INDICATORS OF STRESS IN TILAPIA SUBJECTED TO DIFFERENT STUNNING METHODS*

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ABSTRACT

Nile tilapia is one of the main fish species cultivated in the World. However, there are few studies evaluating the effects of stunning methods on the stress of this fish. The aim of this study was to evaluate the effect of electrical stunning (ES), CO_2 narcosis (CN), and iced water (IW) on indicators of stress in tilapia, e.g., muscle adenosine triphosphate (ATP), enzymatic antioxidants (catalase, lactate dehydrogenase and glutathione reductase) and plasma cortisol. The muscle ATP was highest ($P<0.05$) in fish stunned by CN. However, the cortisol level was higher ($P<0.05$) in tilapia stunned by IW and CN. The levels of catalase in the white muscles were higher ($P<0.05$) in fish stunned by ES than in fish stunned by CN and IW. For the red muscles, the highest levels ($P<0.05$) of catalase were observed in tilapia stunned by ES and IW. Lactate dehydrogenase levels in the white muscles were higher ($P<0.05$) in fish stunned by ES and lower ($P<0.05$) in fish stunned by CN. In the red muscles, lactate dehydrogenase levels were higher ($P<0.05$) in fish stunned by IW in comparison to ES and CN. Therefore, tilapia stunned using CN causes less stress compared to ES or IW and is in accordance with good practices of animal welfare.

Keywords: adenosine triphosphate; animal welfare; cortisol; *Oreochromis niloticus*; oxidative stress; slaughter

INDICADORES DE ESTRESSE EM TILÁPIAS SUBMETIDAS A DIFERENTES MÉTODOS DE ATORDOAMENTO

RESUMO

A tilápia do Nilo é uma das principais espécies cultivadas no mundo. No entanto, existem poucos estudos avaliando os efeitos de métodos de atordoamento no estresse deste peixe. O objetivo deste estudo foi avaliar o efeito do atordoamento elétrico (AE), narcose por CO_2 (NC) ou água e gelo (AG) nos indicadores de estresse de tilápias, tais como: adenosina trifosfato (ATP), enzimas antioxidantes (catalase, lactato desidrogenase e glutationa redutase) e cortisol plasmático. O ATP muscular foi mais alto ($P<0.05$) nos peixes atordoados por NC. No entanto, o nível de cortisol foi mais alto ($P<0.05$) nos peixes atordoados por AG e NC. Os níveis de catalase no músculo branco foi mais alto ($P<0.05$) nos peixes atordoados por AE em relação aos peixes atordoados por NC e AG. Para o músculo vermelho, os níveis mais altos ($P<0.05$) de catalase foram observados nas tilápias atordoadas por AE e AG. Os níveis de lactato desidrogenase no músculo branco foram mais altos ($P<0.05$) nos peixes atordoados por AE e mais baixos ($P<0.05$) nos peixes atordoados por NC. No músculo vermelho, os níveis de lactato desidrogenase foram mais altos ($P<0.05$) nos peixes atordoados por AG em comparação ao AE e NC. Portanto, tilápias atordoadas usando NC causam menos estresse em comparação ao AE e AG e está de acordo com as boas práticas de bem estar animal.

Palavras chave: adenosina trifosfato; bem estar animal; cortisol; *Oreochromis niloticus*; estresse oxidativo; abate

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INTRODUCTION

Fish have the ability to feel pain (nociception) (BRAITHWAITE and BOULCOTT, 2007). Consequently, stunning and slaughter are the most important stages of fish production in terms of animal welfare (LINES and SPENCE, 2011). Moreover, stunning of fish can also cause changes in sensorial characteristics such as color, flavor and texture; and bacterial development (VIEGAS *et al*., 2012).

Stress is a physiological, biochemical, and behavioral adaptative response to a stimulus (IWAMA *et al*., 2004). Stress is quantified by changes in levels of circulating cortisol and catecholamines, manifested in the flesh of the metabolic changes with increasing demand for energy as adenosine triphosphate (ATP) and glucose and also indicates problems with growth, reproductive ability, disease resistance, and survival (BARTON, 2002). Moreover, these factors increase the generation of reactive oxygen species (ROS). Oxidative stress is characterized by an imbalance between the formation of ROS and the capacity for cellular defense against these species, favoring the ROS (CADENAS and DAVIES, 2000). The consequences are manifested in molecular damage to cellular structures that affect vital functions in various tissues and organs. However, fish cells have defense mechanisms including superoxide dismutase, catalase, glutathione peroxidase, and glutathione reductase (FERREIRA *et al*., 2005). The determination of the muscle concentration of these enzymes can be considered as biomarkers of oxidative stress in animals. Nevertheless, the effects of stunning methods on these major antioxidant enzymes have not been evaluated in fish.

Iced water is one of the most used stunning commercial methods in Brazil. However, this method has been challenged, because it causes a slow death and therefore great stress (LINES and SPENCE, 2011). Stunning by CO_2 narcosis is relatively recent and the responses are contradictory. For some fish species, it appears to be a good method, since the method anesthetizes the animal pre-slaughter and therefore decreases pain sensitivity (GIUFFRIDA *et al*., 2007; ACERETE *et al*., 2009). For other species, it has been reported that CO_2 gas causes discomfort such as agony and escape attempt (ROTH *et al*., 2002; ERIKSON, 2011). Electrical stunning consists mainly of passing an electric current in the water where the fish are residing or directly in the body until they lose complete consciousness (VAN DE VIS *et al*., 2003). This technique is considered to be fast and humane, causing apparently little suffering.

Nile tilapia is one of the main fish species cultivated in the world. However there are few studies evaluating methods of stunning/slaughter on the stress and welfare of this fish. Therefore, the aim of this study was to evaluate the effect of electrical stunning, CO_2 narcosis, and iced water on indicators of stress in tilapia, e.g., muscle adenosine triphosphate (ATP), enzymatic antioxidants (catalase, lactate dehydrogenase and glutathione reductase) and plasma cortisol.

MATERIAL AND METHODS

Fish and stunning methods

Nile tilapia (n = 10 per treatment, mean weight = 493.30 ± 132.50 g) were purchased at a fish farm near the laboratory and transported alive and housed in three masonry tanks (capacity of 5,000 L) (dissolved oxygen = 6.20 ± 0.50 mg L^{-1}, temperature = 21.30 ± 0.10 °C and pH = 5.60 ± 0.10). The fish rested for 48 hours before the experiment, with fasting time of 24 h before stunning. All procedures were approved by the Ethics Committee on Research of FZEA (Process CEP-FZEA 123.1.1251.74.4)

Three methods of stunning were performed on the same day to avoid the influence of time. The test methods used in this study were compared to commercial method of tilapia stunning in Brazil, which is the thermal shock with iced water. On the day of the experiment, the water in the three tanks was reduced by half; the fish were quickly caught with a net and transferred to three plastic boxes (120 L) in the laboratory, with one box for each type of stunning. All fish in each treatment (n = 10) were submitted to stunning at the same time. For electrical stunning (ES), water from the plastic box was previously adjusted with salt to achieve conductivity of 700 µS. For the formation of the electric field, two aluminum plates (65 cm long x 35 cm wide) were placed near the walls of the box with a distance of 49 cm between them. One of the plates was insulated with a polyethylene fabric to avoid direct contact with the fish, which could have caused a short circuit. In each plate, an electrode was attached to a machine capable of performing the electrical discharge of up to 220V, which was developed specifically for this purpose. Fish were subjected to electric currents (DC) of 50 Hz, 154 V and 8 A for 180 sec (SCHERER *et al.*, 2005) to avoid the recovery consciousness. For CO_2 narcosis (CN) tilapia were placed in a plastic box with 120 L of water and immediately injected with CO_2 gas (pressure of 2 Kgf cm^{-1}) to complete stunning. For stunning by iced water (IW) (1:1) (commercial method), the fish were placed at the same time a mixture of 60 L of ice and 60 L of water to apparent stunning. The water quality parameters from each treatment were measured after fish death.

For each stunning method, the reaction to the stimulus on the sideline with a pin in the cranial-caudal direction and reflection of the eye rotation with inversion of the fish was monitored for determinate the death time (KESTIN *et al.*, 2002). There were annotations from the moment that started the method to the absence of any physical reaction, indicative of stunning and/or death. After confirmation of death, each fish was taken for analysis of ATP, antioxidant enzymes, and plasma cortisol.

ATP determination

It was used the white muscle fillet of freshly killed fish for quantification of ATP. The concentrations of ATP were determined by the HPLC method used by BURNS and KE (1985).

Plasma Cortisol

Blood were collected from the tail vein immediately after killing fish and plasma cortisol was measured by enzyme immunoassay using commercial kits (Diagnostic System Laboratories, Webster, TX, USA).

Enzymes assays

The activities of catalase (CAT), lactate dehydrogenase (LDH) and glutathione reductase (GR), were evaluated in white and red muscle homogenates. Muscle tissues were homogenized at a ratio of 1:5 (10 mM sodium phosphate pH 7.5 cold buffer) using a universal homogenizer and centrifuged at 8,000 g for 10 min at 4 °C. The supernatant was used for enzyme activity determination. Catalase activity was measured at 240 nm following H_2O_2 reduction (BEERS and SIZER, 1952). Lactate dehydrogenase activity was evaluated by the rate of NADH oxidation at 340 nm in the presence of pyruvate (BERNT and BERGMEYER, 1974). The activity of GR was determined by measuring the rate of NADPH oxidation at 340 nm with the concurrent reduction of oxidized glutathione (CALBERG and MANNERVICK, 1985). All enzyme activity determinations were performed in a spectrophotometer (Beckman-DU800), and the results were expressed in terms of specific enzyme activity. The protein content of the samples was measured according to BRADFORD (1976) using bovine serum albumin as a standard.

Experimental design and statistical analysis

The experimental design was completely randomized with three treatments (electrical stunning - ES, CO_2 narcosis - CN and iced water - IW) and 10 replicates (fish) for each analysis. Data were analyzed by ANOVA, and the significant difference Tukey test ($P<0.05$) was applied with the aid of the statistical program Jandel Sigma Stat® 2.0.

RESULTS

The water quality results after each stunning method of tilapia are shown in Table 1. Dissolved oxygen and temperature of the water tanks of tilapia subjected to electrical stunning (ES) and CO_2 narcosis (CN) were very similar. The stunning iced water (IW) had higher dissolved oxygen and lower temperature than the other techniques. Water salinity was adjusted to promote the conduct of electricity, so water conductivity was observed only in the tank of tilapia subjected to ES. The pH levels of the water after stunning by ES and IW were very close and higher than with CN.

The time required to stun the tilapia was a parameter for the assessment of welfare. Electrical stunning caused the fastest loss of vital signs at just 30 seconds. The tilapia subjected to CN and IW took longer to be stunned at 30 and 20 minutes, respectively. Blood and other biochemical indicators were also used to confirm the results of stress.

Table 1. Water quality after each stunning method of Nile tilapia.

Water parameters	Stunning methods		
	Electrical stunning (ES)	**CO_2 narcosis (CN)**	**Iced water (IW)**
Dissolved oxygen (mg L^{-1})	6.15	6.25	19.90
Temperature (°C)	21.70	21.60	0.80
Conductivity (µS)	700.20	2.00	3.00
pH	6.14	4.12	5.95

It was observed that the expenditure of muscle ATP was lower ($P<0.05$) in fish subjected to CN, higher in those undergoing IW, and intermediate in fish submitted to the ES. The plasma cortisol levels of tilapia stunned by IW were higher ($P<0.05$) than those stunned by ES. In fish killed by CN, plasma cortisol levels were intermediate between the ES and IW (Table 2).

Table 2. Muscle ATP and plasma cortisol of Nile tilapia stunned by electrical stunning (ES), CO_2 narcosis (CN) or iced water (IW). Different letters in the same line indicate significant difference (*P*<0.05; Tukey test) (mean ± standard deviation**)**.

Stress analysis	Stunning methods		
	Electrical stunning (ES)	**CO_2 narcosis (CN)**	**Iced water (IW)**
ATP (µmol g^{-1})	4.52 ± 0.13 ^{ab}	5.90 ± 0.11 ^{a}	3.59 ± 0.49 ^{b}
Plasma cortisol (ng mL^{-1})	15.89 ± 0.22 ^{b}	17.51 ± 2.07 ^{ab}	22.16 ± 0.20 ^{a}

The levels of the antioxidant enzyme CAT in white muscle after death were higher ($P<0.05$) in fish stunned by ES. For the red muscle, the highest levels ($P<0.05$) of CAT were observed in tilapia stunned by ES and IW. Lactate dehydrogenase (LDH) levels were higher ($P<0.05$) in white muscles ($P<0.05$) of fish stunned by ES and lowest in fish stunned by CN. Red muscle LDH levels were higher ($P<0.05$) in fish stunned by IW. The levels of GR did not differ ($P>0.05$) in white and red muscle in tilapia stunned by ES, CN, and IW (Table 3).

Table 3. Enzymes activity: catalase (CAT), lactate dehydrogenase (LDH) and glutathione reductase (GR) of white and red muscle of Nile tilapia stunned by electrical stunning (ES), CO_2 narcosis (CN) and iced water (IW). Different letters in the same line indicate significant difference (*P*<0.05; Tukey test) (mean ± standard deviation).

Enzymes activity	Muscle	Stunning methods		
		Electrical stunning (ES)	**CO_2 narcosis (CN)**	**Iced water (IW)**
CAT (µmol min^{-1} mg of $protein^{-1}$)	White	1.34 ± 0.16 ^{a}	0.59 ± 0.08 ^{b}	0.92 ± 0.15 ^{b}
	Red	3.96 ± 0.32 ^{a}	2.67 ± 0.29 ^{b}	3.98 ± 0.62 ^{a}
LDH (µmol min^{-1} mg of $protein^{-1}$)	White	8.19 ± 1.02 ^{a}	5.79 ± 0.83 ^{b}	7.71 ± 0.82 ^{ab}
	Red	7.88 ± 0.88 ^{b}	6.31 ± 0.92 ^{b}	11.54 ± 0.66 ^{a}
GR (nmol min^{-1} mg of $protein^{-1}$)	White	2.00 ± 0.27 ^{a}	1.64 ± 0.22 ^{a}	1.96 ± 0.09 ^{a}
	Red	4.20 ± 0.67 ^{a}	3.85 ± 0.31 ^{a}	4.44 ± 0.68 ^{a}

DISCUSSION

According to the method of stunning, differences occurred in the quality of water in tanks. Consistent with this study, other fish killed by CN, ES, and IW have also shown variations in water quality parameters (ERIKSON *et al*., 2006; FOSS *et al*., 2012). This can interfere with the degree of stress during stunning, as each species of fish requires certain water quality conditions for their survival.

For the cultivation of Nile tilapia, dissolved oxygen must be above 3 mg L^{-1}; pH should be close to 7; and temperature should be around 25 °C (POPMA and LOVSHIN, 1995). For CN and ES, the dissolved oxygen was in the comfort range for tilapia and was probably not the cause of death. In the IW treatment, the concentration of dissolved oxygen increased due to the decrease in water temperature (ERIKSON *et al*., 2006). In addition, another effect may have been high stress caused by thermal shock. It is believed that CO_2 gas was the main factor for the death of tilapia by CN due to the sharp drop in the pH of the water.

The decrease in pH with the addition of CO_2 occurred due to the combination of water molecules (H_2O) with CO_2, causing the dissolution of H^+ and HCO^{-3}. In some fish, this acidification causes a rapid and violent attempt to escape (POLI *et al*., 2005). Although the tilapia in the present study did not show this behavior, there were signs of breathing on the surface of water, but the fish kept swimming despite the apparent insensitivity.

As expected, according to other studies of ES in the same species (BARRETO and VOLPATO, 2004; LAMBOOIJ *et al*., 2008), tilapia quickly lost their vital signs. This can be positive, because fast-acting methods mean less suffering. In addition, fast methods of stunning can be beneficial for the fish processing industry, which needs to kill large numbers of fish at the same time. However, the stress caused must also be determinated by biochemical and blood analysis. The delay in tilapia subjected to CN was not a surprise, since other studies have also observed this. This delay is undesirable.

Time variations in CN are frequently found. In sea bass (*Dicentrarchus labrax*), stunning by CN occurs after 16 min (ACERETE *et al*., 2009); in Atlantic salmon (*Salmo salar*), after 5 to 10 min (ERIKSON *et al*., 2006); carp (*Cyprinus carpio*), after 9 min; rainbow trout (*Oncorhynchus mykiss*), after 3 min; and eels (*Anguilla anguilla*), after 109 min (MARX *et al*., 1997). These variations may be caused by exposure of fish to the method of stunning, for example, immersing the fish in water saturated with CO_2 as gas injection or after the fish have already been placed at the site where they will be stunned. Moreover, the time of stunning by CN may also depend on the resistance characteristics of each species. Although tilapia took a long time to stun, there were no signs of pain or panic, suggesting that CO_2 causes an anesthetic effect for gradual stunning to complete unconsciousness. Like CN, stunning by IW also caused much delay in unconsciousness of the tilapia. In other species of fish, delayed unconsciousness has also been observed using this method (LINES and SPENCE, 2011).

This is a challenge for animal welfare, because the fish take a long time to be unconsciousness. However, it is a method widely used in the industry due to the ease of execution and the preservation of good meat quality. However, its efficacy is lower when used in warm water fish. For example, Atlantic salmon (*S. salar*) subjected to iced water at 1 °C require more than 40 minutes to lose vital signs (ROTH *et al*., 2006). Therefore, this technique of stunning is not the most suitable for tilapia in accordance with good animal welfare.

The amount of ATP in the muscles of fish at the time of stunning is usually associated with several factors, particularly the stress of capture, water temperature, and time between capture/killing and storage (KNOWLES *et al*., 2007). Although CN causing slower death, it provided higher levels of ATP. This may be due to the anesthetic effect of CO_2 (ERIKSON *et al*., 2006), which can stun tilapia without any signs of stress. Because ES in fish usually causes a sharp contraction of the muscle (ROTH *et al*., 2007), it decreased ATP reserves. Grass carp (*Ctenopharyngodon idella*) submitted to ES have also shown smaller amounts of ATP (SCHERER *et al*., 2005). It was expected that the slaughter of tilapia by IW would present severe and rapid decline of ATP reserves due to the large delay of stunning, attempted escapes, and signs of pain during the stunning procedure. Rainbow trout (*O. mykiss*) (OZOGUL and OZOGUL, 2004) and sea bass (*D. labrax*) (KNOWLES *et al.*, 2007) also show spent of ATP after stunning by IW.

The study shows that the CN causes fewer attempts to escape; therefore, less energy is spent. Although fast and without much stress, ES may cause some degree of pain in the tilapia, as illustrated by the expenditure of ATP.

The stunning methods caused differences in the primary response to stress as evidenced by the levels of plasma cortisol. The plasma cortisol level is widely used as an indicator of stress in fish (BERTOTTO *et al.*, 2010). However, the establishment of baseline levels can be highly variable, because they depend on several factors such as species, season, time of blood collection, etc. Hypoxia (low dissolved oxygen in the water) as well as hyperoxia (too much oxygen dissolved in water) can induce acute stress in fish as seen by the increase in plasma cortisol levels (LEFÈVRE *et al.*, 2008). This could be one explanation for why stunning by IW presented higher values of plasma cortisol, since dissolved oxygen levels were 19 mg L^{-1}. Moreover, the IW caused delays in stunning and apparently much pain. The lowest plasma cortisol in fish subjected to ES may have been due to the speed with which the fish were stunned.

Although the fish stunned by CN took longer to die, their cortisol values were relatively lower than for IW. The plasma cortisol concentrations in Nile tilapia from 5 to 50 ng mL^{-1} indicate low levels of stress (BARRETO and VOLPATO, 2004). Thus, all treatments in this study show low stress. For comparison, sea bass (*D. labrax*) stunned by CN or IW do not show differences in plasma cortisol levels (ACERETE *et al.*, 2009). In Atlantic salmon (*S. salar*), stunning by IW causes stress, demonstrated by increased levels of plasma cortisol (SKJERVOLD *et al.*, 2001; FOSS *et al.*, 2012). Conversely, in Senegal sole (*Solea senegalensis*), stunning by CN showed more stress than IW, as measured by plasma cortisol levels (RIBAS *et al.*, 2007). This shows that the values of cortisol could be vary between species and methods of stunning. Furthermore, the data suggest that IW causes higher values of cortisol levels and thus increased expression of the primary stress response.

The highest levels of catalase (CAT) were observed in tilapia stunned by ES and IW. Catalase is considered the main antioxidant enzyme, since it constitutes the first line of defense against the reactive oxygen species (ROS) by converting hydrogen peroxide into oxygen and water (PANDEY *et al.*, 2003). According to HALLIWELL and GUTTERIDGE (2007) the lower the activity of CAT, the better the cellular redox state, the less stressed the fish, and thus the less need for action of this enzyme against stressors. For this enzyme, higher values were observed in red muscles, this occurred because aerobic metabolism predominates in these muscles, whereas energy generation occurs through anaerobic metabolism in the white muscle (HALLIWELL, 1994). The more active the aerobic metabolism, is the higher the intrinsic antioxidant mechanisms of the cell in response to ROS generated.

The increased activity of antioxidant enzymes resulting from stunning reflects the increased aerobic metabolism in fish during this procedure. On the other hand, the stress of stunning may also induces the anaerobic metabolism measured by increased activity of lactate dehydrogenase (LDH), as observed in this study. The lowest values for both white and red muscles were observed in fish stunned by CN. This may indicate that the CO_2 gas had an anesthetic effect, causing less oxidative stress. The highest value of LDH activity reflects a secondary response of tilapia to the stressor or intense muscular activity (LEFÈVRE *et al.*, 2008). This enzyme is responsible for the formation of lactic acid during anaerobic metabolism. The results of this study are in agreement with those of WENDELAAR BONGA (1997), which observed an accumulation of lactic acid in fish muscle and blood (hyperlactataemia) in response to extreme activity. Other authors have shown that white muscle in fish is mainly anaerobic glycolysis to meet energy needs, while red muscle aerobic pathways include high activity of the citric acid cycle and oxidative phosphorylation (BERNAL *et al.*, 2003; MCKENZIE *et al.*, 2004; CLUTTERHAM *et al.*, 2004; DALZIEL *et al.*, 2005).

The activity of the enzyme glutathione reductase (GR) did not vary (*P*>0.05) among the methods tested. The enzyme GR is also considered a biomarker of oxidative stress and is responsible for maintaining the GSH to GSSG ratio under conditions of stress (WINSTON and DI GIULIO, 1991). Studies in tilapia show that the activity of this enzyme significantly increases upon exposure to stressors such as cyanobacteria (JOS *et al*., 2005; ATENCIO *et al*., 2009), cadmium (ALMEIDA *et al*., 2002), paraquat (FIGUEIREDO-FERNANDES *et al*., 2006) and other pesticides (PEIXOTO *et al*., 2006). As for the enzyme CAT, the expression of GR was higher in red muscles. The explanation for these higher levels of GR may be the same as for CAT.

The relation confirmed in this study between the methods used to stun tilapia and stress parameters and welfare, may produce recommendations for best practices and future legislation that may be adopted by the Ministry of Agriculture, Livestock and Food Supply of Brazil.

Future studies are needed to reduce the time of CN stunning without causing great stress, such as immersing the fish in already-saturated water or testing different levels of CO_2 in the water. Despite the fast stunning of tilapia by ES, this method causes some stress; therefore, more studies are needed to evaluate different protocols for stunning, such as varying the strength, type of current, time, and conductivity of water.

CONCLUSION

Nile tilapias stunned by CN show minor signs of stress as evaluated by ATP, plasma cortisol and activity of enzymes catalase and lactate dehydrogenase. The major signs of stress occurred in tilapia stunned by IW (commercial method), determined mainly by ATP and plasma cortisol. Thus, stunning of tilapia CN is in accordance with good practices of post harvesting and animal welfare.

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