transport and the method itself alter the physiological homeostasis, influencing the post mortem biochemical process, principally anaerobic glucose and ATP (adenosine triphosphate) degradation (POLI et al., 2005). Humanitarian slaughter methods can improve some meat quality indicators. Atlantic salmon stunned with CO_2 showed an earlier onset and resolution of *rigor mortis* than when spiking or electrical stunning methods were used (ROTH et al., 2002). In turbot (*Scophthalmus maximus*) live bleeding without stunning led to a fall in muscle pH and the immediate installation of *rigor mortis*, so the use of spiking or electrical stunning could be alternatives for slaughter (ROTH et al., 2007). Furthermore, muscle pH values were significantly lower in seabass (*Dicentrarchus labrax*) stunned by spiking than in fish chilled in ice/water slurry or on ice (SIMITZIS et al., 2014).

South American catfish (*Rhandia quelen*) stunned by hypothermia showed a slight delay in the onset of *rigor mortis* as indicated by the lower *rigor* index values at 6 h *postmortem* as compared to the use of electrical stunning (128 V, 300 Hz for 5 s) (VEIT et al., 2018). The use of electrical stunning and CO_2 stunning at pre-slaughter for matrinxã (*Brycon cephalus*) maintained the meat quality similar to that produced using hypothermia (VARGAS et al., 2013). In Nile tilapia, the use of CO_2 stunning and hypothermia at pre-slaughter did not decrease the meat quality (OLIVEIRA FILHO et al., 2014).

Thus the objective of this research was to evaluate the influence of different pre-slaughter methods (hypothermia in ice/water; anesthesia with the essential oils *Ocimum americanum* or *Lippia alba* and mechanical spiking) on the meat quality parameters of un-gutted Nile tilapia stored under refrigeration (4-6 °C) for 15 days.

5.2 Material and methods

5.2.1 Animals

All the procedures were approved by the Ethics Commission for the Use of Animals on October 4th 2017, under protocol nº 4446150817.

Nile tilapia (n=56) with a mean slaughter weight of 598.7 ± 112.0 g and mean length of 31.3 ± 2.3 cm were acquired from a producer near Pirassununga, SP, Brazil and

transported to the Aquiculture Laboratory of FZEA/State University of São Paulo (USP), Brazil. In the laboratory fish were allowed to acclimatize in 5000 L concrete tanks for 3 weeks. The water parameters were maintained in a range normal of water parameters for this species (pH: 7.4 ± 0.3; dissolved oxygen: 5.1 ± 1.3 mg/L; T: 25.9 ± 2.7 °C). The fish were fed with commercial fish feed (28% protein, Laguna® Sport 28, São Paulo, Brazil) twice a day, and fasted for 24 h prior to the experiments. After decreasing the water level in the tanks, the fish were captured using hand-held fish nets (5 fish per time) and taken to the laboratory in a process that took no longer than 2 minutes. In the laboratory the fish were maintained in 250 L plastic boxes with constant aeration (10 fish at a time).

5.2.2 Pre-slaughter stunning methods

The Nile tilapia were divided into four (n=14/group) pre-slaughter stunning method groups, i.e. anesthesia with essential oils (2 groups), mechanical spiking and hypothermia.

For the methods using anesthesia with the essential oils *Ocimum americanum* (OA) or *Lippia alba* (LA), the fish were captured individually and placed in glass aquariums (15 L) with the essential oil at 500 µL/L until the fish reached deep anesthesia. The oil was previously diluted in 98.9% ethanol in a proportion of 1:9 to prepare a stock solution of 100 µL/mL. After anesthesia and verification of the absence of behavior indicating consciousness (swimming, equilibrium, response to handling, response to painful stimuli and vestibulo-ocular reflex - VOR), the fish were submitted to cutting of the gills and placed in a water and ice slurry during 3 minutes for bleeding. The anesthetic mixture was used for a maximum of 10 fish and then exchanged with a new mixture.

Mechanical spiking (SP) was carried out using a commercial fish gun (Ikigun®, New Zealand). The fish were captured individually from the maintenance tank using a net and restrained on the Ikiboard and the shot fired through the side access, a process which took approximately 28 s, time during which the fish was exposed to the air. After the shot, any behaviour suggestive of consciousness was verified (breathing, reaction to a painful stimulus and VOR). In the absence of such behaviour, the gills were cut and the fish bled in the water and ice slurry for 3 minutes.

For hypothermia (HP) live chilling, the fish were captured from the concrete tank and placed directly in water and ice slurry (1:2) in a 250 L capacity tank. After 20 minutes of immersion in the hypothermia tank, their gills were cut and they were replaced in the water and ice slurry for 3 minutes to bleed. HP was chosen since it is the main pre-slaughter method used for fish in Brazilian slaughterhouses.

5.2.3 Meat quality indicators

After slaughter the fish were identified with labels, weighed, measured and stored in a cold chamber in Styrofoam boxes in alternate layers with ice. The boxes had valves at the bottom to make it easy to remove melted ice, and the soiled and melted ice was replaced daily. The Styrofoam boxes were stored in a cold chamber at approximately 6 ± 2ºC for 15 days.

The muscle pH value and the rigor mortis index (RMI) were measured at 0, 3, 6 hours and 1, 2, 3, 4, 8, 11 and 15 days after slaughter in 8 fish from each group. A cut was made in the skin caudal to the head to insert the pH electrode in the epaxial musculature, using a spade-shaped electrode specific for foods (Sensoglass, model SC03, SP, Brazil). The same cut was used for all measurements. The RMI was measured in the same fish from pH, according to BITO et al. (1983) with the following formula:

$$RMI\ (\%) = [(L_0 - L_T)/L_0] \times 100$$

where L0 (cm) is the vertical distance between the base of the caudal fin and the table surface, measured immediately after slaughter, and LT (cm) is the vertical distance between the base of the caudal fin and the table surface at selected time intervals after slaughter (CONCOLLATO et al., 2014).

The total volatile basic nitrogen (TVB-N) values were measured in the muscle of other eight fish per group, each fish was sampled at 0, 1, 3, 8, 11 and 15 days after slaughter, approximately 10 g of muscle without bones or skin each time was sampled according to FOGAÇA et al. (2009). Each un-gutted fish was identified and refrigerated in a cold chamber in Styrofoam boxes in alternate layers with ice and reused for all times of sampling.

The samples were cut into small pieces and homogenized with 60 mL 10% trichloroacetic acid in an ultraturrax homogenizer (IKA-T25). The homogenized samples were filtered (Whatman 150 mm) after 4 hours of rest at 22 ºC, and 50 mL of the filtrate reserved for distillation. Two aliquots of each sample were distilled in Kjeldahl tubes, adding 1 g of magnesium oxide to each 25 mL aliquot. The distillate was received in 15 mL of mixed indicator until completing a volume of 75 mL, using a TE-0363 nitrogen distiller. The mixture was titrated with 0.02 N HCl until the color changed to pink and the volume in mL of HCl used noted. The TVB-N, expressed in mg / 100g of muscle was calculated using the following formula:

$$TVB - N\ (mg/100\ g) = \frac{\text{HCl (ml)} * (\text{HCl normality}) * 14 * \text{Extraction (ml)} * 100}{20 * \text{sample weight (g)}}$$

Three fish per group were fillet for the instrumental color was measured on the lateral surface of the fillets without skin at 0, 1, 3, 8, 11 and 15 days after slaughter. The left side fillet of each fish was used for the analyses during the first week and the right side fillet for those of the second week, thus avoiding rapid deterioration due to handling. Fillets were stored in identified plastic bags, and refrigerated under the same storage conditions of un-gutted fish. The color was measured using a Miniscan EZ colorimeter (Hunterlab, USA) at three points of the fillet after calibrating the apparatus using a white standard. A D65 light source was used with a hue angle of 10º and cell aperture of 30 mm (VARGAS et al., 2013). Three measurements were made at each point and the means calculated for the L*, a* and b* values. The L* values evaluate lightness from black to white (0-100), a* the color green (negative) to red (positive), and b* the colorsblue (negative) to yellow (positive). The whiteness (W) was calculated using the following equation (SUEMITSU; CRISTIANINI, 2019):

$$W = 100 - ((100 - L^*)^2 + (a^*)^2 + (b^*)^2)^{0.5}$$

The total color difference between the hypothermia group (HP) and treatments (SP, OA and LA) at the same time is given by ΔE, which was calculated as (HERATH; HAGA; SATOH, 2016):

$$\Delta E^* = \sqrt{(L2^* - L1^*)^2 + (a2 - a1^*)^2 + (b2^* - b1^*)^2}$$

Where difference from ΔE means <1 = not perceptible by human eyes, 1-2 = perceptible through close observation, 2-10 = perceptible at a glance, 11-49 = colors are more similar than opposite and 100 = colors are more similar than opposite.

To determine ATP (Adenosine triphosphate) and its metabolites, three fish were used per group taking samples at 0, 1, 2, 3, 4, 8, 11 and 15 days after slaughter. Each un-gutted fish was identified and refrigerated in a cold chamber in Styrofoam boxes in alternate layers with ice and reused for all times of sampling.

One gram of muscle without skin was sampled at each time according to BURNS; KE, (1985), homogenized with 10 mL 0.6 M perchloric acid using an ultraturrax (IKA-T25), filtered (Unifil 80 g/m²), and 1 mL of the filtrate pipetted into an Eppendorf tube plus 1 mL phosphate buffer (pH 7.6). The Eppendorf was centrifuged for 5 min and the supernatant frozen at -18 ºC before injecting into the HPLC. The supernatant was filtered

through a nylon filter with a pore diameter of 0.45 µm and the HPLC used was a Shimadzu Prominence with a binary injection pump equipped with a 250 mm x 4.6 mm reverse phase C18 column with a particle diameter of 5 µm and detection at 254 nm (Shim-pack GIST).

The mobile phase was ultrapure water (Direct-Q®3) with the pH value adjusted to 7.0 with 0.1 N NaOH. The injection volume was 10 µL, the flowrate 1 mL/min, and the LCSolution software was used to observe the data of the chromatogram. The ATP standard and those of its metabolites (ADP adenosine diphosphate; AMP adenosine monophosphate; IMP inosine 5'-monophosphate; Ino inosine; Hx hypoxanthine) were acquired from Sigma-Aldrich. The standards were prepared at 1 mg/mL in the mobile phase. Knowing the molecular weight and purity of each standard, one can inject volumes with known concentrations, and each standard was injected individually to confirm the retention time. Five mixture with its six standards in increasing concentrations was also read to correlate the values of the area of each peak with the known concentration. The values obtained for these metabolites allowed the calculation of the freshness index of the fish known as the K-value. The K-value is defined as the percentage of the amount of Ino and Hx to the total amount of ATP-related compounds (TEJADA, 2009):

$$K - value\ (\%) = [\left(\frac{Ino + Hx}{ATP + ADP + AMP + Ino + Hx}\right) * 100]$$

In addition to the meat quality analyses, samples of the gills were taken from five fish per group at the moment of bleeding, in order to carry out histological analyses with simple dyeing using hematoxylin and eosin. The gills were cut and fixed in 10% neutral buffered formalin and the tissues processed in a way similar to the procedure described by GENTEN et al. (2009). The histopathological analyses were carried out by a veterinary surgeon with more than 5 years of experience in fish pathology.

5.2.4 Statistical analyses

Prior to a data comparison between the groups, a normality test of each variable was carried out using the Shapiro-Wilk test. The variables pH, RMI, TVB-N, L^* and a^* did not follow a pattern of normality, but the values for b^* did. The concentrations of ATP, ADP, AMP, IMP, INO and Hx also failed to show a pattern of normality. When significant differences were found, the Kruskal-Wallis test was used followed by Dunn's test to make a comparison amongst the groups and also compare the nonparametric variables. One-way ANOVA was applied to the b^* data considering equal variances,

followed by Tukey´s test to compare the groups at the same time. The data were presented as the median ± IQR.

5.3 Results and discussion

Figure 5.1 shows the values obtained for muscle pH. Immediately after slaughter (0 h) the values were similar for all groups (7.13 ± 0.43 SP, 6.86 ± 0.55 HP, 7.33 ± 0.57 OA, 7.23 ± 0.48 LA), but significant differences ($p<0.05$) were found at 3, 6 and 24 hours after slaughter, being higher in group LA than in group SP (Figure 5.1 A). Similar values were reported immediately after slaughter for tilapia slaughtered using CO_2 or immersion in iced water, with mean values of 7.0 ± 0.09 (OLIVEIRA FILHO et al., 2014). For *Rhamdia quelen* submitted to different pre-slaughter methods using anesthesia with 300 µL/L of the essential oil *Lippia alba* combined with electric stunning or hypothermia, the post-slaughter pH values were close to 7.0 with no difference between the groups (VEIT et al., 2018). In tambaqui (*Colossoma macropomum*) allowed to rest (48 h) or slaughtered immediately after transport to the slaughterhouse and slaughtered by either hypothermia or asphyxia, the muscle pH values soon after slaughter were 6.2, with no difference between the groups (MENDES et al., 2017), suggesting that slaughter methods considered inhumane can alter the pH of the muscle.

The muscle pH values found 3, 6 and 24 h after slaughter suggest that immersion in the essential oil *Lippia alba* as a pre-slaughter anesthetic preserved greater muscle energy up to 24 h compared to HP and SP groups. The natural pH value of live fish is just above 7.0, typically about 7.3, but this falls markedly after death as the fish goes through *rigor mortis* and glycogen is converted to lactic acid (HUSS, 1995; POLI et al., 2005). As from 2 days the pH values were similar for all groups, suggesting that the humane slaughter methods did not negatively alter the pH values of chilled Nile tilapia meat during storage (Figure 5.1 B). After 15 days of refrigerated storage, the pH values of the fish were below 7.0, in agreement with Brazilian legislation for fresh fish (BRASIL, 2017), which states that the pH value of fish meat should be below 7.0. This result agrees with that reported for tilapia, but without information concerning the slaughter method, and stored under refrigeration (VÁZQUEZ-SÁNCHEZ et al., 2020).

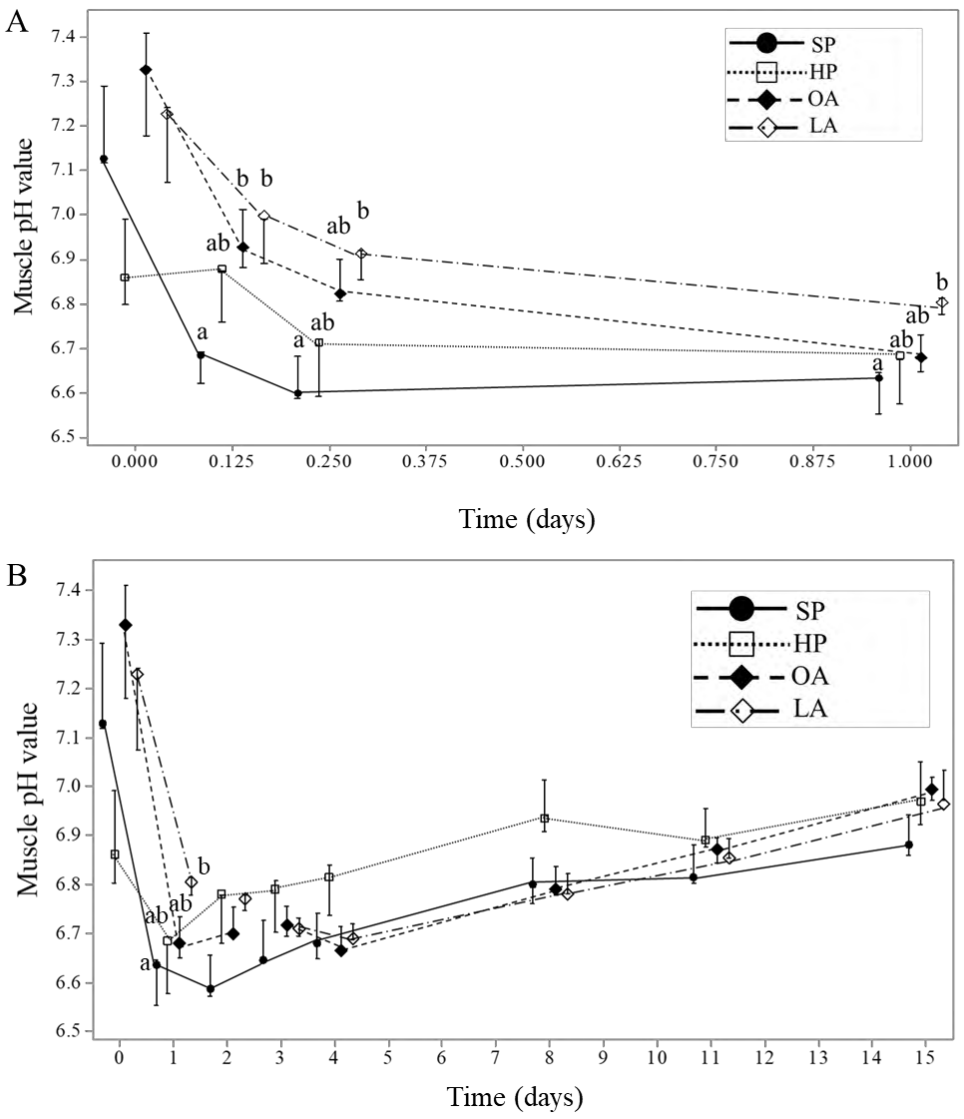

Figure 5.1 - Muscle pH value in Nile tilapia (n=8 fish/group). (A) First 24 hours (0.125 = 3 h; 0.25 = 6h; 1 = 24 h) (B) 15 days. The letters indicate differences amongst the groups for the same point in time according to the Kruskal-Wallis test followed by Dunn's test (P<0.05).

Figure 5.2 shows the progress of the entrance into the state of *rigor mortis*. The values for the RMI obtained at 0, 1, 2, 3 and 4 days after slaughter cannot be compared due to an absence of variance in the data. Significant differences ($p<0.05$) were found between the groups at 3h after slaughter. The values for the HP group (Figure 5.2 B) indicate a quicker installation of *rigor mortis* process as compared to the OA and LA groups after 3 h (45 ± 59 % SP, 78 ± 44 % HP, 30 ± 14% OA, 33 ± 12 % LA). Hypothermia induced a rapid installation of *rigor mortis*, and other papers have reported maximum (100%) *rigor mortis* 7 h after slaughter in Nile tilapia slaughtered by hypothermia and maintained under refrigeration (OLIVEIRA FILHO et al., 2014). Therefore, the results of the present study show a delay in the installation of maximum

rigor mortis using humane methods at pre-slaughter in contrasts to the traditional methods used in fish slaughterhouses, hypothermia live chilling. Hence, the essential oils used before slaughter can improve the quality delaying the installation *rigor mortis* in un-gutted Nile tilapia under refrigeration storage. The filleting process can be less efficient and take longer when the *rigor mortis* of the fish is complete. Thus an increase in the time taken to install *rigor mortis* can make the manual fish filleting process easier, optimizing the worker's time and the procedure (POLI et al., 2005).

The RMI values were similar at 4, 11 and 15 days after slaughter, but the OA group maintained a mean *rigor mortis* index of 100% up to 15 days' post-slaughter. Thus the stunning and humane slaughter methods did not differ from hypothermia in relation to the resolution of *rigor mortis*. Similar to the present results, according to OLIVEIRA FILHO et al. (2014). the resolution of *rigor mortis* started after day 12 (288 h) in Nile tilapia slaughter by hypothermia or CO2 immersion. In addition, the resolution of *rigor mortis* in Nile tilapia slaughtered by hypothermia and stored at 5 ºC started 200 h post-slaughter as compared to 126 h post-slaughter when stored at 0 ºC (MONTOYA-CAMACHO et al., 2020). *Rigor mortis* may last for several days before the fish flesh becomes tender due to the action of endogenous proteases, without, however, reaching the same elasticity as before *rigor mortis*. Several proteolytic systems, consisting of enzymes and inhibitors, are involved in the degradation of the structural proteins of fish muscle, these being acid cathepsins located in the lysosomes, alkaline proteinases, proteosomes (multicatalytic proteinase complexes), calpains, aminopeptidases, collagenases and elastases (OEHLENSCHLÄGER; REHBEIN, 2009).

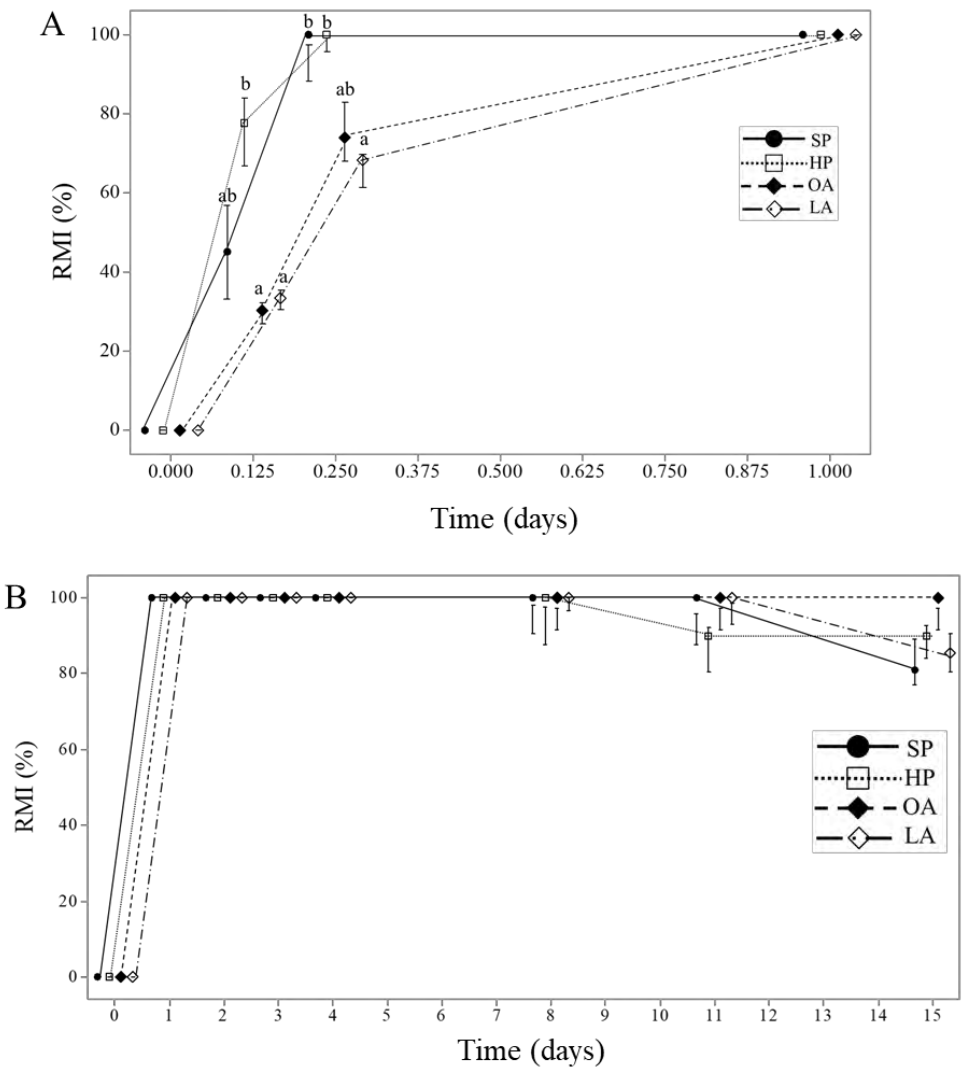

Figure 5.2 - RMI (%) in Nile tilapia (n=8 fish/group). (A) First 24 hours (0.125 = 3 h; 0.25 = 6h; 1 = 24 h) (B) 15 days. The letters indicate differences amongst groups at the same point in time according to the Kruskal-Wallis test followed by Dunn´s test (P<0.05).

Figure 5.3 shows the values obtained for TVB-N, which were similar for the groups at 0, 1, 3, 8 and 15 days post-slaughter. After 11 days, larger amounts were found in HP (14.7 ± 1.6 mg/100g) than in the other groups (12.3 ± 2.9 SP, 12.8 ± 1.3 OA, e 12.6 ± 2.9 LA). However, after 15 days the values were similar for all the groups. Nile tilapia slaughtered by hypothermia or CO2 and stored under refrigeration, as reported by OLIVEIRA FILHO et al. (2014), showed similar values for TVB-N (10.98-15.22 mg/100g) to those found in the present study. TVB-N analyses have traditionally been used as an indicator of quality in fishery products stored on ice, including the measurement of trimethylamine, dimethylamine and ammonia (HUSS, 1995). According to Brazilian norms, the limit for such products to be considered spoiled and unfit for human consumption is >30mg of TVB-N/100 g of muscle (BRASIL, 2017). Working

with Nile tilapia, but giving no information on the slaughter method used, VÁZQUEZ-SÁNCHEZ et al. (2020) in un-gutted Nile tilapia refrigerated under conditions similar to those used in the present study, obtaining higher TVB-N values of 18.62 ± 0.2 (mg/100g) after 13 days of storage.

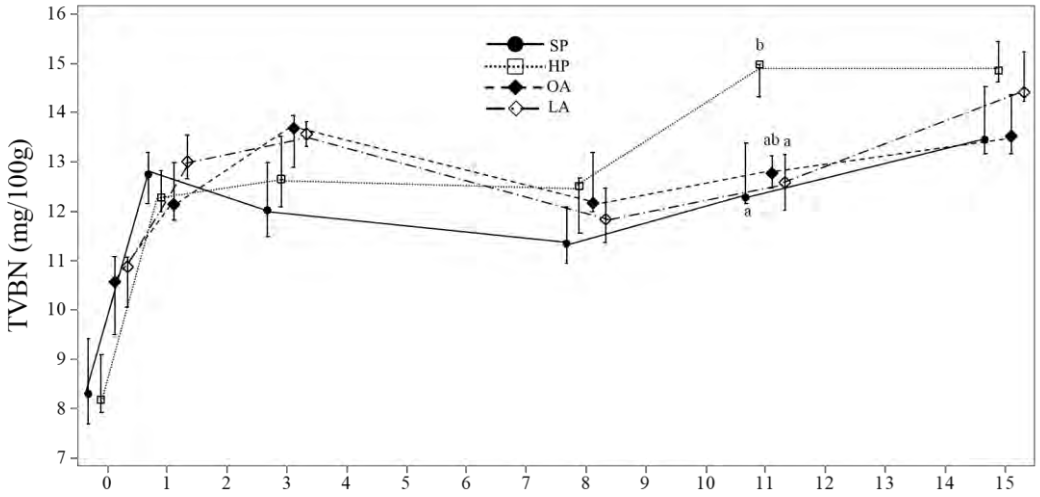

Figure 5.3 - TVB-N (mg/100g) values Nile tilapia (n=8 fish/group). The letters indicate difference amongst the groups at the same time point, according to the Kruskal-Wallis test followed by Dunn´s test (P<0.05).

Table 5.1 shows the results obtained for the instrumental color of the Nile tilapia fillets, including *L**, *a**, *b** and W. The HP fillets showed higher values for lightness (*L**) (62.9 ± 5.9) than SP (59.5 ± 2.5) and OA (57.9 ± 2.9) (P<0.05). These values for *L** were similar to those previously found for Nile tilapia slaughtered by hypothermia (OLIVEIRA FILHO et al., 2014) and by asphyxia (61.7 ± 5.6) (SUEMITSU; CRISTIANINI, 2019). The values obtained for *L** for the fillets refrigerated for 360 h (15 days) showed no differences between the groups, but lower values for *L** (57.35 ± 1.69) were reported by MONTEIRO et al. (2019) in Nile tilapia, with no information about the slaughter method.

The values for *a** were different between the groups at the different times evaluated, except at 264 h (11 days) (P<0.05). Similar values for *a** were observed in fillets from Nile tilapia slaughtered by hypothermia and stored under refrigeration (3.0 ± 0.4) (OLIVEIRA FILHO et al., 2014), but in another study with the same species, slaughtered by asphyxia, lower values were found for *a** in the fillets (1.67 ± 2.05) (SUEMITSU; CRISTIANINI, 2019). The values for *b** were different between the groups at all times evaluated (p<0.05). SUEMITSU & CRISTIANINI (2019) reported similar values for *b**

(11.61 ± 1.96) for Nile tilapia fillets, but MONTEIRO et al. (2019) reported lower values (5.63 ± 0.47).

The values for *W* showed differences between the groups at all evaluation times (Table 5.1). Initially higher values were reported for the HP fillets, similar to those reported previously (59.9 ± 2.8) by (SUEMITSU; CRISTIANINI, 2019). However, after 15 days of evaluation, higher values were observed in the OA fillets, in contrast to the values found in LA and with no difference between SP and HP.

Table 5.1 Color parameter (*L**, *a**, *b**, *W* and *ΔE*) values in Nile tilapia fillets (n=3 fillets/group). The letters indicate differences amongst the groups for the *L** and *a** values at the same time of evaluation, according to the Kruskal-Wallis test followed by Dunn´s test (P<0.05), and for the *b** values according to the one-way ANOVA followed by Tukey´s test (P<0.05).

Color	Groups	Time					
		0	24	72	192	264	360
*L**	SP	59.5 ± 2.5 ab	60.2 ± 1.9 a	59.2 ± 3.9 a	61.3 ± 4.8 a	65.0 ± 3.6 a	66.0 ± 4.3
	HP	62.9 ± 5.9 c	66.0 ± 3.2 b	62.6 ± 2.4 ab	67.5 ± 2.8 ab	69.0 ± 7.5 b	68.3 ± 5.2
	OA	57.9 ± 2.8 a	61.3 ± 1.4 a	64.3 ± 0.9 bc	69.4 ± 5.2 b	71.4 ± 0.8 b	69.6 ± 1.6
	LA	63.1 ± 6.0 bc	66.4 ± 1.1 b	68.5 ± 2.9 c	70.2 ± 2.1 c	70.8 ± 7.5 ab	68.8 ± 4.8
*a**	SP	2.9 ± 1.8 a	4.1 ± 0.9 bc	4.0 ± 1.8 b	3.0 ± 2.1 b	2.7 ± 2.0	3.0 ± 2.0 b
	HP	2.9 ± 2.2 a	2.9 ± 2.1 ab	5.3 ± 1.3 b	3.0 ± 2.7 b	3.1 ± 2.7	2.8 ± 1.3 b
	OA	5.3 ± 3.1 b	5.2 ± 1.1 c	3.8 ± 0.7 b	3.7 ± 2.2 b	1.3 ± 0.2	0.4 ± 0.9 a
	LA	1.6 ± 2.0 a	2.1 ± 1.6 a	0.9 ± 1.9 a	0.8 ± 0.4 a	3.3 ± 2.9	3.5 ± 0.9 b
*b**	SP	12.8 ± 1.0 a	12.7 ± 1.0 a	13.6 ± 1.1 ab	16.6 ± 2.8 c	13.7 ± 1.0 a	13.2 ± 2.1 a
	HP	12.7 ± 4.5 a	12.6 ± 0.8 a	15.0 ± 1.0 c	14.5 ± 1.1 bc	14.3 ± 1.3 a	15.3 ± 2.5 b
	OA	17.1 ± 1.6 c	16.2 ± 0.8 c	13.8 ± 1.3 b	14.4 ± 0.7 b	14.3 ± 0.7 ab	13.4 ± 2.0 ab
	LA	15.2 ± 2.7 b	14.9 ± 2.6 b	13.2 ± 3.4 a	12.6 ± 0.8 a	14.9 ± 0.6 b	16.4 ± 2.0 c
W	SP	58.0 ± 2.4 ab	58.8 ± 1.8 a	57.3 ± 3.9 a	58.9 ± 5.5 a	63.5 ± 3.8 a	64.8 ± 4.3 ab
	HP	61.8 ± 5.4 c	64.7 ± 3.3 b	60.4 ± 2.4 ab	65.9 ± 3.4 ab	67.6 ± 7.5 b	66.8 ± 5.1 ab
	OA	55.1 ± 3.4 a	58.7 ± 1.5 a	62.5 ± 1.2 bc	67.8 ± 5.6 b	69.9 ± 0.6 b	68.3 ± 1.5 b
	LA	61.0 ± 5.7 bc	65.1 ± 0.8 b	67.7 ± 2.8 c	68.8 ± 2.1 b	69.2 ± 7.8 ab	66.7 ± 4.5 a
ΔE	SP	3.42	5.97	3.99	6.53	4.10	3.08
	OA	7.13	6.35	2.56	2.02	3.03	3.27
	LA	2.52	2.43	7.55	3.91	1.94	1.39

The differences in color between the Nile tilapia fillets submitted to different stunning methods were not clear. Hypothermia leads to peripheral vasoconstriction (CURRAN et al., 1986; DONALDSON et al., 2008) and the blood flow decreases and is distributed to the essential organs such as the gills, heart, kidneys, brain and liver (VAN DEN BURG et al., 2005). Thus the muscle receives less irrigation as compared to a physiological situation, presenting higher values for *L**, lower values for *a** and *b** and higher values for W. The principal change in color (perceptible at a glance) (*ΔE*) was

observed in Nile tilapia fillets submitted to LA anesthesia in contrast to HP at 72 hours, and OA anesthesia in comparison to HP at 0 and 24 hours. In addition, studies determining the haemoglobin and myoglobin concentrations in the muscles would be useful in order to understand the color changes in the fillets.

Figure 5.4 shows the values determined for ATP and its metabolites (ADP, Adenosine diphosphate; AMP, Adenosine monophosphate; IMP, Inosine 5'-monophosphate; Ino, Inosine; Hx Hypoxanthine) and also the *K-values*. After 192 h (8 days), the values for ATP, ADP and AMP were no longer detected on the chromatogram, so the K-values were only calculated up to 196 h. The values for AMP and IMP were different between the groups at 0h and 72h, respectively ($P<0.01$). However, the values for ATP, ADP, INO, Hx and the *K-value* were similar for the different groups. After 8 days (192 h) of storage, the *K-values* were similar among groups (46.2% SP; 50.0% HP; 52.5% OA; and 55.8 LA).

The decrease in ATP, ADP and AMP with time is in agreement with the degradation of these metabolites and consequent increase in IMP, INO and Hx. The catabolism of ATP to IMP has been reported to be essentially caused by endogenous enzymes. Nevertheless, the hydrolysis of INO and formation of Hx may also result from bacterial enzymes (HONG; REGENSTEIN; LUO, 2017; JONATHAN; MAINA; and MUSA, 2013). The fresh quality of meat is highly correlated with the biochemical changes taking place during the post-mortem period. Of the chemical methods used to determine freshness, the determination of the concentrations of ATP and its breakdown products, ADP, AMP, IMP, INO, and Hx, are extensively used to calculate a number of different specific freshness indexes in a wide variety of fish. These freshness indicators are derived from measurements of the relative concentrations of ATP and its breakdown products (HONG; REGENSTEIN; LUO, 2017; SAITO; ARAI; MATSUYOSHI, 1959; and TEJADA, 2009). The values for ATP in Nile tilapia slaughtered by hypothermia or CO_2 were detected up to 24 h after slaughter, and were highest in the first hours after slaughter in the group slaughtered by CO_2 (OLIVEIRA FILHO et al., 2014).

The *K-value* is considered to be one of the most effective indicators of fish freshness. Fish products with *K-values* below 20% are categorized as very fresh, those with K-values below 50% as moderately fresh, and those with *K-values* above 70% as un-fresh (SAITO; ARAI; MATSUYOSHI, 1959; and TEJADA, 2009). However, a shortcoming of the K-value as a freshness index is its dependence on species, seasons, handling conditions, slaughtering methods, temperature during storage and the presence

of ice. Maximum *K-values* for the rejection of the fish have been reported for several species ranging from: 80% for European white fish (*Coregonus wartmanni*); 70–80% for Atlantic salmon (*Salmo salar*); 60–70% for European seabass (*Dicentrarchus labrax*), farmed turbot (*Psetta maxima*) and wild turbot (*Scophthalmus maximus*); and farmed gilthead seabream (*Sparus aurata*) (TEJADA, 2009).

Sensory (Quality Index Method - QIM) and microbiological analyses and the determination of the ATP values during storage are required to establish the maximum acceptable levels for the K-value. Nile tilapia slaughtered by hypothermia and refrigerated for 12 days were processed in three ways: un-gutted, gutted and filleted, and the maximum acceptability times estimated at 12, 10 and 6 days, respectively (LALY et al., 2017). In addition, Brazilian scientists determined the rejection point of un-gutted tilapias stored under refrigeration as 10.8 days, using the QIM scheme, physicochemical changes and microbiological analyses (VÁZQUEZ-SÁNCHEZ et al., 2020). The *K-value* of un-gutted Nile tilapia after 12 days of refrigerated storage was 61.2% (RONG et al., 2009). Thus, according to the results of the present study, the humane slaughter methods did not negatively affect the quality parameters of the meat (pH, TVB-N and K-values) of Nile tilapia (un-gutted) stored under refrigeration for 8 days, as compared to the traditional method of hypothermia.

The biomass used in this study was 43.1 g/L for OA and 41.2g/L for LA. During these experiments the anaesthetics solution was refreshed at each 10 fish, more research is necessary to determinate if the same solution can be used in more than ten fish. Future studies can test lipidic oxidation and microbiology in Nile tilapia stunned under different methods at slaughter, as a complement to our results. In 2019, the unitary price (5 mL) of *Ocimum americanum* was R$33,00 (8.3 USD) and *Lippia alba* was R$44,00 (11.0 USD). Additionally, calculating the residual of essential oils in the meat in future trials can be useful for food safety. The increase in the production of essential oils can reduce the price for industrial use during the pre-slaughter of fish.

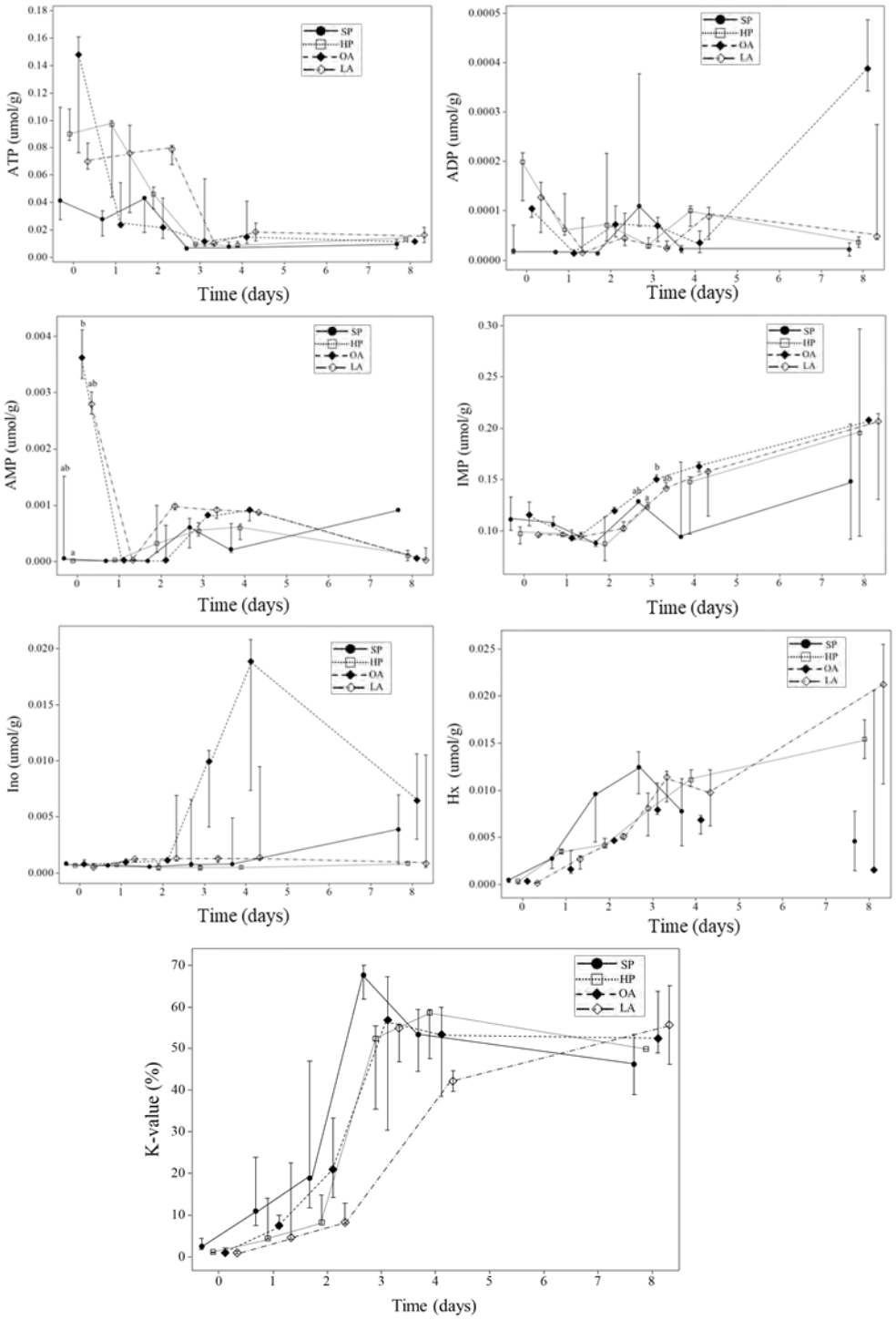

Figure 5.4 - Values for ATP, ADP, AMP, IMP, Ino and Hx (µmol/g) and the K-values (%) in Nile tilapia (n=3 fish/group). The letters indicate differences amongst the groups at the same time according to the Kruskal-Wallis test followed by Dunn's test ($P<0.05$).

Slight and slight to moderate wounds were observed in the gills by way of the histological analyses, mainly classified as circulatory changes: edema (1/5 SP, 1/5 OA), congestion (3/5 SP; 4/5 HP; 4/5 OA; 3/4 LA), telangiectasia (2/5 SP; 2/5 HP; 1/5 OA; 1/4 LA) and hemorrhage (1/4 LA).

One sample from the LA group was not analyzed due to problems with the cut which impeded adequate visualization. Figure 5 shows the images of gills normal (A), with congestion (B) and with telangiectasia (C).

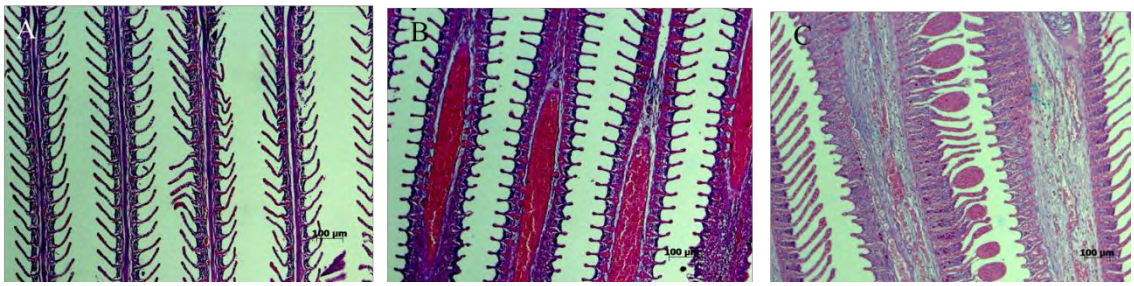

Figure 5.5 - Representative histology of the gills in Nile tilapia: (A) normal, (B) with congestion and (C) with telangiectasia, 10x.

The most frequent wound found in the gills was congestion, observed in 74% of the individuals (n=14/19). Telangiectasia was the second most frequent alteration, observed in 42% (n=8/19), edema in 11% (n=2/19) and hemorrhage in 5.3% (n=1/19).

The changes found in the SP group can be explained by the mechanical impact of the slaughter method, since the fish receive the impact with lateral access to the brain, and hence the kinetic energy is distributed through the gills. ROTH et al. (2007) observed hemorrhages in the eyes of Atlantic salmon (*Salmo salar*) slaughtered by spiking, but no hemorrhages were found on the fillet surfaces (LAMBOOIJ et al., 2010). In the HP group, the heat shock induced peripheral vasoconstriction (DONALDSON et al., 2008) in an attempt to centralize the blood flow to the essential organs. Thus, it is expected that in fish submitted to hypothermia the blood flow to the gills increased in an attempt to optimize the exchange of oxygen. Similar changes were observed in the gills of carp (*Cyprinus carpio*) slaughtered by hypothermia where hemorrhages were observed as well as primary and secondary separations between the lamellas (SUWANDI et al., 2020). The changes observed in the OA and LA are not well understood and should be studied in the future. The changes observed in the gills were probably related to the pre-anesthetic process (fasting, crowding, and transference from the maintenance to the experimental tanks).

5.4 Conclusion

The use of mechanical spiking and anesthesia with the essential oils *Ocimum americanum* (OA) or *Lippia alba* (LA) in un-gutted Nile tilapia under refrigeration storage did not negatively change the following quality parameters of the meat: pH value,

rigor mortis, TVB-N and K-value as compared to the traditional method of hypothermia. The essential oils can improve the *rigor mortis* process, delaying the installation. Humane slaughter methods can be adopted for Nile tilapia without prejudicing the quality of the final product. Research support for the development of humane slaughter methods for fish on an industrial scale is urgent and very necessary in Brazil.

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6 Final considerations

The methods used at pre-slaughter process in fish industry in Brazil are not considered humane and should be replaced by humane slaughter methods. Our results refute the idea that hypothermia (live chilling in ice/water) is an efficient stunning method for Nile tilapia. In this thesis, alternative methods for pre-slaughter of Nile tilapia were studied. The use of *Ocimum americanum* or *Lippia alba* essential oils as anaesthetics were effective to induce unconsciousness from both behaviour recordings and brain activity observations through electroencephalography (EEG). The concentration of essential oils at 500 µL/L induced anaesthesia during immersion of less than 180 s, with a duration of unconsciousness higher than 200 s. Furthermore, the use of essential oils did not decrease sensory quality of Nile tilapia fillets.

The density or biomass (g of fish / litre of anaesthetic solution) of fish used during anaesthesia with *Ocimum americanum* or *Lippia alba* essential oils was estimated. On chapter 2 was 31.4 g/L OA and 37g/L for LA. On chapter 4 the density was 20.0 g/L for OA, and 17.6 g/L for LA during EEG analysis and 36.2 g/L for OA and 37.6 g/L for LA during behavioural analysis. On chapter 5 the biomass was 43.1 g/L for OA and 41.2g/L for LA. During these experiments the anaesthetics solution was refreshed each 10 fish. For the use of essential oils in industrial scale more research is necessary.

The use of mechanical spiking with the commercial gun needs refinement, as firing must be precise and accurate to induce an effective stunning. Guns adapted for use in Nile tilapia, weighing more than 655 g must be developed and tested.

Research is necessary to advance knowledge and establish parameters for electrical stunning in species of interest in Brazil. Such parameters should be initially tested from behavioural studies with a maximum duration of exposure to electric currents of 1 s. Longer exposure can cause paralysis and apparent unconsciousness. After researching behavioural responses, electrical parameters must be validated for unconsciousness through EEG studies.

The use of anaesthesia with essential oils of *Ocimum americanum* or *Lippia alba* at pre-slaughter in Nile tilapia kept meat quality under refrigeration for 8 days, not being an impediment to replace hypothermia. Hypothermia is not a method that causes unconsciousness, but a high degree of suffering in fish, therefore its replacement by humane slaughter methods in Brazil is urgent and necessary.

We are open to discuss the results presented here to update the "*Instrução Normativa 03 de 2000 do Ministério da Agricultura, Pecuária e Abastecimento - MAPA"*. Hypothermia at pre-slaughter of fish, commonly used in the Brazilian industry, consumes electricity, water and time. Such investment could be redirected to the development of humane slaughter methods at the industrial scale in Brazil, thus reducing fish suffering. The changes in the Brazilian fish industry should include all the links involved in the chain, from producers, processing industry, traders and consumers. Implementing humane fish slaughter methods will put Brazil at the vanguard of fish welfare discussions.

ANNEX 1 Poster presented in "Latin American and Caribbean Aquaculture 2019 Lacqua 2019, São José, Costa Rica, 19-22 November 2019"

Óleos essenciais de *Ocimum americanum* e *Lippia alba* como anestésicos no pré-abate de tilápia do Nilo: análise sensorial dos filés

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Introdução

A normativa de abate humanitário do Brasil não inclui peixes. A hipotermia em água e gelo é o método utilizado no pré-abate de peixes no Brasil, embora tal método não seja recomendado pela OIE. O objetivo deste estudo foi avaliar os óleos essenciais de *Ocimum americanum* e *Lippia alba* como anestésicos para tilápia do Nilo e sua influência sob o aroma e sabor dos filés.

Material e métodos

Todos os procedimentos foram aprovados pelo Comitê de Ética no Uso de Animais da FZEA/USP (4446150817) e pelo Comitê de Ética em Pesquisa com Seres Humanos (3.326.401).

Tratamentos (n=20/grupo)

Tilápias do Nilo (574.0 ± 170.8 g; 30.9 ± 3.1 cm)

Controle C
Hipotermia na água e gelo

Peixe anestesiado

Ocimum americanum OA 500uL/L

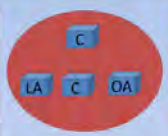
Lippia alba LA 500 uL/L

Processamento

Abate
Filetagem
Refrigeração 2-4 °C (24h)
Preparação

Análise Sensorial com 120 provadores

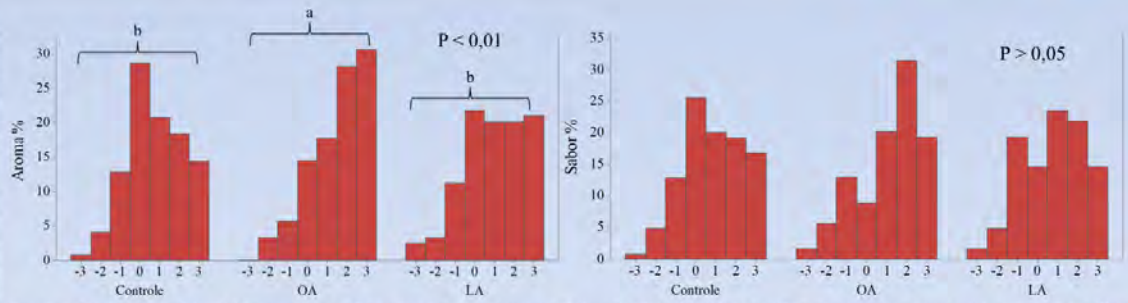
Cabines individuais para a análise sensorial



Controle identificado, três amostras codificadas incluindo um controle escondido

Comparação com o controle	Escala
Muito melhor que o controle	3
Moderadamente melhor que o controle	2
Ligeiramente melhor que o controle	1
Sem diferença do controle	0
Ligeiramente pior que o controle	-1
Moderadamente pior que o controle	-2
Muito pior que o controle	-3

Resultados e conclusão



Distribuição dos scores dos 120 provadores não treinados para Aroma e Sabor de filés de tilápia do Nilo abatidas por hipotermia (controle) e óleos essenciais de *Ocimum americanum* (OA) e *Lippia alba* (LA). Letras diferentes indicam diferença significativa entre os tratamentos (P<0,05, Teste de Kruskal-Wallis, seguido do teste de Dunn).

O uso do óleo essencial de *O. americanum* no pré-abate melhora o aroma no filé de tilápia do Nilo. Por não causar diminuição da qualidade sensorial dos filés, a anestesia com os óleos essenciais de *O. americanum* e *L. alba* pode ser uma alternativa ao uso de hipotermia no pré-abate de peixes no Brasil.

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ANNEX 2 Poster presented in "53rd Congress of the ISAE International Society for Applied Ethology ISAE 2019, Bergen Norway, 5-9 August 2019"

Mechanical spiking as a killing method in Nile tilapia (*Oreochromis niloticus*) – A pilot study

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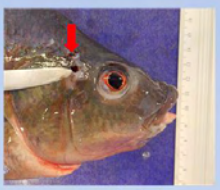
Introduction

Hypothermia in ice and water is historically used in Brazil in the slaughter process in most fish slaughterhouses (Oliveira Filho et al., 2014), although not recommended by OIE. Additionally, humane slaughter regulation does not include fish. Nile tilapia is the most produced and consumed species in Brazil, with 350.000 tons/year. The study aimed to assess mechanical spiking in the induction of unconsciousness as a killing method in Nile tilapia.

Material and Methods

All procedures were approved by the Ethics Committee on Animal Use at the University of São Paulo (4446150817). Nile tilapia was divided in lateral (L) and frontal (F) access of the captive bolt gun, using a commercial gun for fish – Ikigun® (Fig 1).



n= 40 fish
574.0 ± 170.8 g; 30.9 ± 3.1 cm

- Number of attempts for an efficient penetration
- Immediate loss of consciousness (swimming, equilibrium, handling, painful stimulus, vestibulo-ocular reflex (VOR) and breathing) (Kestin et al., 2002)
- Number of fish with recovery indicators
- *Rigor mortis* index (RMI) evaluation at 0, 3, 6, 24, 48, 72 and 120 h after bleeding (n=10 per group)

Fig 1. Representation of methodology and point of access used to assess mechanical spiking in Nile tilapia, February 2019, State of São Paulo.

Results and Discussion

All L fish lost consciousness immediately after the shot, in contrast with 95% F fish (P=1.0, df=39). All fish that lost consciousness immediately after the shot did not swim, lost equilibrium, did not respond to painful stimuli or handling, did not show any VOR and ceased breathing. Additionally, brain hemorrhages were noted (Fig 2).

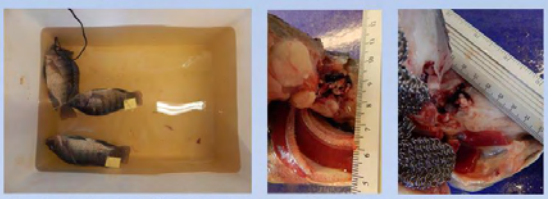


Fig 2. Behavioural evaluation and dissection of skull to assess mechanical spiking in Nile tilapia, February 2019, State of São Paulo.

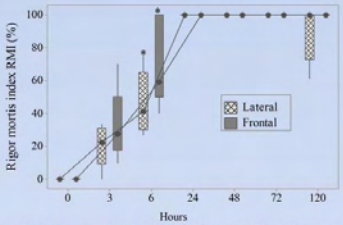


Fig 3. Median of *rigor mortis* index (%), 10 Nile tilapia per group, February 2019, State of São Paulo, differences between groups (*P<0.05*, Mann-Whitney test).

Attempts for an effective shot did not differ between groups (1.15 ± 0.49 L; 1.05 ± 0.22 F) (P=0.5536, df=39). One L fish (5%) showed indicators of recovery, different from 25% F fish, which showed signs of recovery (P=0.182, df=39). The progress of RMI was delayed in L fish (median, min-max) (41%, 27-75%) at 6h post-slaughter, in contrast with F fish (59%, 40-100%) at the same period of time (P=0.0445, df=19) (Fig 3). However, previous studies from our research team showed that all tilapia submitted to hypothermia reached full *rigor mortis* (100%) at 6h.

Conclusion

Mechanical spiking is effective to induce unconsciousness without recovery, delaying the onset of rigor mortis in Nile tilapia, and its use in an automatic system may be an alternative to slaughter.

References

Kestin, S., Van de Vis, J., Robb, D., 2002. Protocol for assessing brain function in fish and the effectiveness of methods used to stun and kill them. Vet. Rec. 150, 302–307.

Oliveira Filho, P., Oliveira, C., Sobral, P., Balieiro, J., Natori, M., Viegas, E., 2014. How stunning methods affect the quality of Nile tilapia meat. CyTA - J. Food 13, 56–62. doi:10.1080/19476337.2014.911211

ANNEX 3 Approval by the animal research ethics committee

UNIVERSIDADE DE SÃO PAULO
Faculdade de Zootecnia e Engenharia de Alimentos
Comitê de Ética em Pesquisa da FZEA

CERTIFICADO

Certificamos que a proposta intitulada "Avaliação de inconsciência, por meio de eletroencefalografia (EEG), no processo de insensibilização, por eletronarcose, percussão e anestesia para abate humanitário de tilápia do Nilo", protocolada sob o CEUA nº 4446150817, sob a responsabilidade de **Daniel Santiago Rucinque Gonzalez** - que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa científica ou ensino - está de acordo com os preceitos da Lei 11.794 de 8 de outubro de 2008, com o Decreto 6.899 de 15 de julho de 2009, bem como com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi **aprovada** pela Comissão de Ética no Uso de Animais da Faculdade de Zootecnia e Engenharia de Alimentos da Universidade de São Paulo - FZEA/USP (CEUA/FZEA) na reunião de 04/10/2017.

We certify that the proposal "Unconsciousness assessment through Electroencephalografy (EEG), in the process of stunning, for electronarcosis, percussion and anesthesia for humane slaughter of Nile tilapia", utilizing 343 Fishes (343 males), protocol number CEUA 4446150817, under the responsibility of **Daniel Santiago Rucinque Gonzalez** - which involves the production, maintenance and/or use of animals belonging to the phylum Chordata, subphylum Vertebrata (except human beings), for scientific research purposes or teaching - is in accordance with Law 11.794 of October 8, 2008, Decree 6899 of July 15, 2009, as well as with the rules issued by the National Council for Control of Animal Experimentation (CONCEA), and was **approved** by the Ethic Committee on Animal Use of the School of Animal Science and Food Engineering - (São Paulo University) (CEUA/FZEA) in the meeting of 10/04/2017.

Finalidade da Proposta: Pesquisa (Acadêmica)

Vigência da Proposta: de 11/2017 a 07/2021 Área: Zootecnia

Origem: Animais provenientes de estabelecimentos comerciais

Espécie:	Peixes	sexo:	Machos	idade:	5 a 8 meses	N:	343
Linhagem:	Tilápia do Nilo			Peso:	500 a 900 g		

Resumo: Introdução. Recentemente a World Organization for Animal Health - OIE, por meio do Código de Animais Aquáticos, recomenda inserir práticas de bem-estar no transporte e abate de peixes destinados ao consumo humano. No entanto, os métodos tradicionais de abate de peixes não são considerados humanitários. Por exemplo, a imersão no gelo ou hipotermia é conhecida com um método que causa um alto grau de sofrimento, pois não provoca uma perda imediata da sensibilidade à dor. A hipotermia é utilizada pela maioria de frigoríficos no pré-abate de peixes no Brasil. O objetivo do trabalho é determinar a inconsciência de tilápias do Nilo por meio de Eletroencefalografia (EEG) no processo de insensibilização por eletronarcose, percussão e anestesia pré-abate. Material e métodos. Os peixes utilizados nos experimentos serão tilápias do Nilo com peso de abate aproximado de 700 g. No Subprojeto 1 (n=40) serão testados dois óleos essenciais para avaliação anestésica, seguido de análise sensorial. No subprojeto 2 (n=103) serão testados três tratamentos de eletronarcose e um tratamento de percussão com o objetivo de conhecer a duração da insensibilização por meio de avaliação comportamental. No subprojeto 3 (n=60) a inconsciência por meio de eletroencefalografia (EEG) será avaliada em 5 tratamentos, no melhor tratamento de anestesia (subprojeto 2), no melhor tratamento de eletronarcose (subprojeto 2), o tratamento de percussão, o controle negativo de inconsciência (hipotermia) e o controle positivo de inconsciência (anestesia com benzocaína). Prévio os estudos de eletroencefalografia (EEG) as cabeças de 10 peixes serão utilizadas para saber a posição adequada dos eletrodos. No subprojeto 4 (n=140) será avaliada a qualidade da carne dos peixes insensibilizados por eletronarcose, percussão, anestesia e hipotermia. Conforme a prática comum nos frigoríficos de tilápia que realizam hipotermia, a participação dos peixes do experimento de percussão, eletronarcose e anestesia tende a causar uma diminuição no sofrimento dos animais, em relação àqueles que não participaram do experimento. Isso se deve ao fato da hipotermia causar sofrimento agonizante e prolongado. Forma de análise dos resultados. Os dados serão avaliados por meio de estatística descritiva. A normalidade de cada variável será avaliada com o teste de Shapiro-Wilk. Para a comparação dos grupos das variáveis de distribuição normal será utilizado o teste de ANOVA One-Way seguido do teste de Tukey para resultados ($P<0.05$). Para comparar as variáveis que não cumprem com a distribuição normal será utilizado o teste de Kruskall-Wallis seguido do teste de Dunn para resultados ($P<0.05$). Resultados esperados. As perspectivas para o desenvolvimento deste trabalho envolvem a colaboração para o entendimento dos critérios da eletronarcose como método de insensibilização de peixes. Esses resultados serão importantes tanto no sentido de auxiliar no estabelecimento de normativas para o abate humanitário de peixes, quanto em relação à diminuição do sofrimento dos animais envolvidos nos processos realizados atualmente. Em seguida, almeja-se estudar os indicadores de insensibilização de tilápias, conhecimento este que auxiliará no estudo e determinação da inconsciência. Espera-se que a prática de abate humanitário provoque diminuição do sofrimento dos animais e que o método seja viável para a insensibilização de grupos de animais em cenários comerciais de frigoríficos. O estabelecimento de diretrizes para a implantação

Av. Duque de Caxias Norte, 225 - CEP 13635-900 Pirassununga/SP - tel: 55 (19) 3565 4254 / fax:
Horário de atendimento: 2ª a 6ª das 8h as 17h : e-mail: cepfzea@usp.br

UNIVERSIDADE DE SÃO PAULO
Faculdade de Zootecnia e Engenharia de Alimentos
Comitê de Ética em Pesquisa da FZEA

do método de abate humanitário poderá trazer muitos benefícios em termos de bem-estar animal no abate e de qualidade do produto. Espera-se que a prática de abate humanitário propicie uma diminuição das consequências negativas do estresse pré-abate sobre a qualidade da carne. A determinação de diretrizes para o abate humanitário poderá gerar conhecimento que forneça subsídios para uma demanda já existente, oriundas do MAPA. Esta demanda está principalmente relacionada à construção de normativas relacionadas ao abate humanitário de peixes no Brasil. Com o avanço do conhecimento, pretende-se produzir três artigos completos para serem submetidos a revistas científicas internacionais.

Local do experimento: Laboratório de aquicultura

Pirassununga, 04 de outubro de 2017

Profa. Dra. Daniele dos Santos Martins
Coordenadora da Comissão de Ética no Uso de Animais
Faculdade de Zootecnia e Engenharia de Alimentos da
Universidade de São Paulo - FZEA/USP

Profa. Dra. Cristiane Gonçalves Titto
Vice-Coordenadora da Comissão de Ética no Uso de Animais
Faculdade de Zootecnia e Engenharia de Alimentos da
Universidade de São Paulo - FZEA/USP

ANNEX 4 Approval by the human research ethics committee

USP - FACULDADE DE ZOOTECNIA E ENGENHARIA DE ALIMENTOS DA

PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: Análise sensorial de filés de tilápia do Nilo, utilizando óleos essenciais como anestésicos no pré-abate

Pesquisador: Daniel Santiago Rucinque Gonzalez

Área Temática:

Versão: 2

CAAE: 10443019.1.0000.5422

Instituição Proponente: UNIVERSIDADE DE SAO PAULO

Patrocinador Principal: UNIVERSIDADE DE SAO PAULO

DADOS DO PARECER

Número do Parecer: 3.326.401

Apresentação do Projeto:

A World Organization for Animal Health - OIE, por meio do Código de Animais Aquáticos, recomendou inserir práticas de bem-estar no transporte e abate de peixes destinados ao consumo humano OIE. No entanto, os métodos tradicionais de abate de peixes no Brasil não são considerados humanitários, e o método mais utilizado é a hipotermia na imersão de uma mistura de água e gelo. Percussão mecânica, insensibilização elétrica e o uso de anestésicos naturais podem ser alternativas para o abate de peixes no Brasil. Os óleos essenciais são um produto derivado da hidro-destilação de folhas, flores ou outras partes da planta. Óleos essenciais Lippia alba e Ocimum americanum possuem efeitos anestésicos em peixes e recentemente foi observado que os óleos essências utilizados no pré-abate de peixes podem retrasar a degradação microbiana em filés de Jundiá (Rhamdia quelen). Além disso, o óleo essencial de Lippia alba utilizado no pré-abate de tilápia do Nilo não induziu nenhuma diferencia de aroma ou sabor detectável pelos avaliadores na análise sensorial em comparação a amostras de peixes não expostos.

Objetivo da Pesquisa:

Objetivo da Pesquisa:

Objetivo primário: Avaliar os atributos sensoriais de aroma e sabor de filés de tilápias do Nilo, usando óleos essências de Lippia alba e Ocimum americanum no pré-abate. Objetivo secundário: Comparar os atributos sensoriais de filés provenientes do uso anestesia no pré-abate com filés de tilápia provenientes do abate por hipotermia

Endereço: Avenida Duque de Caxias Norte, 225
Bairro: Campus Universitário da USP **CEP:** 13.635-900
UF: SP **Município:** PIRASSUNUNGA
Telefone: (19)3565-4299 **Fax:** (19)3565-4114 **E-mail:** cepfzea@usp.br

Avaliação dos Riscos e Benefícios:

Considerando que o consumo de alimentos envolve riscos, neste projeto os eventuais riscos que podem ocorrer estão contaminação física, química e microbiológica do pescado durante o processo de abate, processamento, embalagem, transporte e cozimento. Tais riscos serão minimizados utilizando as Boas Práticas de Fabricação de Alimentos, Avaliação dos Riscos e Benefícios: os métodos tradicionais de abate de peixes no Brasil não são considerados humanitários, e o método mais utilizado é a hipotermia na imersão de uma mistura de água e gelo.

Comentários e Considerações sobre a Pesquisa:

Comentários e Considerações sobre a Pesquisa:

O uso de anestésicos naturais podem ser alternativas para o abate de peixes no Brasil, além inserir práticas de bem-estar no transporte e abate de peixes destinados ao consumo humano

Considerações sobre os Termos de apresentação obrigatória:

Comentários e Considerações sobre a Pesquisa: O TCLE informa claramente aspectos sobre a pesquisa e possíveis riscos que possam ocorrer na análise sensorial.

Recomendações:

Todas as recomendações foram atendidas.

Conclusões ou Pendências e Lista de Inadequações:

NDA.

Considerações Finais a critério do CEP:

Este CEPH FZEA aprova o desenvolvimento do projeto, a partir desta data.

Este parecer foi elaborado baseado nos documentos abaixo relacionados:

Tipo Documento	Arquivo	Postagem	Autor	Situação
Informações Básicas do Projeto	PB_INFORMAÇÕES_BÁSICAS_DO_PROJETO_1321570.pdf	15/04/2019 08:57:25		Aceito
Parecer Anterior	PB_PARECER_CONSUBSTANCIADO_CEP_3254104.pdf	15/04/2019 08:57:02	Daniel Santiago Rucinque Gonzalez	Aceito
Projeto Detalhado / Brochura Investigador	Projeto_CEPH_FZEA_v1.docx	15/04/2019 08:56:53	Daniel Santiago Rucinque Gonzalez	Aceito
TCLE / Termos de Assentimento / Justificativa de Ausência	TCLE_v3_ass.pdf	15/04/2019 08:50:21	Daniel Santiago Rucinque Gonzalez	Aceito

Endereço: Avenida Duque de Caxias Norte, 225
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Telefone: (19)3565-4299 **Fax:** (19)3565-4114 **E-mail:** cepfzea@usp.br

Folha de Rosto	Folhaderosto_ass.pdf	26/03/2019 18:02:43	Daniel Santiago Rucinque Gonzalez	Aceito

Situação do Parecer:
Aprovado

Necessita Apreciação da CONEP:
Não

PIRASSUNUNGA, 15 de Maio de 2019

Assinado por:
Judite das Graças Lapa Guimarães
(Coordenador(a))

ANNEX 5 Approval by the animal research ethics committee

UNIVERSIDADE DE SÃO PAULO
Faculdade de Zootecnia e Engenharia de Alimentos
Comitê de Ética em Pesquisa da FZEA

CERTIFICADO

Certificamos que a proposta intitulada "Eletroencefalografia (EEG) como ferramenta de avaliação de inconsciência de tilápia do Nilo, no processo de abate. ", protocolada sob o CEUA nº 4021010719 (ID 001342), sob a responsabilidade de **Elisabete Maria Macedo Viegas** *e equipe; Daniel Santiago Rucinque Gonzalez* - que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa científica ou ensino - está de acordo com os preceitos da Lei 11.794 de 8 de outubro de 2008, com o Decreto 6.899 de 15 de julho de 2009, bem como com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi **aprovada** pela Comissão de Ética no Uso de Animais da Faculdade de Zootecnia e Engenharia de Alimentos da Universidade de São Paulo - FZEA/USP (CEUA/FZEA) na reunião de 14/11/2019.

We certify that the proposal "Electroencephalography (EEG) like tool for assessment unconsciousness of Nile tilapia on slaughter process. ", utilizing 225 Fishes (225 males), protocol number CEUA 4021010719 (ID 001342), under the responsibility of **Elisabete Maria Macedo Viegas** *and team; Daniel Santiago Rucinque Gonzalez* - which involves the production, maintenance and/or use of animals belonging to the phylum Chordata, subphylum Vertebrata (except human beings), for scientific research purposes or teaching - is in accordance with Law 11.794 of October 8, 2008, Decree 6899 of July 15, 2009, as well as with the rules issued by the National Council for Control of Animal Experimentation (CONCEA), and was **approved** by the Ethic Committee on Animal Use of the School of Animal Science and Food Engineering - (São Paulo University) (CEUA/FZEA) in the meeting of 11/14/2019.

Finalidade da Proposta: Pesquisa (Acadêmica)

Vigência da Proposta: de 08/2019 a 08/2022 Área: Zootecnia

Origem:	Animais provenientes de estabelecimentos comerciais						
Espécie:	Peixes	sexo:	Machos	idade:	4 a 36 meses	N:	225
Linhagem:	Tilapia do Nilo			Peso:	500 a 2000 g		

Local do experimento: Laboratório de aquicultura, Faculdade de Zootecnia e Engenharia de Alimentos FZEA/USP, Pirassununga, São Paulo, Brasil

Pirassununga, 21 de novembro de 2019

Profa. Dra. Daniele dos Santos Martins
Coordenadora da Comissão de Ética no Uso de Animais
Faculdade de Zootecnia e Engenharia de Alimentos da
Universidade de São Paulo - FZEA/USP

Profa. Dra. Cristiane Gonçalves Titto
Vice-Coordenadora da Comissão de Ética no Uso de Animais
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