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Effects of different stunning methods on blood markers and enzymatic activity of stress responses of tilapia (*Oreochromis niloticus*)

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ABSTRACT

The objective of this study was to evaluate less stressful stunning methods for tilapia (*Oreochromis niloticus*). A preliminary experiment was performed to elect a chemical anaesthetic to be used as control in the following electronarcosis assays. Fish (498.8 ± 16.7 g) were submitted to four treatments: 1 (T1) and 2 minutes (T2) of electronarcosis at 205 volts and 10.8 amperes; and immersion in benzocaine (TB) and eugenol (TE), both at 100 mg L^{-1}. After plasma stress biomarkers evaluation, that is, ammonia, glucose and lactate, and considering use safety, eugenol was elected as control anaesthetic method. In the second experiment, fish were submitted to three electronarcosis frequencies: 400 (T400), 800 (T800) and 1200 (T1200) Hz, all of them at 203 V, ~3.3 A for 15 sec. After electronarcosis, plasma lactate was increased by 121 and 146% in fish submitted to T400 and T800, respectively, when compared to control. Ammonia and glucose did not depict significant differences. White muscle catalase (CAT), glutathione reductase (GR) and lactate dehydrogenase (LDH) activities were also assessed. Catalase activity augmented in the measure of 25% in T400 and T800, while GR activity was increased in the measure of 41, 43 and 29% for T400, T800 and T1200, respectively. Boosts of 50 and 38% in LDH activity were observed in T400 and T800 groups. Our findings suggest that higher frequency (T1200) electronarcosis improves *O. niloticus* welfare during slaughter.

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Introduction

The care taken with the management of the animals at the time of slaughter has significant consequences both for the welfare of the animals and for the quality of the final product destined to the consumers. According to Poli et al. (2005), if the slaughter and pre-slaughter procedures are not prudent regarding the minimisation of stress conditions, changes in the quality and time of storage of the final product may occur. Thus, according to the same authors, the concern for wellbeing is an ethical aspect closely related to the quality of the meat.

Electrical stunning is a common humane slaughter practice to ensure unconsciousness in many domestic animals. This method causes epileptiform activity or complete suppression of nerve function, resulting in immediate unconsciousness and insensibility to pain (Llonch et al. 2012). Many factors may influence the effectiveness of this technique, such as the type of electrical current, frequency, voltage, water conductivity, fish species and time to reach unconsciousness (Lambooij et al. 2008).

Tilapia, *Oreochromis niloticus,* is the most widely cultivated fish species worldwide, and especially in Brazil. However, there are few studies evaluating how the stress provoked by stunning methods affects the quality of tilapia meat (Lambooij et al. 2008).

The aim of this study was to evaluate electronarcosis parameters that would account for the lowest stress responses in *O. niloticus*, aiming the animal welfare and good meat quality. Moreover, the feasibility of two chemical anaesthetics (benzocaine and eugenol) to be used as control in the electronarcosis experiment was previously assessed.

Material and methods

Two experiments were performed: the first one for the choice of the chemical anaesthetic to be used as

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control in the electronarcosis tests; and the second experiment, modifying exposure frequency in electronarcosis.

Choice of chemical anaesthetic

Experimental design

Forty-eight male and female Nile tilapia (498.8 ± 16.7 g) were purchased at a fish farm and acclimated in 5000 L tank, under natural photoperiod, for 10 days. Fish were fed twice a day, *ad libitum,* until the beginning of experimental span. On the day of the experiment, fish were transferred to two plastic boxes (120 L, one box for each treatment) and kept indoors. Fish ($n = 12$) were submitted to benzocaine (TB) or eugenol (TE), diluted in ethanol P.A. 0.01%, at the concentration of 100 mgL^{-1} for 3 minutes, following Delbon and Paiva (2012). In the electronarcosis stunning, fish were simultaneously subjected to an electric current of 205 V, 10.8 A for 1 minute in treatment T1 and for 2 minutes in treatment T2.

After narcosis (chemical or electric), blood was withdrawn for plasma separation, which was immediately frozen for further analyses. Fish were then slaughtered by gill cutting.

Plasma analyses

Ammonia was quantified according to Gentzkow and Masen (1942); glucose quantifications used the LabTest® Kit, following the enzymatic method of glucose oxidase (Trinder 1969); lactate analysis was performed as described by Harrower and Brown (1972).

Electronarcosis parameters test

Experimental design

O. niloticus specimens (554.0 ± 61 g) were submitted to four treatments ($n = 12$ per treatment). In this evaluation, the tension (203 volts) and the desensitisation time (15 seconds) were maintained, while frequency (Hz) was modified, as presented in Table 1.

Eugenol was established as control in the previous experiment. Fish were submitted to the same conditions described for the first experiment. After stunning, plasma and white muscle were collected for further analyses.

Plasma analyses: they were performed as previously described.

Enzymatic activities in white muscle

Catalase (CAT) activity was assayed according to Beers and Sizer (1952); glutathione reductase (GR) was analysed according to Carlberg and Mannervik (1985); and lactate dehydrogenase (LDH) activity was assayed following Bergmeyer (1974).

Statistical analyses

Data normality was verified by the Kolmogorov–Smirnov test. ANOVA one-way test was then applied, followed by Tukey's test for comparison of means, considering $p < .05$ as level for significance. For the first experiment, all treatments were compared amongst them; while in the second experiment, electronarcosis treatments were compared to control. Evaluations were performed by Graph Pad Prism 5.

Ethics

The experiments were approved by the Ethical Committee of the University of São Paulo (Brazil), under the process number 1454110615.

Results

Choice of chemical anaesthetic

The plasma ammonia concentration of the fish submitted to electronarcosis for 1 minute (T1) and 2 minutes (T2) presented an increase of 26% and 34%, respectively, when compared to those anaesthetised in benzocaine (TB). In fish submitted to eugenol (TE), a 35% increase in plasma ammonia was observed in relation to TB. The treatments TE, T1 and T2 did not present significant differences (Table 2). No significant differences were observed in the plasma glucose concentration of the fish submitted to any of the four treatments (Table 2).

The plasma lactate of fish submitted to T1 and T2 treatments showed an increase of 78% and 106%, respectively, in comparison with TB treatment fish. Compared to TE, this metabolite was increased by 126% after electronarcosis for 1 minute (T1) and by 161% after electronarcosis for 2 minutes (T2). The plasma lactate levels of the fish submitted to the TE and TB treatments did not show significant differences between them (Table 2).

Table 1. Parameters of electronarcosis to which *O. niloticus* specimens were submitted.

Treatment	Frequency (Hz)	Voltage (V)	Current (A)	Time (s)
T400	400	203	3.28	15
T800	800	203	3.35	15
T1200	1200	203	3.47	15

Hz: hertz; V: volts; A: amps; s: seconds.

Parameters of electronarcosis

Plasma ammonia and glucose concentrations did not show significant differences in fish submitted to electronarcosis when compared to eugenol control (Table 3). Plasma lactate was increased by 121% in tilapia submitted to the T400 treatment and by 146% in those submitted to the T800 treatment, when compared to the control tilapia. The fish of the T1200 treatment did not present significant differences in the plasma lactate concentration.

The activities of tilapia muscle CAT, GR and LDH depicted significant differences after electronarcosis, which are brought by Table 4. Fish submitted to T400 and T800 treatments depicted a 25% increase in muscle CAT activity when compared to those anaesthetised in eugenol, whereas the T1200 treatment fish showed no significant differences. In tilapia submitted to the 3 different forms of electronarcosis, GR activity increased 41%, 43% and 29% for T400, T800 and T1200 treatment fish, respectively, when compared to the control ones. In *O. niloticus* submitted to T400 and T800 treatments, the activity of muscle LDH increased by 50% and 38%, respectively. The T1200 treatment did not cause significant differences in this enzymatic activity when compared to control.

Discussion

Narcosis methods exhibit great influence in the animal welfare, besides being crucial in the meat quality and in the final product shelf life. Studies concerning reductions in stress are pivotal in the assurance of these parameters, especially in fish, one of the most consumed food worldwide. Our data shed light on better electronarcosis parameters, a suitable and low cost method ready to be used in the meat industry.

In our assessments, both in the experiment to choose the chemical anaesthetic and in the different parameters of electronarcosis assay, no significant differences were observed in the plasma glucose concentrations of *O. niloticus*. One of the observed responses when there is an increase in circulating catecholamines is the beginning of processes for extra-energy availability, such as gluconeogenesis, in order to perfuse vital organs in stress situations. Thus, plasma glucose concentration is widely used as a biomarker of responses to stress. Both adrenaline and cortisol are related to increased glucose production in fish, playing a key role in stress-induced hyperglycaemia in these animals (Iwama et al. 2004).

Despite being relatively easy to obtain and being widely used as an indicator of stress, plasma glucose faces some criticisms, as some authors observed a delay in the release of this metabolite into the bloodstream (Poli et al. 2005), which may explain the absence of significant differences in *O. niloticus* submitted to chemical anaesthetics and to different parameters for electronarcosis in this study.

Plasma ammonia concentration provides insights on protein metabolism, which can also be altered by cortisol, which stimulates proteolysis and transaminations, and consequently increasing plasma levels of this metabolite (Inoue et al. 2011). The data obtained in the first experiment show that the electronarcosis parameters tested (T1 and T2) and eugenol (TE) provoked an increase in the plasma ammonia concentration of *O. niloticus* when compared to the treatment with benzocaine (TB). Ammonia was the only parameter that exhibited significant differences between fish from TB and TE treatments. This suggests that nitrogen metabolism was less affected by the benzocaine exposure. However, eugenol was the elected control

Table 2. Plasma intermediary metabolites of *O. niloticus* submitted to chemical anaesthetics and electronarcosis. Data are presented as mean ± standard deviation ($n = 12$).

Intermediates	TB	TE	T1	T2
Ammonia	0.89 ± 0.03a	1.20 ± 0.03b	1.12 ± 0.06b	1.19 ± 0.06b
Glucose	0.60 ± 0.03a	0.69 ± 0.02a	0.62 ± 0.04a	0.57 ± 0.04a
Lactate	1.81 ± 0.11a	1.43 ± 0.07a	3.23 ± 0.29b	3.74 ± 0.25b

Different letters indicate significant difference between treatments ($p < .05$).
TB = 100 mg benzocaine L^{-1}; TE = 100 mg eugenol L^{-1}; T1 = 205 V, 10.8 A, 1 min; T2 = 207 V, 10.6 A, 2 min.

Table 3. Plasma metabolic intermediates of *O. niloticus* submitted to different parameters of electronarcosis.

Intermediates	Control	T400	T800	T1200
Ammonia	1.32 ± 0.12	1.12 ± 0.95	1.45 ± 0.10	1.24 ± 0.13
Glucose	0.90 ± 0.17	0.89 ± 0.22	0.82 ± 0.19	0.92 ± 0.16
Lactate	1.55 ± 0.32a	3.43 ± 0.53b	3.82 ± 0.86b	1.49 ± 0.28a

The concentrations of the intermediates are expressed in µmol mL plasma^{-1}. Data are presented as mean ± standard deviation ($n = 12$).
Different letters indicate significant difference between treatments ($p < .05$).
Control =100 mg L^{-1} eugenol; T400 = 400 Hz, 203 V, 3.28 A, 15 s; T800 = 800 Hz, 203 V, 3.35 A, 15 s; T1200 = 1200 Hz, 203 V, 3.47 A, 15 s.

Table 4. Activity of catalase enzymes (CAT), glutathione reductase (GR) and lactate dehydrogenase (LDH) in white muscle of *O. niloticus* submitted to different parameters of electronarcosis.

Enzimatic activity	Control	T400	T800	T1200
CAT	0.12 ± 0.02a	0.15 ± 0.02b	0.15 ± 0.03b	0.14 ± 0.03a
GR	0.41 ± 0.05a	0.58 ± 0.09b	0.59 ± 0.09b	0.53 ± 0.08b
LDH	2.18 ± 0.33a	3.28 ± 0.47b	3.01 ± 0.35b	2.47 ± 0.30a

The enzymatic activities are expressed in mmol min mg of protein^{-1}. Data are presented as mean ± standard deviation ($n = 12$).
Different letters indicate significant difference between treatments ($p < .05$).
Control = 100 mg L^{-1} eugenol; T400 = 400 Hz, 203 V, 3.28 A, 15 s; T800 = 800 Hz, 203 V, 3.35 A, 15 s; T1200 = 1200 Hz, 203 V, 3.47 A, 15 s.

anaesthetic for the following electronarcosis experiment, due to two main reasons: it presents reliably safety both for fish and for the operator in industry in the dose used and its sedation is lighter than that produced by the synthetic anaesthetics, which is often preferable in fish aquaculture (Pattanasiri et al. 2017). Moreover, eugenol is more economically affordable when compared to benzocaine.

In the second experiment, there were no significant differences in ammonia concentrations between the three parameters of electronarcosis tested and the eugenol control. These data indicate that the evaluated treatments do not interfere in the protein metabolism of the animals, as well as in nitrogen excretion mechanisms, being therefore suitable for the minimisation of stress in pre-slaughter management.

In the first experiment of the present study, plasma lactate levels did not differ between the groups exposed to eugenol (TE) and benzocaine (TB); however, both treatments of electronarcosis exhibited hyperlactemia when compared to chemical anaesthetics. It is worth mentioning that increases in plasma lactate of *O. niloticus* submitted to electronarcosis were more pronounced when compared to animals treated with eugenol (TE). According to Barton (2002), the evaluation of a response may suggest a higher degree of stress in a particular species, whereas when we evaluate another response in the same species under the same conditions, the results may be contradictory.

In the experiment where the electronarcosis parameters were changed, the animals submitted to the treatments T400 and T800 depicted higher plasma lactate concentrations, concomitantly to increases in the activity of white muscle LDH. Considering that this enzyme is responsible for the lactate-pyruvate interconversion, these levels are suggestive of modifications in carbohydrate metabolism, possibly arisen from the anaerobic metabolism activation due to the vigorous muscular contraction occurring in electronarcosis. Augmented LDH activity in *O. niloticus* submitted to electronarcosis was also observed by Oliveira Filho et al. (2015), although the results obtained by these authors came from 6 minutes exposure under electric current of 50 Hz, 154 V and 8 A. The maintenance of this enzymatic activity in the T1200 treatment, the highest frequency tested, enabled the inference that higher frequencies at shorter exposure times are less harmful, both regarding stress parameters and meat quality, considering that increased muscle lactate may interfere with these parameters. This hypothesis gives rise to future studies, where an adequate pre-slaughter management for this specie will be established.

The activity of CAT is responsible for the transformation of hydrogen peroxide ($2\,H_2O_2$) into oxygen and water ($O_2 + 2H_2O$). According to our observations, this enzymatic activity was augmented in the T400 and T800 treatments, suggesting raises in the production of potentially dangerous free radicals arisen from these electronarcosis parameters. In *O. niloticus* submitted to the T1200 treatment, muscle CAT activity was unchanged, corroborating the previous hypothesis that higher frequencies of electronarcosis are advantageous regarding fish meat quality. The data obtained by Oliveira Filho et al. (2015), where *O. niloticus* were subjected to lower frequencies for a longer time, substantiated our hypothesis, as seen the increases in muscle CAT activity observed by these authors, when compared to other methods of pre-slaughter management, *i.e.* CO_2 narcosis and immersion in ice water.

There was an increase in the muscle GR activity of *O. niloticus* after the three tested electronarcosis methods. However, T1200 exhibited the less pronounced boost, highlighting the lower production of free radicals in the muscle of the animals submitted to this treatment. Future investigations are imperative for a better understanding of the action of electronarcosis on the welfare of pre-slaughter and during slaughter, as well as on the preservation of the meat quality of this fish.

Conclusions

The use of eugenol (100 mgL^{-1}) or high-frequency electronarcosis (1200 Hz) as stunning methods prior to bleeding presented lower plasma lactate concentration, muscle catalase and glutathione reductase activities, suggesting diminished stress levels in *O. niloticus* during slaughter.

Disclosure statement

No potential conflict of interest was reported by the authors.

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