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Effects of Clove Oil on Behavior and Flesh Quality of Common Carp (*Cyprinus carpio* L.) in Comparison with Pre-slaughter CO_2 Stunning, Chilling and Asphyxia

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Abstract

This study evaluates different pre-slaughter stunning methods for common carp (*Cyprinus carpio* L.) using behavioral responses and product quality investigations. A total of 32 fish were stunned using CO_2, clove oil (CL), hypothermia via chilling (HY) and asphyxia (AS) in addition to a control group killed by a percussive blow to the head. Behavioral and quality measurements including pH, rigor mortis and flesh and skin color were used to determine the level of effects on behavior and quality. The results were indicative of aversive behavior associated to asphyxia, CO_2 and a cold shock (chilling). Flesh quality measurements revealed a rapid pH decline and earlier onset and resolution of rigor mortis in CO_2 and asphyxia group compared to clove oil and chilling groups. Using colorimetric assays of skin, asphyxia and CO_2 caused lighter, less red and yellower color with higher Hue. The asphyxia caused darker, yellower flesh with higher Hue and CO_2 caused lighter and yellower flesh with less reddish than other groups. The lowest flesh color saturation was observed in CO_2 group. Regarding to welfare issue and meat quality the less causative pre-slaughter method of aversive behavior which provides higher product quality is clove oil stunning.

Keywords: Behavior; flesh quality; stunning; slaughter; clove oil.

Kesim Öncesi CO_2 Şoklama, Soğutma ve Asfiksi ile Kıyasla Karanfil Yağının Sazanın (*Cyprinus carpio* L.) Davranışlarına ve Et Kalitesi Üzerine Etkisi

Özet

Bu çalışma, sazanlarda (*Cyprinus carpio* L.) kesim öncesi farklı şoklama yöntemlerinin balık davranışları ve ürünün kalitesi üzerine etkisi incelenmektedir. Karanfil yağı (CL), CO_2, soğutarak hipotermi (HY) ve asfiksi (AS) yöntemleri kullanılarak toplam 32 balık şoklanmış, kontrol grubu ise baş kısmına darbe ile öldürülmüştür. Et sertliği (Rigor motris), pH ve et ve deri rengi dâhil olmak üzere kalite ölçümleri kullanılarak, davranış ve kalite üzerindeki etkinin seviyesini tespit etmek amaçlanmıştır. Sonuçlar; asfiksi, CO_2 ve soğuk şok (soğutma) ile ilişkili aversif davranışları işaret etmiştir. Karanfil yağı grubu ve soğutma yönteminin kullanıldığı grupla karşılaştırıldığında; CO_2 ve asfiksi grubunda pH'ın hızlı düştüğü ve rigor motris'in erken başladığı ve çözüldüğü, et kalitesi ölçümleriyle ortaya konmuştur. Deride kolorimetrik analizle asfiksi ve CO_2 yöntemi; daha açık renkte, daha az kırmızı ve daha sarı rengin (daha yüksek Hue değeri) ortaya çıkmasına neden olmuştur. Asfiksi ise daha koyu, daha yüksek Hue değeri olan daha sarı et renginin ve CO_2 ise daha açık ve diğer gruplarınkinden daha az kırmızımsı olan daha sarı et renginin ortaya çıkmasına neden olmuştur. En düşük et rengi satürasyonu CO_2 grubunda gözlenmiştir. Hayvan refahı ve et kalitesi ile ilgili olarak, karanfil yağı ile şoklama, daha yüksek kalitede ürün kalitesi sağlar ve kesim öncesinde daha az davranış semptomlarına yol açan uygulamadır.

Anahtar Kelimeler: Behavior; flesh quality; stunning; slaughter; clove oil.

Introduction

The welfare issue is a relatively new concept for fish. Although some reviwers do not cathegorize fish as sentient animal (Chandroo *et al.*, 2004) and believe that they can not experience pain and fear (Rose, 2002) but according to some anatomical, physiological and behavioral evidences fish can suffer from pain and fear (Portavella *et al.*, 2003; Sneddon, 2003).

One of the most important steps to promote welfare of fish is humane slaughtering which is obtained via pre-slaughter stunning (Lambooij *et al.*, 2002) and is based on the principle that the animal must be killed quickly with minimum fear and pain or suffering (FAWC, 1996). Electrical stunning, percussive stunning, thermal shock, asphyxiation in air, carbon dioxide narcosis, exsanguinations, anesthesia and spiking are common methods for killing or stun/killing fish (EFSA, 2006). Stunning with CO_2 as a highly soluble gas in water, labor saving and easy method to mechanize is used for salmon stunning in countries with expensive labor (EFSA, 2009). Marx *et al*. (1997) found that stunning fish using carbon dioxide (CO_2) causing death by asphyxia was a very rapid method for trout about 3.2 min, but very slow in hypoxia-resistant species such as eel (*Anguilla anguilla*) (109.7 min), and probably would be for carp species, which show signs of increased mucus production during CO_2 narcosis that consequently affects muscle quality.

Clove oil; a relatively new inexpensive anesthetic for fish with no withdrawal period and with the major component of Eugenol (2-methoxy-4-(2-propenyl) phenol) is listed by the FDA as safe for consumers (Harper, 2003) although its impacts on behavior, physiology and meat quality of different fish species need to be more understood. Ribas *et al*. (2007) found that clove oil is a good anesthetic to stun/killing Senegal sole (*Solea senegalensis*). Another way for stunning fish is hypothermia with the aim of chilling, sedation and killing the fish simultaneously (EFSA, 2004). Asphyxia (keeping the fish out of water) is probably the most common and the oldest method used for killing fish around the world (Robb and Kestin, 2002).

The current slaughter process that is used in Iran (asphyxia) takes a long time for fish to be dead prior to which vigorous movements and agitating behavioral responses of fish are observed.

Although there is no single criteria to measure welfare, a wide range of physiological, biochemical and behavioral measurements are used to assess welfare. Behavioral observations have been used to assess the welfare of terrestrial animals (Fraser and Broom, 1990) and fish (Lambooij *et al*., 2007; Lambooij *et al*., 2008) combined with brain and heart activity assays as approval. Also biochemical and physical measures like rigor index, muscle pH and ATP/AMP ratio are widely used for evaluating effects of slaughtering methods on imposed stress levels and meat quality of fish (Acerete *et al*., 2009; Ribas *et al*., 2007; Bagni *et al*., 2007). However for many farmed fish species, there have been few of investigation into humane killing methods (EFSA, 2004).

First, rigor mortis (RM) was examined, as it is widely used as an indicator of pre mortem stress both in duration and intensity (Ribas *et al*., 2007; Lowe *et al*., 1993). Second, muscle pH was measured, as changes in pH help to assess the expected shelf-life of the product. Several studies revealed that pre mortem stress caused lower muscle pH immediately after death in white sturgeon (*Acipenser transmontanus*) (Izquierdo-Pulido *et al*., 1992) and rainbow trout (Robb *et al*., 2000). In addition Skin and color analysis was measured as it changes depending on the time after death and different stun/slaughtering methods and freshness conditions can be different.

This study evaluates the effectiveness of some stunning procedures in carp with clove oil, CO_2, hypothermia and asphyxia, to determine the best method that minimizes vigorous pre mortem movements and achieves good product quality.

Materials and Methods

Experimental Animals

90 Common carps of 1,042±196 g (mean±SD) were fasted for 48 h **(**Voshmgir Carp Farm, Inche Borun, Golestan, Iran) and were transported in aerated water to the Institute of Aquaculture Science of Gorgan University, where the fish were acclimatized for 1 month in fiberglass tanks (fish density: 1.5 kg/m^3) supplied with aerated well water (temperature: 23±1°C, pH: 8.18±0.2, dissolved O_2: 7±1 mg L^{-1}, nitrite nitrogen: 0 ppm; total ammonia nitrogen <1 ppm). Fish were fed once a day at 2% body weight.

Stun/Killing Protocols

A total of 32 randomly selected (n=32) carps were divided into 4 different Stun killing groups. Each individual fish from first three groups (mentioned below) was delivered to an aquarium (60×35×30 cm) for separate stun and behavioural observations until body equilibrium and opercular movements were vanished, then fish were killed percussively (EFSA, 2004) (Table 1).

Clove Oil (CL)

1 ml L^{-1} of clove oil bath (Barij Essans Co., Tehran, Iran) was prepared 30 min before for fish immersion.

Table 1. Average body measurements and characteristics of water quality during stunning process (mean±SE)

	Clove oil	CO_2	Hypothermia	Asphyxia
Dissolved Oxygen (mg L^{-1})	7.06±0.04	1.62±0.09	7.24±0.04	---
Temperature (°C)	21±0.0	16±0.8	1.1±0.1	---
pH	7.23±0.06	4.81±0.06	7.27±0.04	---
Total weight (g)	1034±86	1029±68	1180±84	1036±76
Standard length (cm)	31.9±1.1	32.4±0.8	33.2±0.7	32.8±0.8

CO_2

The carp were netted directly from the tank into a bath which CO_2 was injected through the water for CO_2 to be maintained at saturation level.

Hypothermia (HY)

The fish were directly immersed into an ice water tank (temperature: 0.6–1.5°C).

Asphyxia (AS)

The fish were removed from water and allowed to die with suffocation in air.

Behavioral Assays

Each carp induced in one aquarium under identical water condition. The behavior of the animals was recorded on digital video camera recorder (DCR-DVD 850E, Touch Panel, Japan) and analyzed afterwards for:

1- Normal swim: swimming around in the water mostly at the middle of column water.

2- Escape behavior: increased activity in particular agitated swimming, violent reaction or startle-escape activity.

3- Slight equilibrium disturbance: loss of equilibrium.

4- Inhibition of reflex activity: breathing but showing no other reflex to all strong stimuli like touching. The fish lies on the bottom or surface of aquarium.

5- Medularly Collapse (respiratory failure): showing no opercular movements.

Flesh Physicochemical Assays

After behavioral observation recordings, the dead fish were transferred to polystyrene boxes with ice for biochemical measurements performed at 0, 3, 9, 12, 24, 36, 48, 60 and 72 h post mortem and fish kept on ice during all period of experiment.

Rigor mortis

The rigor index (I_r) was obtained using tail-bending measurement method (Cuttingers Method). $I_r= [(L_0-L_t) /L_0]\ 100$ (Bito *et al.*, 1983). L represents the vertical drop (cm) of the tail, when two thirds of the fish fork length is placed on the edge of a table. L_0 is the tail drop at the beginning of the experiment, while L_t represents measurements throughout the experiment.

Flesh pH

The pH was measured at 0, 3, 9, 24, 36, 48, 60 and 72 h post mortem using a penetration electrode (Testo 206, pH2, Germany) through incisions made in the thickest part of the white muscle of each fish. At 12 h post mortem pH could not be evaluated (Ribas *et al*., 2007).

Color Measurement

Skin and fillet color were measured by means of a colorimeter (Lovibond, CAM-System 500, England). The color parameters were: L*, lightness (from 0 for black and 100 for white), a* for red/green chromaticity and b* for yellow/blue chromaticity (CIE, 1976). From the a* and b* values, the Hue and chroma values were calculated. Hue ($H°_{ab}$) is determined by the dominant wavelength and is the name of a color as found in its pure state in the spectrum and expressed by the equation $H^{°}_{ab}= \text{Arctan} (b^*/a^*)$. Chroma is an expression of saturation or intensity of the color attained and is expressed by the equation, $C^*_{ab}= (a^{*2}+b^{*2})^{1/2}$ (Pavlidis *et al.*, 2006). External and fillet color were measured above the lateral line, behind the head. Skin color assayed at 0 h after death and fillet color evaluated at 72 h after death.

Statistics

Statistical analysis was carried out using SPSS 16.0 for windows (SPSS Inc.). For physicochemical parameters studied, samples were tested for differences using one-way analysis of variance (ANOVA) with method and time separately (Zar, 1999). This means that the variables on each sampling time after each stun-killing method were analyzed compared to their respective initial values and differences among the stun-killing methods in each sampling time were analyzed as well. First of all homogeneity of variances were checked and if they were homogeneous, differences among groups were assessed by means of ANOVA. Subsequently, for all parameters, comparisons between means were performed with TUKEY test. Statistical significance was taken as $P<0.05$, indicated in figures and tables by different letters. Values (n=8 for all groups) were depicted as mean ± standard error (SE).

Results

Behavioral Observations

CO_2

The normal swimming behavior was not observed after transportation to the aquarium containing dissolved CO_2. The fish swam steadily and showed strenuous avoiding reactions and aversive behavior at first and tried to keep their mouths and operculum close and collided with the aquarium wall by flashing swimming. After 185±11.3 s from the beginning of the experiment, carps lost their equilibrium, showing aversive behavior and violent reactions without equilibrium. For 447±19 s carps continued dart aversive movements. At 500±18 s after stunning carps had no reflex to stimulus like touching. Opercular movements of fish were stopped at 915±74 s. Scale diffusion, increased mucus secretion, blackened gills and pale appearance of fish were also observed during stunning in CO_2.

Hypothermia

Carps were swimming normally at the beginning of transportation into ice slurry (temperature: 0.6-1.8°C) and continued with slow swimming on the bottom of the aquarium for 675±34 s. Then carps exhibited energetic erratic and rapid swimming accomplished with remarkable time intervals. After darting movements, some strong tremors were observed and at 967±63 s no aversive movements were observed in the fish of hypothermia that lost their equilibrium. During 1974±49 s of chilling process, carps had not any reflex to strong stimulus and opercular movements of carps stopped after 2932±172 s. Other behavior of fish in HY was gasping at the surface of water.

Asphyxia

Fish in AS group removed from water and left until opercular movements cease at 293 min after the start of the experiment. Because of violent scramble of fish out of water no behavioral response was recorded.

Clove Oil

Carps in CL group stunned steadily and had a rapid anesthesia. Carp exhibited normal swimming without any aversive movement and steadily missed their equilibrium after 65±6.3 seconds. After 125±5.2 s, carps had no response to strong stimuli and after 225±24 s opercular movements stopped. The behavior of carp in clove oil bath seemed calm and they had normal post mortem appearance (Figure 1).

Physicochemical Analysis of Flesh

Rigor Mortis

Different stunning procedures before slaughtering had a significant impact on rigor development in carps (Figure 2). AS and CO_2 stunned fish exhibited a faster onset and resolution of rigor mortis compared to HY and CL groups while CL stunning group showed a later onset and resolution of rigor mortis compared to other groups of fish, except for individuals showed full rigor until 60 h after death. The rigor index of carps slaughtered by AS was significantly higher than other groups. In addition, the resolution was faster, and the intensity of contraction was higher in AS, HY and CO_2 groups. The peak value for rigor mortis was observed in AS, CO_2 and HY fish within 3-6, 12-24 and 36-48 h post mortem respectively, showing gradual decrease of contraction intensity during the 72 h of observation.

Flesh pH

Initial muscle pH development differed between the groups ($P<0.05$) (Figure 3). AS and CO_2 caused an immediate post mortem pH decrease compared to HY and CL groups ($P<0.05$). The slowest reduction rate of muscle pH was observed in CL and HY groups ($P<0.05$) reaching an ultimate pH after 36-48 h of storage while the ultimate pH was recorded after 3 h for the AS, and after 9 h for the CO_2 groups. The lowest ultimate pH for AS group was 6.19, whereas the lowest pH obtained for the groups CO_2, HY and CL were 6.36, 6.36 and 6.37 respectively. After 48 h of storage, all groups showed no significant differences in muscle pH.

Skin and Fillet Color

The colorimetric assays exhibited immediate and significant post mortem differences in skin color parameters. Prolonged death in AS group caused differences in skin color ($P<0.05$). Stunning method had no significant effect on chroma* value. Carps in AS group showed significant higher L* (lightness), b* (yellowness) and Hue* value in skin and lower a* (redness) value compared to all other groups of fish (Table 2).

Significant differences in fillet color were observed among the pre-slaughter stunning procedures (Table 3). Carps in the AS group had darker and in the CO_2 group had lighter fillets (higher L* value than all other groups). There were no significant differences in redness (a*) among CL, CO_2 and HY groups, whereas CO_2 group had lower a*-values in fillet ($P<0.05$). Yellowness (b*) and Hue* of fillet in groups AS and CO_2 were higher than groups CL and HY ($P<0.05$). The saturation of fillet color (chroma*) was significantly affected by pre-slaughter stunning methods as follows: CL and HY>AS>CO_2 groups.

Discussion

One of the most important steps in the management of fish welfare is pre-slaughter stunning procedures (Anne Brown *et al*., 2010). Various methods are used to slaughter fish that many of them may not be in accordance with animal welfare (Hastein *et al.*, 2005). In order to evaluate aspects of fish welfare related to stunning-killing procedures, behavioral responses (fear, pain and suffering) physiological and physico-chemical parameters (rigor index, muscle pH, eye refraction index, reactive oxygen metabolites (ROMs) and k-value) are investigated in many researches (Acerete *et al*., 2009;

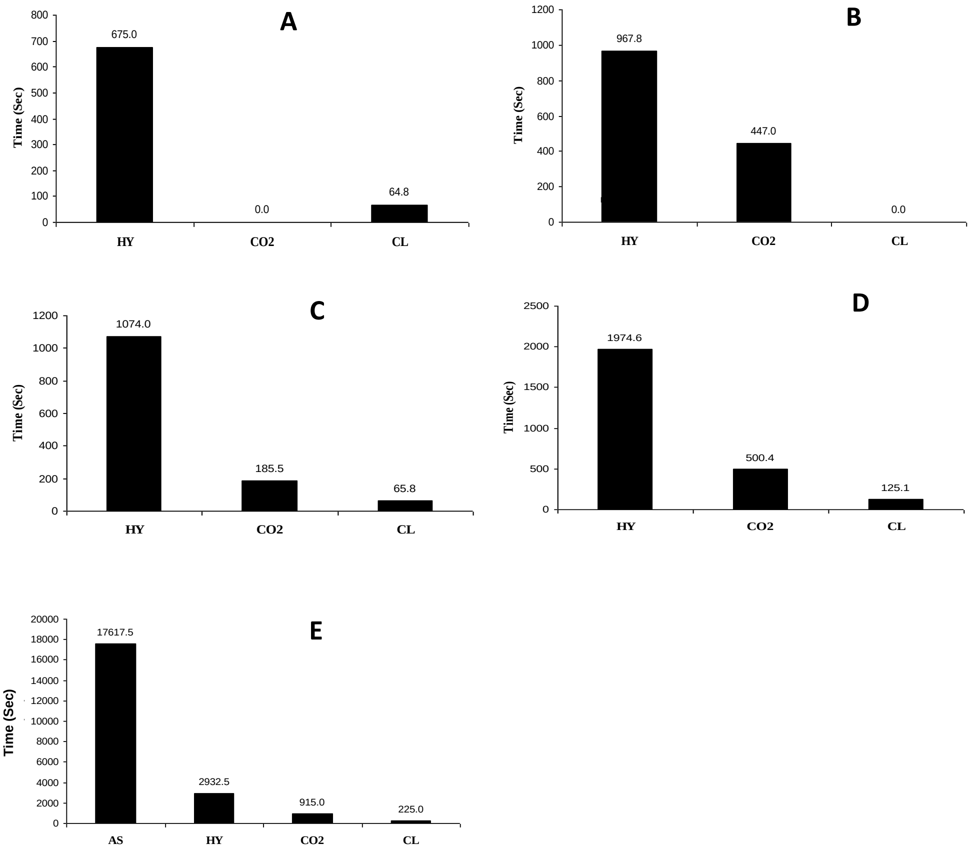

Figure 1. The duration of (s) different behavioral responses included: normal swimming (a), escape behavior (b), slight equilibrium disturbance (c), inhibition of reflex activity (d), medullary collapse (e) with different stunning pre-slaughter procedures. The time calculated since the beginning time of stunning process until to the beginning time each of the stage. Values are expressed as mean ± SE.

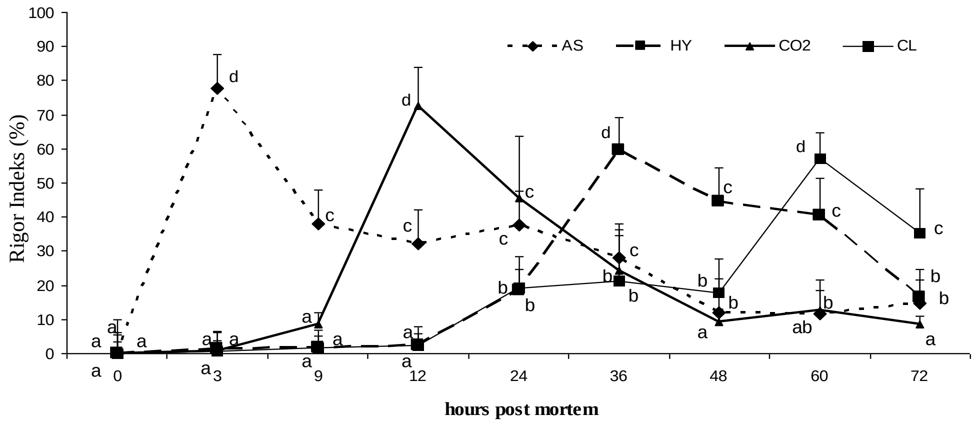

Figure 2. Rigor development during 72 h of ice storage of common carp treated with different pre-slaughter stunning procedures: clove oil (CL), CO_2, hypothermia (HY) and asphyxia (AS). Values are expressed as mean ± SE. different letters (a, b) represent significant differences (P<0.05, n = 8).

Roth *et al.*, 2009; Lambooij *et al.*, 2007; Bagni *et al.*, 2007; Marx *et al.*, 1997).

In this study substantial differences in behavioral and biochemical assays were observed during different stunning methods in carp. In CO_2 group, the fish that seemed to be suffered from procedure had abnormal activity and at the beginning of the induction tried to keep their mouth and operculum close in CO_2 saturated water. After losing their equilibrium in CO_2, the fish showed evasive behavior and aversive movements with increased mucosal discharge. This occurrence of abnormal behavior is a

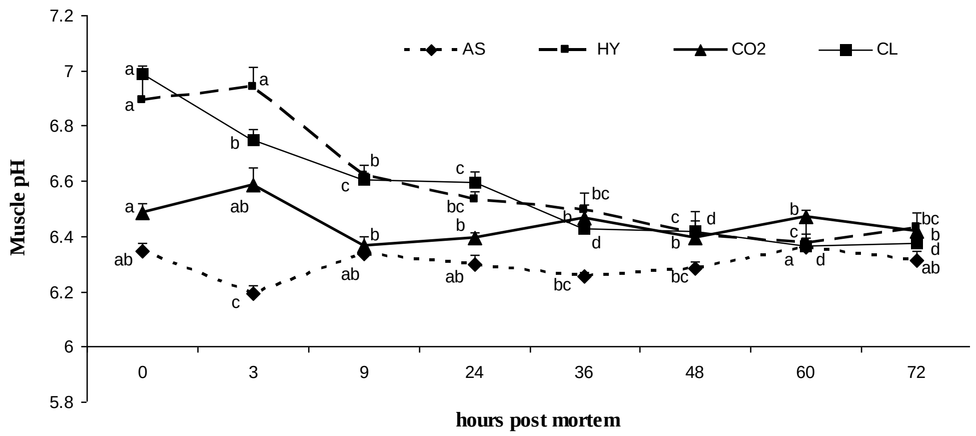

Figure 3. Muscle pH (mean ± SE) in common carp during 72 h storage in ice after different pre-slaughter stunning procedures: clove oil (CL), CO_2, hypothermia (HY) and asphyxia (AS). Different letters represent significant differences (P<0.05, n=8).

Table 2. Skin color (L*, a*, b*, Hue* and chroma*, Mean ± SE) in common carp at 0 h post mortem treated with different pre-slaughter stunning methods: clove oil (CL), CO_2, hypothermia (HY) and asphyxia (AS)

Stunning Method	L*	a*	b*	Hue*	Chroma*
CL	60.82±1.36 ^{b}	4.30±0.26 ^{a}	1.41±0.19 ^{b}	17.75±2.00 ^{b}	4.54±0.29 ^{a}
CO_2	63.28±0.88 ^{b}	4.30±0.30 ^{a}	1.58±0.25 ^{b}	20.51±3.71 ^{b}	4.64±0.26 ^{a}
HY	60.33±0.88 ^{b}	4.70±0.15 ^{a}	1.58±0.36 ^{b}	18.53±4.11 ^{b}	5.05±0.13 ^{a}
AS	69.60±0.88 ^{a}	3.30±0.13 ^{b}	3.37±0.40 ^{a}	44.39±3.24 ^{a}	4.77±0.32 ^{a}

Different letters (a, b) within each column denote significant differences (P<0.05, n = 8).

Table 3. Average fillet color (L*, a*, b*, Hue* and chroma* (mean±SE) in common carp 72 h post mortem stunned with different pre-slaughter procedures: clove oil (CL), CO_2, hypothermia (HY) and asphyxia (AS)

Stunning method	L*	a*	b*	Hue*	Chroma*
CL	45.25±0.83 ^{b}	12.25±0.42^{a}	-11.38±0.55^{b}	-42.82±2.13^{b}	16.75±0.59^{a}
CO_2	50.20±0.83 ^{a}	9.47±0.24 ^{b}	-1.58±0.73^{a}	-9.51±1.35^{a}	9.62±0.24^{c}
HY	45.58±1.45^{b}	11.67±0.69^{a}	-10.0±0.25^{b}	-40.40±1.45^{b}	15.40±0.94^{a}
AS	40.21±2.02^{c}	11.85±0.51^{a}	-1.27±0.45^{a}	-6.36±1.27^{a}	11.98±0.51^{b}

Different letters (a, b) within each column denote significant differences (P<0.05, n=8).

symptom of stress and irritation (Wiepkema and Koolhaas, 1993) and is similar to agitated swimming behavior observed in CO_2 narcotized trout and salmon (Roth *et al.*, 2002; Robb *et al*., 2002). In this study the time of death for CO_2 stunned carp was 15 min which is longer than time of death (9.3 min) reported by Marx *et al*. (1997) that observed the unsuitable effects of CO_2 on welfare and meat quality of common carp. The time for death can be affected by conditions of the experiment like temperature. It is suggested by Robb *et al.* (2002) that other physicochemical parameters in the water such as elevated ammonia levels and water pH are also likely to be aversive to the fish. The low water pH decreases blood pH (Wiepkema and Koolhaas, 1993) and subsequently causes a decrease in brain pH which disrupts nervous system and establishes immobility and eventual insensibility (Robb *et al.*, 2002). This can be the probable mechanism for the long and inhumane CO_2 caused immobility before the real unconsciousness. Therefore there is a certain risk that the fish remain conscious but unable to move, prior to actual death and during the stunning. Scale diffusion, increased mucus secretion and hemorrhaging of the gills were also seen in CO_2 stunned carps. These reactions reflect that being in CO_2 saturated water is not a desirable condition for fish. The phenomenon is in accordance with the gill hemorrhages of the hyperactive salmon in carbon dioxide bath (Robb and Kestin, 2002) and injuries and scale loss in trout and salmon (Robb *et al*., 2000; Roth *et al*., 2007).

Hypothermia (HY) is used for stun-killing of many of economic fish spices. In the present study carps in HY groups showed prolonged immobility and loss of equilibrium compared to other stunning methods. Although the fish seemed comfortable at the beginning and were swimming normally and slowly but they showed abnormal behavior afterwards and aversive behaviours and violent reactions accompany with muscle tremors and gasping at the surface of water occurred. Similarly, it is reported that carps during hypothermia swim normally before getting slow and becoming immobilized as their muscles cool but conversely other species like eel (Lambooij *et al*., 2002), African catfish (Lambooij *et al*., 2006) and Turbot (Roth *et al*., 2009), after the start of the experiment show evasive behaviour. The hypothermia for Sea bream induced immobilization before unconsciousness (Van de Vis *et al*., 2003). Using physiological measurements Arends *et al.* (1998) found hypothermia very stressful for carp. Similar results were obtained from live chilling of Atlantic salmon compared to percussive stunning (Skjervoldt *et al*., 2001).

In this study carps in HY had abnormal swimming after several minutes of introduction which is mentioned as an indicator of a poor welfare in farmed fish by Close *et al.* (1997). Hypothermia is assumed to affect metabolic rate, movements, behaviour and oxygen consumption via temperature decrease and finally immobilizes the fish (Hovda and Linley, 2000). Also periods of hyperactivity followed by slow movements, compromised swimming ability, reduced responsiveness, loss of equilibrium, onset of cold coma and respiratory failure are believed as the consequences of hypothermia (Smith *et al*., 2003). The present results suggest that hypothermia with prolonged induction time and provoked evasive movements and abnormal behaviour of fish is not acceptable stunning procedure.

Carps in AS group agonized and scrambled vigorously and finally died after about 5 h. This method was previously found to cause maximal stress responses, maximal aversive reactions and physical activities (EFSA, 2009; Acerete *et al*., 2009; Ribas *et al*., 2007). Removing fish from water is highly aversive for fish and violent attempts to escape impose a maximal stress response to fish (Robb and Kestin, 2002). Asphyxia in air for the long duration of 5 h until actual death happens in itself is inhumane and substantially affects fish welfare. In comparison to other groups, immobility and loss of equilibrium in the carps in Clove oil (CL) occurred more rapidly and behaviour of fish seemed to be normal during stunning stages. Behavioural evaluations of stunned groups revealed that stunning with 1 $ml \cdot L^{-1}$ of CL, decreases pre-slaughter caution of carp. Although there is a paucity of information on the behavioural responses of fish to different pre-slaughter procedures but many of defined behavioural indicators such as duration of escape behaviour, slight disturbance of equilibrium, reflex inhibition and medullary collapse(s) were observed in HY group. However swimming attempts and aversive movements of carp in CO_2 group were more intense and steady.

The development of rigor mortis is widely used as indicator of pre-mortem stress (Kiessling *et al*., 2006; Roth *et al*., 2006). Rigor mortis is accompanied with the acidification process resulted from production of lactic acid in the muscle tissue during the pre mortem phase (Bate-Smite, 1948). Pre-slaughter stress, combined with killing methods that includes greater physical activity prior to death, causes depletion of the glycogen sources through adenosine three phosphate (ATP) consumption and simultaneously a production of lactic acid occurs in the muscle which is usually further increased by a passive post mortem production (Thomas *et al*., 1999). In this study rigor mortis started earlier in carp subjected to pre mortem stress similar to Senegal sole, Sea bass, Sea bream and Rainbow trout (Acerete *et al*., 2009; Ribas *et al*., 2007; Bagni *et al*., 2007; Robb *et al*., 2000) which might be related to struggling and therefore higher ATP consumption before death in this group. In this study, resolution of rigor for both CL and HY was relatively slow compared to the AS and CO_2 groups. The resolution of rigor is mainly due to the action of endogenous proteolysis and microbial enzymes which result in the demolition of myofibrillar proteins (Sebastio *et al.*, 1996). The delayed rigor resolution is probably because of less physical damages to myotomes and muscular tissue due to less vigorous muscle contractions in CL and HY stunned carp that leads to minimal release of endogenous enzymes and subsequent proteolysis. The initial pH of post mortem in AS and CO_2 groups was higher than HY and CL groups. Also, between 0 and 24 h post mortem, the mean muscle pH in the groups HY and CL was higher than the groups AS and CO_2. On the other hand ultimate pH was dissimilar among different stunning methods. Findings reported here that the ultimate muscle pH is affected by pre-slaughter activity is similar to the results reported for other species (Kiessling *et al*., 2004; Ruff *et al*., 2002; Thomas *et al*., 1999). Exogenous factors such as catching, transport, confinement, anesthesia and slaughter in itself do influence on flesh pH (Sneddon, 2003). One reason for the reduction of pH during or immediately after slaughter is the decomposition of glycogen followed by increasing values of lactic acid. Such a decrease usually results in damage to the flesh texture and a fall in the fish fillet quality as previously observed in some other species (Sattari *et al*., 2010; Wilkinson *et al*., 2008). The color of skin and fillets was affected by the pre mortem stress. Results reveal that significant differences in skin lightness, redness, yellowness and Hue values were observed among treatments. The AS carp showed the skin color of lighter, yellower and less red than other groups which is surprisingly in accordance to the color criteria for stressed fish defined by Robb *et al.* (2000). In addition chromaticity parameters of fillet determined after 72 h post mortem indicated that pre-slaughter stunning procedures affected the fillet color. It has been shown that fish hyperactivity at slaughter (stress) causes the flesh to be significantly lighter, less red, more yellow (Robb *et al.*, 2000). However flesh color recorded in this study revealed that in several points chromaticity parameters do not have distinct and regular pattern related to imposed stress. In order to evaluate flesh color in fish, lightness, Hue and chroma value are more preferred to be used than a* and b* values (Pavlidis *et al*., 2006). Accordingly, it is obvious that AS and CO_2 groups had higher Hue value than HY and CL groups. Furthermore paler fillets in group CO_2 may be because of biochemical effects of hypercapnea on carp blood and flesh which can be more investigated through next studies.

Conclusion

Unsuitable pre-slaughter stunning procedures provoke the behavioral responses. The behavioral responses result in alterations in homeostasis that leads to physiological stress responses. On the other words, all responses have the interacting effects. Behavioral and physicochemical comparisons between AS and the other groups reveal that long duration of procedure, makes AS an inhumane method to slaughter fish which dissatisfies market demands for fish flesh of high quality. However with respect to the physicochemical factors, HY in comparison to CO_2 had better quality but both of the methods from welfare point of view are not suitable. HY was a prolonged procedure for the stunning carp and a prolonged procedure is inhumane in itself. HY decreases opecular rate and consequently decreases cold water flow through the gills which probably can result in a slower decrease in the brain temperature and delayed loss of consciousness. For further studies it is suggested that level of consciousness be investigated via monitoring of brain and heart activity during and after different pre-slaughter methods. Generally, this study shows that CL is not an aversive method for stunning the carp and from welfare point of view it can be a suitable procedure.

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