J Food Sci Technol (May 2015) 52(5):2585–2597
DOI 10.1007/s13197-014-1324-8

ORIGINAL ARTICLE

Evaluation of different methods of stunning/killing sea bass (*Dicentrarchus labrax*) by tissue stress/quality indicators

Giulia Zampacavallo · Giuliana Parisi · Massimo Mecatti · Paola Lupi · Gianluca Giorgi · Bianca Maria Poli

Revised: 28 February 2014 / Accepted: 14 March 2014 / Published online: 8 April 2014

Abstract The aim of the study was to evaluate the effect on the final product quality of certain innovative stunning/killing methods for sea bass as substitutes for the most common methods used by European farmers. The changes in tissue stress/quality parameters were monitored from the first hours after death and during the shelf life of the fish. Two trials were conducted in July and November on n. 231 sea bass stunned/killed by ice-water slurry, by single gas or mixture of gases in ice-water and by single- or two-stage electrical stunning/killing methods. Behavioural responses, stun/death time, *rigor index*, muscular and ocular pH, lactic acid, ATP and catabolites at death and within the 24 h after death were determined. In the November trial, the sensorial evaluation, *rigor index*, IMP, inosine, hypoxanthine, and K_1 values were also evaluated during refrigerated storage until spoilage. The stunning/killing in ice-water appeared to induce low effects on the analysed parameters and preserve a good product quality as indicated by the highest pH and ATP values at death, the delayed full *rigor* onset and the 1 day longer shelf life (14 days) in comparison with the single- or two-stage electrical stunning/killing. The gas mixture addition provided a 40 % shortening of the time to obtain stunning/killing and 14 days of shelf life. The actual level of quality loss with the different killing conditions and the actual impact of a significant shortage of *rigor mortis* onset and pH drop on the possible *pre-rigor* filleting remain to be studied in depth.

G. Zampacavallo · G. Parisi · M. Mecatti · P. Lupi · G. Giorgi · B. M. Poli
DISPAA (Department of Scienze delle Produzioni Agroalimentari e dell'Ambiente), Università degli Studi di Firenze, Firenze, Italy

B. M. Poli (✉)
DISPAA, Scuola di Agraria, Università degli Studi di Firenze, via delle Cascine 5, 50144 Firenze, Italy
e-mail: biancamaria.poli@unifi.it

Keywords Quality changes · Sea bass (*Dicentrarchus labrax*) · Stunning/killing methods · Stress indicators

Introduction

The killing procedure has been proven to induce an acute stress experience in fish that develops into a response in which hormonal, biochemical, osmoregulatory, and energetic alterations occur (Tort et al. 1998; Barton 2002). High levels of stress at death have been reported to cause violent reactions in fish, including increasing muscular activity and influencing the *post mortem* changes in *rigor mortis* onset and resolution, drop of muscle pH immediately after death (Izquierdo-Pulido et al. 1992; Nakayama et al. 1992; Sigholt et al. 1997), and decrease of ATP reserves (Sikorski et al. 1990; Tornberg et al. 2000). As with terrestrial animals, a close relation between the stress suffered before and during slaughter and the quality of the final product are found in fish (Azam et al. 1989; Erikson 1997; Morzel et al. 2003). Stress at death affects the quality and freshness during shelf life, with the consequent variation of parameters during storage, such as *rigor* release, ATP catabolites evolution and general aspects of the fish (Pottinger 2001; Tejada et al. 2001; Poli et al. 2005). These changes are widely considered as good indicators to evaluate the stress degree and the quality changes both in terrestrial species such as pigs (Warris et al. 2003) and in fish (Lowe et al. 1993; Marx et al. 1997; Robb and Warriss 1997; Poli et al. 2005).

Several stunning/killing procedures are used in aquaculture, and different fish species vary in their response to different methods (Morzel et al. 2003). The direct immersion in containers filled with ice-water slurry (ice flakes and water ranging from 1:2 to 3:1 ratio) is the method most commonly used by fish farmers for stunning/killing the Mediterranean species slaughtered at small size. If the difference in temperature between the rearing water and the ice-water slurry in the

crowding container is great enough (at least 10 °C), the thermal shock may reduce the brain function and shorten the loss of consciousness time compared with air asphyxia (Robb and Kestin 2002; Bagni et al. 2007; Poli 2009). Both the quantity of fish placed in the containers and their initial temperature in relation to the one of the rearing water can affect the chilling efficiency. As highlighted by Hovda and Linley (2000), the low water temperature is able to decrease fish activity, metabolic rate, oxygen consumption, which immobilises fish until death. However, the method is considered stressful by the EFSA panel on Animal Health and Welfare (AHAW) because is not able to provide immediate unconsciousness (EFSA 2006; EFSA 2009a). Recently, Panagiotis et al. (2013), comparing the effects of six stunning/killing procedures on the flesh quality of sea bass, reported that a combination of rapid clove oil anaesthesia followed by chilling in ice/water slurry was able to greatly improve both flesh quality and welfare of sea bass, compared with the use of only chemical anaesthesia prior to slaughter. However, further investigation is needed because of food safety concerns regarding the use of anaesthetic compounds.

Carbon dioxide-saturated water is able to stun fish in a few minutes but produces evident aversive behaviour in sea bass (Poli et al. 2004; Acerete et al. 2009). The CO_2 dissolved in water exerts a lowering effect on blood pH, and, in this way, a toxic effect on the brain (Kestin et al. 1995). A combination of live chilling in ice slurry and the use of moderate levels of dissolved carbon dioxide is considered better than the traditional carbon dioxide narcosis using CO_2-saturated water (Poli et al. 2004, 2005; Erikson et al. 2006).

The use of nitrogen has been suggested as a relatively new and effective stunning method for rainbow trout (Wills et al. 2006). Erikson (2011) tested isoeugenol, nitrogen, and low, medium, and high levels of carbon dioxide as stunning agents for Atlantic salmon. Fish treated with nitrogen displayed the strongest aversive reactions and were the most stressed fish that did not appear to be sedated. At low carbon dioxide level, changes in behaviour and stress were modest. However, only isoeugenol fulfilled all of the chosen fish welfare criteria.

The use of a mixture of gases (60–70 % N_2 and 40–30 % CO_2) during live chilling shortened the time needed to stun sea bass, without large differences in *rigor* onset and muscular pH and ATP levels (Poli et al. 2004, 2005).

Bjørlykke et al. (2011) analysed the effects of carbon monoxide on stress and carcass quality parameters of Atlantic salmon. Carbon monoxide led to the rapid depletion of tissue O_2 concentration and resulted in an earlier onset of *rigor* and faster decrease in pH due to lactate production but appeared to have a relaxing effect on fish and a positive effect on fillet colour.

Different studies have been performed regarding the application of an electrical current of sufficient strength to stun fish, mainly salmonids, and on the choice of proper intensity, magnitude, frequency and time of the current delivery: in rainbow trout and salmon, insensibility length increased with higher intensity (V/m), magnitude (mA) and time of the delivered current at the fixed frequency of 50 Hz (Robb et al. 2002; Robb and Roth 2003; Lines et al. 2003). Higher frequencies resulted in a more rapid but less long-lasting insensibility (Roth et al. 2003). In trout, Lines and Kestin (2005) applied a two-stage stunning method. The first stage had a very high frequency (1,000 Hz, for two seconds) for a rapid stun, and the second stage had a lower frequency (50 Hz) to elongate the duration of the insensibility. The same stunning procedure, followed by killing in ice, was confirmed to be very fast and humane for trout in comparison with asphyxia but resulted in the significant presence of bloodstains in 38 % of fish, downgrading their filleted product (Poli et al. 2007). Electrical current application is considered a rapid stunning method, but the stun lasts for a very short time and needs to be followed quickly by a killing method. Moreover, if not perfectly performed, it may be stressful, causing immobility without stunning or violent reactions of fish, resulting in several and evident haemorrhagic spots in fillets and column breakage (Gregory 1998; Lines et al. 2003). In sea bass, the evaluation of neural and behavioural responses and product quality after electric stunning for 10 s, followed by 15–20 min of chilling in sea water with flake ice (Lambooij et al. 2008) indicated the death of the stunned fish and acceptable flesh quality, as evaluated by fillet pH and colour during a 10-day period of refrigerated storage. The results obtained with Atlantic cod strongly suggest that electrical stunning per se resulted in a drop of pH to a level typical for severely stressed cod, in partial depletion of the high-energy phosphates and in early onset of *rigor mortis* (Digre et al. 2010; Erikson et al. 2012).

In terms of fish welfare, fish should be stunned prior to slaughter, assuring that unconsciousness is maintained until death. Lopes da Silva (1983) suggested that fish are unconscious and insensible when no movement is observed. In particular, the use of eye roll (VOR) has been indicated as a simple field method to assess if fish are unconscious (Kestin et al. 2002). However, Lambooij et al. (2010) suggested that caution is needed regarding the interpretation of VOR. Neurophysiologic measurements, such as EEG (electroencephalogram) and ECG (electrocardiogram), have been utilised in some species to test the time of fish unconsciousness after using ice and water mixture as a stunning/killing method. An irregular heart rate was observed in eels (*Anguilla anguilla*) and catfish (*Clarias gariepinus*) transferred to ice-water (Lambooij et al. 2002; Lambooij et al. 2006). In both these species, low brain activity and no response to pain stimuli both in the EEG and in behaviour were observed with restrained fish after approximately 13 min in ice water, corresponding to a fish body temperature decrease of 9 °C. In the same trial on catfish, Lambooij et al. (2006) indicated, after 5 min, unconsciousness onset for freely moving fish in ice water mixture.

Live chilling has been defined "not humane" by Lines et al. (2003) and the Norwegian Scientific Committee for Food Safety (VKM 2010) because of its effect on slow decreasing of fish activities up to the point of immobility without a sure loss of consciousness and sensibility. For this reason, live chilling is not considered a good stunning/killing practice for cold-water species (EFSA 2009b; Grigorakis 2010) because of the lesser stunning effect when the difference between the water temperature in the rearing tanks and the temperature of the ice-water mixture is moderate. The debate is still open regarding tropical and subtropical species. Concerning the sea bass and sea bream welfare, the EFSA (2009a) has not banned the ice-water method and affirmed that for these species, there are not yet available efficient and reliable alternative techniques, highlighting the need for new research in this field.

Literature on stunning and killing methods of farmed European sea bass is limited and not conclusive (Bagni et al. 2002; Poli et al. 2004, 2005; Bagni et al. 2007; Knowles et al. 2007; Lambooij et al. 2008; Acerete et al. 2009; Panagiotis et al. 2013). Therefore, the screening of new methods has to be continued to obtain a proper stunning/killing method for this species with the preservation of the product quality.

The present study was aimed at contributing to this issue with the evaluation of certain stunning/killing methods for sea bass (*Dicentrarchus labrax*), alternatives to the traditional ice-water slurry method, taken as point of reference, by the use of *post mortem* tissue stress/quality indicators determined at death, within 24 h after death and during shelf life.

Materials and methods

Fish sampling and stunning/killing methods

Two-day-starved sea bass (n. 231) were harvested in two different months of the same year (July and November), each time from the same rearing tank of a commercial farm (Il Padule, Castiglione della Pescaia, GR, Tuscany, Italy). The pre-slaughter procedures were the same for all the subjects: immediately after the contemporary catch, groups of n. 25 (July trial, 28 °C rearing water temperature) or n. 39 (November trial, 10 °C rearing water temperature) sea bass were submitted in parallel to the following stunning/killing methods detailed in Table 1. The higher number of fish in the November trial was because of the addition of the sensorial evaluation analysis.

a) *Water and ice slurry (IW)*

Fish were immersed in a tank (55×35×45 cm) containing 50 L of sea water and flake ice (2:1) at 1±1 °C. IW was present in both trials and used as point of reference.

b) *Gas addition in ice water (IW100N; IW70N)*

In the July trial, two groups of fish were immersed in tanks (55×35×45 cm) containing 50 L of sea water and flake ice (2:1) saturated with i) nitrogen gas (IW100N) or ii) a mixture of N_2 and CO_2 (70 % N_2+30 % CO_2: IW70N).

In the November trial, one group of fish was immersed in a tank (55×35×45 cm) containing 50 L of sea water and flake ice (2:1) saturated with a mixture of N_2 and CO_2 (70 % N_2+30 % CO_2: IW70N).

Before the fish immersion, nitrogen or gas mixtures were bubbled from a cylinder through a perforated pipe anchored to the bottom of the tank until the O_2 level was 0.9 mg/L. The concentration of CO_2, calculated on the basis of water pH value (6.4±0.1) and temperature in the tanks, during the test varied between 120 and 160 mg/L.

c) *Electrical stunning/killing in the November trial (EL1p; EL2p)*

For the electrical stunning/killing, Fishkill® EG200 equipment (Scubla Aquaculture, Remanzacco, UD, Italy) was utilised. The time of current delivery was checked by a timer.

Fish were put in the Fishkill® tank containing a minimum amount of fresh water (1 cm high) to cover the electrodes placed on the bottom, and the side to side passage of electric current was assured by the film of salt water that remained naturally around the animal after the capture.

The fish were electrically treated within 10 s of their transfer in the Fishkill® to limit their air exposition duration.

A single-phase method and a two-phase method were utilised. Frequencies, voltages and times of current delivering are reported in Table 1.

The single-phase method (*EL1p*) simulated the method frequently used in some Italian farms to stun/kill trout.

The two-phase method (*EL2p*) is based on one proposed by Lines and Kestin (2005), after previous preliminary trials carried out on sea bass. The choice of electricity parameters was related to the fact that in salmonids, 400 Hz is more efficient for fast stunning, but the range 50–100 Hz produces a longer stunning duration (Robb et al. 2002; Lines et al. 2003; Roth et al. 2004, 2009).

Evaluations, measurements and analytical methods

The behavioural fish responses and the time needed to achieve insensibility/death status were recorded. Afterwards, the fish were placed in polystyrene boxes (5 per box) and covered with flake ice for 20 min to rapidly reduce the animals' body temperature and prevent their possible recovering.

In both trials, with 5 fish for each treatment, *rigor index* (RI %), muscular and eye liquor pH (pHm and pHe) were measured at 0, 3, 5, and 24 h after death; lactic acid, ATP and

Table 1 Trial 1 (July) and trial 2 (November): water temperature, number and mean body weight of fish and stunning/killing methods

			Trial 1 July	Trial 2 November
	water temperature (°C)		28	10
	sea bass (n.)		75	156
	mean body weight (g)		402.3±74.9	548.5±100.6
Stunning/killing methods				
a) Ice-water			IW	IW
b) Gas addition	70 % N_2+30 % CO_2 mixture		IW70N	IW70N
in ice-water:	100 % N_2		IW100N	
c) Electricity:	Two-phase			EL2p
	phase 1	400 Hz 120 V 1′		
	phase 2	50 Hz 40 V 3′		
	Single-phase	50 Hz 40 V 4′		EL1p

relative catabolites contents in muscle were determined at 0, 3 (only in the November trial) and 5 h after death. In the November trial, RI %, inosine 5′-monophosphate (IMP), inosine, hypoxanthine, freshness index (K_1) value were also determined at 11, 12, 13 and 14 days (264, 288, 312 and 336 h) of storage together with the sensorial evaluation, according to the EU scheme (Council Regulation 2406/96 EEC).

Behavioural observations The behaviour of fish stunned/killed with ice-water slurry and ice-water saturated with gas methods was observed during the treatments. The self-initiated behaviours were evaluated according to Kestin et al. (1995), using behavioural indicators of aversion and indicators of incoming stunning.

The behaviour indicators of fish were noted, and the occurring time or the presence/absence of these actions was registered.

The tank of the Fishkill® device must be closed during the electric application, so no observation of fish was possible. Fish were observed after the 4-min treatment to evaluate their stunned/killed condition and for 20 min in ice.

Evaluation of the time required to stun/kill fish When no movement or activity were observed (with the water and ice-water saturated by gases method), and at the end of the supply of the current (with the electrical stunning/killing methods), fish were examined to determine cessation of breathing movements and the loss of the eye roll reflex (vestibule-ocular reflex–VOR) and then submitted to a tactile stimulation with a metallic point near the lateral line (Kestin et al. 2002; Robb and Roth 2003). The time needed to stun/kill the fish was indicated by the lack of response to the external stimuli.

Rigor index Rigor status was measured by the horizontal displacement method described by Bito et al. (1983).

pH Muscular pH (pHm) was measured by inserting the probe of a pH meter Jenway (Mod. 3100) into the epaxial muscle of the left side of the fish, in correspondence to the cranial insertion of the dorsal fin. Eye pH (pHe) was measured by inserting the probe in the liquor of the left eye, one time for each fish.

Lactic acid Lactic acid was determined in 1 g of muscle sampled from the cranial portion of the epaxial muscle of the left side of the fish. A standard enzymatic test kit (Enziplus® EZA 890+ L-Lactic acid, Raisio Diagnostics, Italy - UV method, for the determination of L-lactic acid in food) was used.

ATP and catabolites From the cranial side of the left epaxial muscle, 1 g of muscle was sampled. The concentrations of adenosine 5′-triphosphate (ATP), adenosine 5′-diphosphate (ADP), adenosine 5′-monophosphate (AMP), inosine 5′-monophosphate (IMP), inosine (Ino) and hypoxanthine (Hx) were determined by a HPLC analysis method based on Burns and Ke (1985). The HPLC apparatus comprised a pump system (Beckman mod.125-S) equipped with a UV detector (Beckman mod. 166) with absorbance fixed at 254 nm, analogic interface (Beckman mod. 406), Ultrasphere ODS Reverse Phase column (Beckman, length 250 mm, internal diameter 4.6 mm; particle size 5 μm; pore size 80 Å), Ultrasphere ODS pre-column (4.6 mm ID, 45 mm length), and 20-μl fixed loop. The mobile phase was KH_2PO_4, 0.5 M, pH 7.0. Standards were purchased from Sigma, St. Louis.

Adenylate Energy Charge AEC = (0.5 ADP + ATP)/(AMP + ADP + ATP) (Atkinson 1968), ATP/IMP ratio (Erikson et al. 1997), and K_1 value = [(Hx + Ino)/(Hx + Ino + IMP)] * 100 (Karube et al. 1984) were also calculated.

Freshness Freshness and shelf life were determined by sensorial analysis according to the EU scheme (Council

Regulation (EEC) No. 2406/96) by an expert panel of five judges. To assign the fish to the freshness classes, a score from 3 to 0 was available for each fish, corresponding to the following classes: E class (Extra), very fresh fish (score 3-2.6); A class, fresh (score 2.5-1.6); B class, stale (score 1.5-0.6); and unfit, not edible (score<0.6).

Statistical analysis

Muscle stress/quality parameters were analysed with SAS statistical software (SAS® Institute Inc. 2003) using ANCOVA (slaughter methods, covariant body weight) within each sampling time. A Tukey multiple comparison test for differences among the stunning/killing methods was performed within each sampling time. Differences were considered significant at $p \leq 0.05$. The correlation of chemical parameters (pH, IMP, inosine, hypoxanthine, and K-value) with sensory evaluation was also performed.

Ethics

The experiments were approved beforehand by a governmental ethical committee.

Results and discussion

Stress/quality indicators at death and within the first 24 h after death

Behavioural observation and times of action Fish in ice-water slurry displayed rapid to normal swimming during the first 2 min. The loss of balance (position on a side, vertical or belly upside-down), symptoms of incoming asphyxia (breathing amplitude decrease), and the loss of movements of fish on the bottom of the tank were noted after 3 min. All fish were motionless, but some were still reacting to external stimuli after 9 min (July) and 11 min (November). Panagiotis et al. (2013) reported that sea bass in ice/water mixture (1:2) were unconscious and insensible within 10 min, at that time displaying an absence of breathing and opercular movement, fixed eyes, absence of response to painful stimuli and loss of balance. However, the fish of the present trials were completely stunned/killed after 23 and 30 min in July and November, respectively (Table 2). Stunning/killing times from 20 to 35 min have been found for sea bass by Bagni et al. (2007) using live chilling. The efficiency of hypothermia for stunning appears to depend on the temperature of the rearing water and to be higher for warm species, such as Mediterranean species (Acerete et al. 2004). The duration of the pre-mortem interval in ice/water slurry may depend on the season or the rearing water temperature being longer in winter (EFSA 2008). The differences obtained in the two trials could be assigned both to the different ($P<0.001$) body weight of fish (Table 1) and to the different seasons and rearing water temperatures.

Table 2 Times (minutes) for the stunning/killing methods in ice-water and with gas addition in ice-water

		July	November
Ice-water	IW	23	30
Gas addition	IW70N	14	19
in ice-water	IW100N	16	-

After the addition of gases into the ice-water slurry, fish attempted to escape with rapid swimming and jumps, followed by slowing of movements in the first 3 min. Balance loss, symptoms of incoming asphyxia and loss of movements on the bottom of the tank occurred after 4 min. After 11 min (July) and 6 min (November), all fish were motionless, but some of them still reacted to the external stimuli. As reported in Table 2, fish in ice water with 100 % nitrogen (0.9 mg L^{-1} DO and 1±1 °C temperature) were completely stunned/killed after 16 min. Fish in ice-water with the gas mixture were completely stunned/killed after 14 min in the July trial and 19 min in the November trial. The addition of gases decreases the time to obtain the stunning/killing. In both trials, the reduction of time with the use of ice-water with 70 % nitrogen/30 % CO_2 was 40 %. Acerete et al. (2009) reported similar results, considering the suitability in term of stress and quality of a method based on CO_2-supersaturated water, with a slightly better performance regarding the time to death (16 vs 34 min for the ice and water mixture alone). Gelwicks et al. (1998) reported a 3-min shorter stunning time for rainbow trout with levels of CO_2 above 155 mg L^{-1} (similar to the level used in our trial). Erikson (2011) monitored Atlantic salmon transferred in a stunning tank with water saturated with nitrogen (5 % DO and 11 °C temperature) for ten minutes and reported fast swimming and escape behaviour during the first 2 min. After 3–4 min, all fish suddenly lost equilibrium and floated at the water surface, belly up. When removed from the tank (10 min), fish did not respond to stimuli, but all displayed VOR_S. These fish displayed even greater signs of stress than those observed with the high levels of carbon dioxide (> 400 mg CO_2 L^{-1}).

All fish treated with one-phase or two-phase electricity methods were stunned/killed after the delivering of the current with the chosen parameters during 4 min, appearing immobile and not reacting to external stimuli at the end of the current delivery. No fish recovered during the following 20 min in ice.

It has also to be underlined that the presence of a little quantity of water in the tank of the electricity application to assure a film of water on the body of the fish and exposing the fish to air should not represent itself a stressor. In fact, the

groups of fish were transferred into the tank immediately before the application of the electrical treatment to minimise the stress as much as possible. The choice to limit the amount of water used for the freshwater just to cover the electrodes was because of the higher conductivity of the salt water in previous trials partially affected the correct functionality of the used device. Limiting the amount of water in the tank to keep the voltage low is useful both for the benefit of animal welfare and the safety of the operators, which allows the use of this method eventually also on boats.

Recently, many trials on electric stunning have used very short times (1–10 s) of current delivery to limit the fish stress and the negative effect on the meat quality, but the fish unconsciousness duration observed has been very short. Electrical stunning in seawater was attempted by Lambooij et al. (2008), who tested the sea bass stunning using an electrical sinusoidal or pulse square wave current in seawater for 10 s, followed by chilling in seawater with ice flakes to kill all fish. However, the Authors reported an unconsciousness effect lasting only 48±34 s when using a 50 Hz current. In a different trial, Sattari et al. (2010) used chilling in flake ice after an electric stunning of 5.2±0.7 s to extend the unconscious state in fish, but the ECG revealed an unconsciousness lasting only 124±20 s. The short duration of the unconsciousness represents potentially an exposure of the recovered fish from stunning to the cold shock (electricity plus cold shock). Currently, considering the European sea bass and sea bream farming systems, even one minute and half of stunning duration appears inadequate when not followed by automatic systems based on pumping. However, pumping itself is a very stressful procedure (EFSA 2009b; Roth et al. 2009, 2012).

On the whole, it is important to remember that slaughter is generally a two-stage process: stunning and killing (Van de Vis et al. 2001; Robb and Kestin 2002; Lambooij et al. 2002). The fish are stunned to make them insensible to pain and successively killed, before they recover, by various methods such as bleeding, stopping the heart by gill cut or cutting off oxygen. In the present trials, it was chosen to stun/kill the fish by electrical current and a precautionary permanence of at least 20 min in flake ice was added to be sure that no fish could recover.

Rigor mortis As shown in Fig. 1, in the July trial at 3 h after death, IW70N sea bass displayed the lowest RI % value (26.8 %), followed by IW (49.4 %) and IW100N (57.0 %) sea bass, but there were no significant differences between them. At 5 h after death, IW fish displayed the lowest value of IR %, whereas IW100N and IW70N already were in full *rigor*. This aspect might have an impact on the possibility of *pre-rigor* filleting of fish. In the November trial, IW and IW70N sea bass displayed significantly lower, then better, RI % values at 3 h after death in comparison with both electric methods, and the differences were not significant among them (14.2 and 18.5 % vs 72.6 and 96.3 %). In this trial, IW and IW70N sea bass also displayed a later full *rigor* onset (near 7 vs 5 h after death). The same results have been found in other studies on sea bass, where also short electrical stunning time accelerated the pattern of onset and resolution of *rigor mortis* when compared with the immersion in ice slurry (Knowles et al. 2007; Lambooij et al. 2008). The actual level of welfare loss in the different killing conditions and the actual impact of a significant shortage of *rigor mortis* onset on the possible *pre-rigor* filleting of fish are not known. Morzel et al. (2003) compared three slaughtering methods for turbot, including whole body electrical treatment (150 V for 2 s followed by 25 V for 5 min), bleeding in ice slurry and percussion with respect to animal welfare and flesh quality. Fish killed by electricity had the shortest *pre-rigor* time. In addition, the flesh was softer, and the flesh colour was redder and darker.

pH In the July trial, muscle pH values were not significantly different among the stunning/killing methods with the exclusion of IW100N pH, which was the lowest at 5 h after death (6.62 vs 6.80). In the November trial, significant differences only emerged at death (0 h), with the highest values in the IW group (6.97) in comparison with the other methods, except EL2p. This last method in fact displayed an intermediate value (Table 3). In partial agreement with the present results, Knowles et al. (2007) reported lower pH in sea bass treated with electrical stunning than in subjects treated with ice-water. In addition, with the gas mixtures methods, pH values were lower than those with IW, but, in that case, acidification of the muscle with carbon dioxide could have contributed. Significantly lower muscle pH values were also found in sea bass stunned by percussive stunning than in fish chilled in ice/water slurry or on ice ($P<0.001$) (Di Marco et al. 2007).

In the July trial, the eye liquor pH (pHe) at death (0 h) was higher in IW and IW100N, whereas the IW70N group displayed the lowest values most likely due to the presence of CO_2 in the gas mixture. In the November trial, the IW group maintained higher pHe values at 0, 3 and 5 h after death (7.48, 7.17 and 7.05), whereas the sea bass killed with the gas mixture displayed the lowest values (7.08, 7.05 and 6.83). Regarding the method involving the use of CO_2 in the gas mixture, the pHm and pHe most likely should not be considered good indicators of stress. At death, the EL1p and EL2p groups displayed pHe values that were not different from the values of the ice-water group. At 24 h after death, no differences were voted in these values for either the July or November trial.

Lactic acid In the July trial, there were no differences in muscular lactic acid content at death. On the contrary, at 5 h after death, the IW group displayed the lowest, then better, value (28.2 μmol/g, $p<0.05$) (Fig. 2). In addition, in the November trial, no differences were noted for muscular lactic

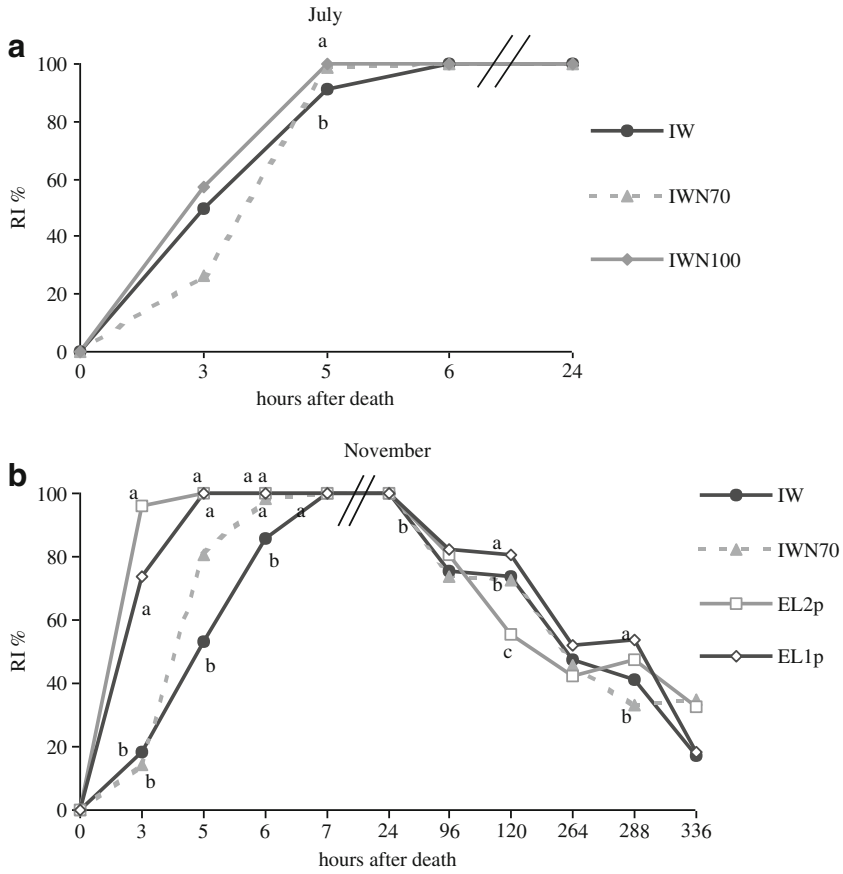

Fig. 1 *Rigor index* (RI %) in the first 24 h after death in the July trial (**a**) and from 0 to 336 h after death in the November trial (**b**). Values with different letters (**a**, **b**, **c**) are significantly different among treatments ($p \leq 0.05$)

acid content at death. The EL2p group displayed the highest value and then the worst values at 3 h. The IW group displayed the lowest, then better, values at 5 h after death, indicating overall low stress and muscular activity at slaughter. In addition, Sebastio et al. (1996), in a study on rainbow trout, found higher values of lactic acid in fish treated with electricity (46.3 μmol/g) than in fish treated with a CO_2 stunning method (37.2 μmol/g). Di Marco et al. (2007) found higher plasma lactate levels in sea bass anaesthetised with clove oil (100 mg/L) than in fish killed by percussive stunning or chilling on ice/water slurry.

ATP and catabolites In July, no differences among ice-water and gases in ice-water methods were found at death, when ATP values ranged from 2.82 to 3.37 μmol/g (Table 4). However, at 5 h after death, the IW group displayed higher ATP values than the other two groups, and IW100N displayed the highest IMP value.

In November, the ice-water group displayed the highest ATP values at each sampling time. In particular, the ice-water group displayed the highest ATP values at death, and the EL2p group displayed the lowest values (8.13 μmol/g vs 1.88 μmol/g; $p<0.05$). At 3 h after death, IW displayed the highest value of ATP (4.93 μmol/g), IW70N intermediate values, and all the electrical stunning/killing groups the lowest values, with no differences among them. At 5 h after death, the ice-water group again displayed the highest ATP values (3.96 μmol/g), and the other three groups the lowest, with no differences among them. The AEC ratio was calculated because it is considered a good indicator of the energetic cost consequent to the stress (Erikson et al. 1997). This parameter always reflected the trend of ATP alone, with no difference in the July trial, and it generally displayed the highest values in the IW and IW70N groups of the November trial. In particular, the electrical stunning method EL2p group, at death and at 3 h after death, displayed lower values of AEC than the ice-water and ice-water with added gas groups. At 5 h after death, both of the electrically stunned groups displayed the lowest AEC values. The high values of ATP at death in fish in the IW group appear to indicate they suffered low stress. These results can be justified by the effect of low temperature on fish. In fact, the fish responded to hypothermia by reducing mobility and most likely decreasing their metabolism. They displayed a lower consumption of high-energy phosphates despite the long time required for killing. The generally worse performance of the methods involving the gas mixture can most likely be justified by the rapid movement of the fish in the first minutes of contact with the gases. These movements, when the temperature of fish and their metabolism were higher, likely caused a larger depletion of muscular energy reserves

Table 3 Muscular pH (pHm) and ocular pH (pHe) from 0 to 24 h after death in trial 1 (July) and in trial 2 (November)

July					
	Hours	IW	IW70N	IW100N	r.s.d.[1]
pHm	0	6.82	6.63	6.74	0.17
	3	6.93	6.89	6.75	0.17
	5	6.80^{a}	6.80^{a}	6.62^{b}	0.08
	24	6.71	6.64	6.63	0.09
pHe	0	7.31^{a}	7.21^{b}	7.35^{a}	0.07
	3	7.08^{b}	7.02^{b}	7.22^{a}	0.12
	5	7.18	7.21	7.15	0.10
	24	7.11	7.06	7.01	0.10
November					
	Hours	IW	IW70N	EL2p	EL1p	r.s.d.
pHm	0	6.97^{a}	6.76^{b}	6.81^{ab}	6.76^{b}	0.14
	3	6.61	6.69	6.55	6.61	0.11
	5	6.57	6.56	6.55	6.58	0.10
	24	6.47	6.53	6.49	6.50	0.08
pHe	0	7.48^{a}	7.08^{b}	7.48^{a}	7.35^{a}	0.10
	3	7.17^{a}	7.05^{b}	7.02^{b}	7.09^{ab}	0.09
	5	7.05^{a}	6.83^{b}	6.92^{ab}	6.91^{ab}	0.10
	24	6.64	6.73	6.70	6.74	0.08

[1] r.s.d.: residual standard deviation

Values with different letters (a, b) are significantly different among treatments ($p \leq 0.05$)

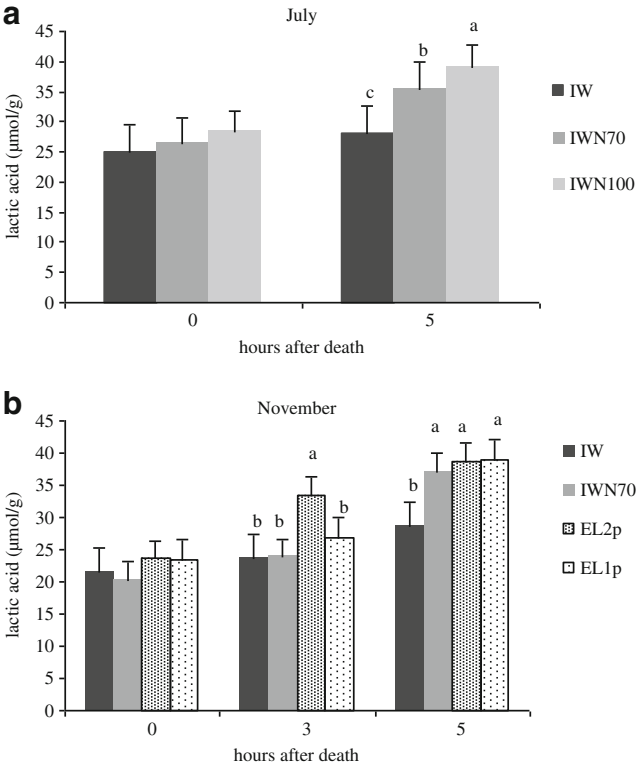

Fig. 2 Lactic acid in sea bass muscle at 0 and 5 h after death in the July trial (**a**) and at 0, 3 and 5 h after death in the November trial (**b**). Values with different letters (**a**, **b**, **c**) are significantly different among treatments ($p \leq 0.05$)

despite the shorter time for killing. The electrical method with two phases appears to more rapidly drain muscular energetic reserves than the single-phase method, possibly because of the high electrical frequency level of the first phase. Electrical stunning per se resulted in a drop of pH to a level typical for severely stressed cod, partial depletion of the high-energy phosphates and early onset of *rigor mortis* (Digre et al. 2010; Erikson et al. 2012).

Differences in ATP values and/or its catabolites among the stunning/killing method groups have hardly been reported or found in other studies. Senegal sole anaesthetised using clove oil and hypothermia displayed similar muscle ATP/IMP ratios throughout a 72-h *post mortem* period (Ribas et al. 2007). In addition, in sea bass (Di Marco et al. 2007), the hypothermia demonstrated a very good capacity to inhibit the stress response compared with anaesthetics during both short and long exposure (6 and 20 min). No significant differences in IMP levels at death between the sea bass groups treated with electrical stunning (ES) and ice-water slurry (IW) were found by Knowles et al. (2007). High initial IMP levels were found in both slaughter treatments, indicating a complete depletion of ATP and then high stressful *pre-mortem* procedures. In agreement with this hypothesis, in the mentioned paper, the ATP values at death in the ice-water treated sea bass were always lower than those found in the present trials, indicating some possible differences in the application of the IW methodology and/or stress in the pre-slaughter events.

Quality indicators

ATP catabolites and K_1 value Differences among the methods were not so homogeneous during the last days of storage (11, 12, 13 and 14 days after death) for the fish of the November trial monitored for fish freshness/quality loss at the end of shelf life. Higher values of IMP and lower values of inosine and hypoxanthine and the lowest K_1 value, indicating a better freshness condition, were found in the IW group at 11 days of storage (K_1=29 % $p<0.05$) (Table 5). At 12 days of storage, no difference in IMP but higher inosine and hypoxanthine values and then the highest and worst K_1 value was found in the EL1p (49 %) group, with no significant difference among the other methods.

On the whole, the last ATP catabolites, such as inosine (r=−0.67, $p<0.001$) and hypoxanthine (r=−0.73, $p<0.001$) and the K_1 value (r=−0.78, $p<0.001$), resulted in the chemical parameters most correlated (in a negative sense), with fish freshness determined by the sensorial EU score. K-value is a widely accepted indicator of fish freshness. Increasing the K-value, a ratio that evaluates the accumulation of inosine and hypoxanthine compared with the total pool of ATP and related

Table 4 ATP, IMP (μmol/g), ATP/IMP and AEC ratio at the death (0 h) and at 5 h after death in trial 1 (July) and at 0, at 3 and at 5 h after death in trial 2 (November)

July					
		IW	IW70N	IW100N	r.s.d.[1]
0 h	ATP	3.37	3.31	2.82	1.42
	IMP	3.39	3.65	4.25	1.68
	ATP/IMP	1.45	1.25	0.84	1.03
	AEC[2]	0.83	0.80	0.80	0.04
5 h	ATP	3.42^{a}	1.09^{b}	1.24^{b}	0.68
	IMP	3.80^{c}	6.37^{b}	8.84^{a}	1.19
	ATP/IMP	0.97^{a}	0.16^{b}	0.14^{b}	0.26
	AEC[2]	0.94	0.85	0.86	0.05
November					
		IW	IW70N	EL2p	EL1p	r.s.d. [1]
0 h	ATP	8.13^{a}	5.90^{b}	1.88^{d}	3.53^{c}	1.10
	IMP	3.54^{b}	5.02^{a}	4.87^{a}	5.41^{a}	1.15
	ATP/IMP	2.44^{a}	1.25^{b}	0.40^{c}	0.65^{c}	0.38
	AEC[2]	0.89^{a}	0.87^{a}	0.82^{b}	0.86^{ab}	0.03
3 h	ATP	4.93^{a}	3.26^{b}	1.19^{c}	1.08^{c}	0.88
	IMP	4.73^{b}	4.88^{b}	6.63^{a}	7.27^{a}	0.69
	ATP/IMP	1.06^{a}	0.68^{b}	0.18^{c}	0.15^{c}	0.20
	AEC[2]	0.87^{a}	0.89^{a}	0.76^{b}	0.81^{ab}	0.07
5 h	ATP	3.96^{a}	1.27^{b}	0.38^{b}	0.22^{b}	1.15
	IMP	5.83^{b}	5.95^{b}	8.70^{a}	6.33^{b}	1.32
	ATP/IMP	0.84^{a}	0.21^{b}	0.04^{b}	0.03^{b}	0.31
	AEC[2]	0.86^{a}	0.85^{a}	0.55^{b}	0.50^{b}	0.16

[1] r.s.d.: residual standard deviation

[2] AEC = (ATP + ADP*0.5)/(ATP + ADP + AMP)

Values with different letters (a, b, c, d) are significantly different among treatments ($p \leq 0.05$)

compounds, have been correlated with loss of freshness in many fish species, and stress at death has been reported to lead to faster ATP degradation and higher K-value (Morzel and Van de Vis 2003).

K_1 index values significantly increased from 5 % at day 1 to 27 % at day 6 and 41 % at day 10 of storage, as reported by Knowles et al. (2007) in a comparison between ice-water and electrical stunning methods used on sea bass. The K_1 value at 10 days of storage was higher than that one found in the present trial at day 11 of storage (41 % vs 29 %), which may indicate some differences in the IW procedure application. Digre et al. (2010), in a comparison of the effect of an industrial and an experimental electrical stunning method in Atlantic cod on handling stress and fish quality, also concluded that electrical stunning increases the ATP-breakdown already occurring at the time of death, leading consequently to a high K-value. However, the authors also concluded that electrical stunning of cod appears to be a promising stunning method, unless filleting is the chosen processing strategy.

Table 5 Trial 2 (November): IMP, inosine (ino), hypoxanthine (Hx) (μmol/g), and K_1 index (%) at 11, 12 and 14 days of storage in ice

		IW	IW70N	EL2p	EL1p	r.s.d.[1]
day 11	IMP	5.53^{a}	4.86^{b}	5.92^{a}	4.33^{b}	0.30
	Ino	1.99^{b}	2.90^{a}	2.88^{a}	2.53^{ab}	0.45
	Hx	0.29^{b}	0.61^{a}	0.55^{a}	0.56^{a}	0.05
	K_1[2]	29^{b}	41^{a}	37^{a}	42^{a}	3
day 12	IMP	5.52	5.20	5.33	4.31	0.69
	Ino	2.52^{ab}	2.42^{b}	2.63^{ab}	3.31^{a}	0.38
	Hx	0.70^{ab}	0.66^{b}	0.74^{ab}	0.84^{a}	0.09
	K_1[2]	37^{b}	37^{b}	39^{b}	49^{a}	5
day 14	IMP	4.67^{a}	3.43^{ab}	3.11^{b}	4.79^{a}	0.70
	Ino	3.21^{b}	2.77^{b}	3.13^{b}	4.29^{a}	0.54
	Hx	1.02^{b}	1.51^{a}	1.04^{b}	1.19^{b}	0.14
	K_1[2]	48	56	57	53	6

[1] r.s.d.: residual standard deviation

[2] K_1=(Ino+Hx) *100/(IMP+Ino+Hx)

Values with different letters (a, b, c) are significantly different among treatments ($p \leq 0.05$).

Freshness classes, shelf life and rigor release According to the data of the sensory freshness/quality evaluation performed in the November trial, the IW fish remained in A class for longer: 1 day longer than the EL1p and EL2p groups and 2 days longer than the IW70N group (Table 6). However, both the IW and IW70N groups had the longest shelf life (14 days), which was 1 day longer than the electrical stunning methods. It was also observed that the EL1p and EL2p groups displayed a slightly darker flesh colour in agreement with Lambooij et al. (2008).

The electrical stunning method with two phases (ELp2) produced also a more rapid onset of *rigor* release (Fig. 1), displaying the lowest value at 120 h from death ($p<0.05$). However, no haemorrhagic spots were found in the fillets of sea bass treated with electrical stunning, as frequently found in electric stunned freshwater fish. In fact, blood spots and damages on the column have been found in 38 % of the trout treated with the same parameters as the current study (Poli et al. 2007).

Table 6 Trial 2 (November): Freshness classes and shelf life at 10, 11, 12, 13, and 14 days of storage. A class, fresh fish; B class, stale; unfit, not edible

	10 d	11 d	12 d	13 d	14 d
IW	*A*	*A*	*A*	B	B
IW70N	A	B	B	B	B
EL2p	A	A	B	B	unfit
EL1p	A	A	B	B	unfit

Knowles et al. (2007) reported, for sea bass, that electrical stunning advanced the resolution of *rigor mortis* compared with live chilling, without affecting any of the quality aspects of the fish, such as carcass damage and sensory quality or freshness, evaluated until 10 days after death. The same pattern of *rigor* onset and release found in the present paper for sea bass stunned by electricity was described by Lambooij et al. (2008). These authors also described some further effects regarding the aspect of the eyes, which may be less dark and convex compared with the fish killed by live chilling. The differences in the muscle *post-mortem* changes can be explained in terms of muscle stimulation by the electrical current, causing the muscle contraction and trigging metabolic processes that accelerate the progress of *rigor*. This is not surprising when we consider that the electric stimulation can be used to shorten the *pre-rigor* period in terrestrial animals (Simmons et al. 2008). Erikson et al. (2012) reported that electrical stunning method applied in air (107 V_{rms} 0.5+0.2 A_{rms} for 0.5 or 15 s) did not affect cod quality, at least not to different extents, apart from some minor (but significant) differences related to the eyes and skin. However, differences in *rigor* onset, evolution and eye aspect should not be underestimated because currently, these parameters are the main criteria used by the customers to judge the whole product's freshness. Electrical stimulation of the cod during stunning (0.5 and 1.5 s) caused the depletion of the white muscle energy reserves, appearing similar to the effect of escape swimming or forced exercise (Erikson et al. 2012). In a trial on farmed Atlantic cod comparing two electrical stunning methods in seawater and in air and investigating the impact of exercise and sedation with AQUI-S™ before electrical stunning, the acceleration of *rigor mortis* occurred in the electrical stunning group without anaesthesia (Digre et al. 2010). Electrical stimulation of the muscle can cause an early onset of *rigor mortis*, and in *rigor* processing of fish should be avoided. Thus, it is of special interest to keep control of *rigor* development when commercial stunning methods based on electricity are devised (Erikson et al. 2012).

The single-phase and two-phase electrical methods used here to stun/kill the fish often produced worse results in quality than the ice water method, with some differences among them. The two-phase treatment produced higher values of lactic acid at 3 h after death and more rapid *rigor* release than the single phase, most likely because of the higher frequency level of the current in the first phase.

On the other hand, live chilling with ice-water, even if not very fast at stunning, especially when the rearing water temperature is low as in winter, induced a sedation effect and reduced muscle activity, partially limiting the stress effects, according to the results of all the studied muscle stress/quality indicators. The fish responded to hypothermia by reducing mobility and decreasing their metabolism and thus lowering the consumption of energy reserves. The additional gas mixture in ice-water produced a 40 % reduction of the time needed to obtain fish stunning/killing with the same shelf life in comparison to IW method but also caused higher values of lactic acid at 5 h after death, lower pH and ATP values and decreased duration for A class freshness. A combination of live chilling in ice slurry and the use of moderate levels of dissolved carbon dioxide is considered better than the traditional carbon dioxide narcosis using CO_2 saturated water (Erikson et al. 2006) and shortens the time required to stun fish (Poli et al. 2004, 2005). The performance of the methods with the gas mixture can most likely be justified by the rapid movement of the fish in the first minutes of the stunning. However, the use of the chosen gas mixture was able to produce a 14-day shelf life, which was better than both methods with electric stunning. However, even if the moderate CO_2 addition in the IW70N treatment caused a lesser adverse reaction compared with previous studies where higher levels of this gas were utilised (Kestin et al. 1995; Robb 2001; Van de Vis et al. 2003; Poli et al. 2004; Erikson et al. 2006), the overall results do not appear particularly promising. The actual level of welfare loss in the different stunning/killing conditions and the actual impact of a significant shortage of *rigor mortis* onset and pH drop during the *pre-rigor* filleting remain to be studied in depth.

Conclusions

The electrical stunning/killing methods tested, even if they reduced the time to stun and were better than the methods applied in several preliminary trials, still do not appear to satisfy all quality requirements for a Mediterranean species, such as sea bass, as revealed by the early *rigor mortis* onset/release and the shortage of shelf life. Electrical stunning could be a promising stunning method for sea bass after improvement, unless *pre-rigor* filleting is chosen as a processing strategy.

The use of water and ice, without any alternative reliable method, still appears to be suitable for sea bass, causing little violent reaction in the fish and reduced struggling, lowering of muscle activity and consumption of energy reserves with consequent extension of *rigor mortis* onset/release phases and of shelf life. An efficient stunning method, even when not rapid, should not cause particular stress before fish unconsciousness. The immersion in water and ice slurry is still the more practical, albeit deficient, method for sea bass. In fact, the critical aspect of this method is the longer time required to achieve complete stunning.

The addition of nitrogen to water and ice to reduce the dissolved oxygen concentration and, moreover, the addition of a moderate quantity of carbon dioxide for its neurotoxic action could be useful in reducing the stunning times, but the stress/

quality parameter results, mostly with 100 % nitrogen stunning, do not appear to justify the increased costs.

However, further studies are needed to find better stunning/killing methods to preserve welfare and quality, with suggested focus on the *pre-rigor* filleting possibility, the freshness status in the period of commercialisation, and the shelf life of the product. It would also be really useful to assess the quality changes related to the stunning/killing methods that could affect the perception of the product quality by the consumer.

Acknowledgments This research was funded by the Ministry of Agricultural, Foods and Forestry Policy, Project number 6 C 151, titled "Study of post mortem indexes of fish quality in relation to stress at death and post harvesting procedures".

The Authors wish to thank the Il Padule farm (Castiglione della Pescaia, Grosseto, Italy) and its owner Mr. Argo Fornaciari for the significant contribution in animals, facilities and experience and the Fondazione Cassa di Risparmio di Firenze, which funded the SAS® statistical software licence.

References

Acerete L, Balasch J, Espinosa E, Josa A, Tort L (2004) Physiological responses in Eurasian perch (*Perca fluviatilis*, L.) subjected to stress by transport and handling. Aquaculture 237:167–178

Acerete L, Reig L, Alvarez D, Flos R, Tort L (2009) Comparison of two stunning/slaughtering methods on stress response and quality indicators of European sea bass (*Dicentrarchus labrax*). Aquaculture 287:139–144

Atkinson D (1968) The energy charge of the adenylate pool as a regulatory parameter. Interaction with feedback modifiers. Biochemistry 7: 4030–4034

Azam K, Mackie IM, Smith J (1989) The effect of slaughter method on the quality of rainbow trout (*Salmo gairdneri*) during storage on ice. Int J Food Sci Technol 24:69–79

Bagni M, Priori A, Finoia MG, Bossù T, Marino G (2002) Evaluation of pre-slaughter and killing procedures in sea bream (*Sparus aurata*). In: "Aquaculture Europe 2002: Sea farming today and tomorrow" Special Publication n 32, pp 135-136

Bagni M, Civitareale C, Priori A, Ballerini A, Finoia M, Brambilla G, Marino G (2007) Pre-slaughter crowding stress and killing procedures affecting quality and welfare in sea bass (*Dicentrarchus labrax*) and sea bream (*Sparus aurata*). Aquaculture 263:52–60

Barton BA (2002) Stress in fishes: a diversity of responses with particular reference to changes in circulating corticosteroids. Integr Comp Biol 42:517–525

Bito M, Yamada K, Mikumo Y, Amano K (1983) Studies on *rigor mortis* of fish. Differences in the mode of *rigor mortis* among some varieties of fish by modified Cutting's method. Bull Tokai Reg Fish Res Lab 109:89–96

Bjørlykke GA, Roth B, Sørheim O, Kvamme BO, Slinde E (2011) The effects of carbon monoxide on Atlantic salmon (*Salmo salar* L.). Food Chem 127:1706–1711

Burns GB, Ke PJ (1985) Liquid chromatographic determination of hypoxanthine content in fish tissue. J Assoc Anal Chem 68:444–447

Council Regulation (EEC) No. 2406/96 laying down common marketing standards for certain fishery products. Official Journal L 334, 23 December 1996, pp 1–15

Di Marco P, Petochi T, Priori A, Finoia MG, Langobardi A, Massari A, Mandich A, Marino G (2007) Effect of anaesthetics on stress physiology and welfare in seabass (*Dicentrarchus labrax*). Aquaculture Europe 2007. Competing claims. European Aquaculture Society Conference. Istanbul, Turkey, 25-27 October 2007. CD-ROM Abstracts, pp 337-338.

Digre H, Erikson U, Misimi E, Lambooij B, Van de Vis H (2010) Electrical stunning of farmed Atlantic cod (*Gadus morhua*): comparison of an industrial and experimental method. Aquac Res 41: 1190–1202

EFSA (2006) EFSA Recommends Conditions and Methods for Effective Stunning and Killing of Animals to Avoid Pain and Minimize Suffering. http://www.efsa.europa.eu

EFSA (2008) Scientific opinion of the panel on animal health and welfare on a request from European commission on animal welfare aspects of husbandry systems for farmed European seabass and gilthead seabream. EFSA J 844:1–21

EFSA (2009a) Scientific opinion of the panel on animal health and welfare on a request from the European commission on welfare aspect of the main systems of stunning and killing of farmed sea bass and sea bream. EFSA J 1010:1–52

EFSA (2009b) Species-specific welfare aspects of the main systems of stunning and killing of farmed fish: rainbow trout. EFSA J 1013:1–55

Erikson U (1997) Muscle quality of Atlantic salmon (*Salmo salar*) as affected by handling stress. Doctoral thesis, Norwegian University of Science and Technology, Trondheim, Norway, p 68

Erikson U (2011) Assessment of different stunning methods and recovery of farmed Atlantic salmon: isoeugenol, nitrogen, and three levels of carbon dioxide. Anim Welfare 20:365–375

Erikson U, Beyer AR, Sigholt T (1997) Muscle high-energy phosphates and stress affect K-values during ice storage of Atlantic salmon (*Salmo salar*). J Food Sci 62(1):43–47

Erikson U, Hultmann L, Steen JE (2006) Live chilling of Atlantic salmon (*Salmo salar*) combined with mild carbon dioxide anaesthesia. I. Establishing a method for large-scale processing of farmed fish. Aquaculture 252:183–198

Erikson U, Lambooij B, Digre H, Reimert HGM, Bondø M, Van de Vis H (2012) Conditions for instant electrical stunning of farmed Atlantic cod after de-watering, maintenance of unconsciousness, effects of stress, and fillet quality – A comparison with AQUI-S™. Aquaculture 324–325:135–144

Gelwicks KR, Zafft DJ, Bobbitt JP (1998) Efficacy of carbonic acid as an anesthetic for rainbow trout. N Am J Fish Manage 18:432–438

Gregory NG (1998) Animal welfare at markets and during transport and slaughter. Meat Sci 8(1):2–11

Grigorakis K (2010) Ethical issues in aquaculture production. J Agr Environ Ethic 23:345–370

Hovda J, Linley TJ (2000) The potential application of hypothermia for anaesthesia in adult Pacific salmon. N Am J Aquacult 62:67–72

Izquierdo-Pulido ML, Hatae K, Haard NF (1992) Nucleotide catabolism and changes in texture indices during ice storage of cultured sturgeon, *Acipenser transmontanus*. J Food Biochem 16:173–192

Karube I, Matsuoka H, Suzuki S, Watanabe E, Toyama K (1984) Determination of fish freshness with an enzyme sensor system. J Agric Food Chem 32:314–319

Kestin SC, Wotton SB, Adam S (1995) The effect of CO_2, concussion or electrical stunning of rainbow trout (*Oncorhynchus mykiss*) on fish welfare. In: Quality in Aquaculture. European Aquaculture Society, Ghent, Belgium. Special Publication n 23, pp 380-381

Kestin SC, Van de Vis JW, Robb DHF (2002) Protocol for assessing brain function in fish and the effectiveness of methods used to stun and kill them. Vet Rec 150:302–307

Knowles TG, Brown SN, Warris PD, Lines J, Tinarwo A, Bravo A, Carvalho H, Gonçalves A (2007) Effect of electrical stunning at slaughter on the carcass, flesh and eating quality of farmed sea bass (*Dicentrarchus labrax*). Aquac Res 38:1732–1741

Lambooij E, Van de Vis JW, Kloosterboer RJ, Pieterse C (2002) Welfare aspects of live chilling and freezing of farmed eel (*Anguilla anguilla*

L.): neurological and behavioural assessment. Aquaculture 210: 159–169

Lambooij E, Kloosterboer RJ, Gerritzen MA, Van de Vis JW (2006) Assessment of electrical stunning in fresh water of African catfish (*Clarias gariepinus*) and chilling in ice-water for loss of consciousness and sensibility. Aquaculture 254:388–395

Lambooij B, Gerritzen MA, Reimert H, Burggraff D, André G, Van de Vis H (2008) Evaluation of electrical stunning of sea bass (*Dicentrarchus labrax*) in sea water and killing by chilling: welfare aspects, product quality and possibilities for implementation. Aquac Res 39:50–58

Lambooij E, Grimsbø E, Van de Vis JW, Reimert HGM, Nortvedt R, Roth B (2010) Percussion and electrical stunning of Atlantic salmon (*Salmo salar*) after dewatering and subsequent effect on brain and hearth activities. Aquaculture 300:107–112

Lines J, Kestin S (2005) Electric stunning of trout: power reduction using a two-stage stun. Aquacult Eng 32:483–491

Lines JA, Robb DH, Kestin SC, Crook SC, Benson T (2003) Electric stunning: a humane method for trout. Aquacult Eng 28:141–154

Lopes da Silva HF (1983) The assessment of consciousness: general principles and practical aspects. In: Eikelboom G (ed) Stunning of animals for slaughter. Martinus Nijhoff, The Hague, pp 3–12

Lowe T, Ryder JM, Carrager JF, Wells RMG (1993) Flesh quality in snapper, *Pagrus auratus*, affected by capture stress. J Food Sci 58: 770–796

Marx H, Brunner B, Weinzier W (1997) Methods of stunning freshwater fish: impact on meat quality and aspects of animal welfare. Z Lebensm Unters Forsch A 204:282–286

Morzel M, Van de Vis H (2003) Effect of the slaughter method on the quality of raw and smoked eels (*Anguilla anguilla* L.). Aquac Res 34:1–11

Morzel M, Sohier D, Van de Vis H (2003) Evaluation of slaughtering methods for turbot with respect to animal welfare and flesh quality. J Sci Food Agr 83:19–28

Nakayama T, Da-Jia L, Ooi A (1992) Tension changes of stressed and unstressed carp muscle isometric rigor contraction and resolution. Nippon Suisan Gakk 58:1517–1522

Panagiotis ES, Tsopelakos A, Charismiadou MA, Batzina A, Deligeorgis SG, Miliou H (2013) Comparison of the effects of six stunning/killing procedures on flesh quality of sea bass (*Dicentrarchus labrax*, Linnaeus 1758) and evaluation of clove oil anaesthesia followed by chilling on ice/water slurry for potential implementation in aquaculture. Aquac Res 1–12. doi:10.1111/are.12120

Poli BM (2009) Farmed fish welfare-suffering assessment and impact on product quality. Ital J Anim Sci 8(1):139–160

Poli BM, Scappini F, Parisi G, Zampacavallo G, Mecatti M, Lupi P, Mosconi G, Giorgi G, Vigiani V (2004) Traditional and innovative stunning slaughtering methods for European seabass compared by the complex of the assessed behavioural, plasmatic and tissue stress and quality indexes at death and during shelf life. Proceedings of the 34th WEFTA Conference, Lübeck, Germany, 12-15 September 2004, pp 58-63

Poli BM, Parisi G, Scappini F, Zampacavallo G (2005) Fish welfare and quality as affected by pre-slaughter and slaughter management. Aquacult Int 13:29–49

Poli BM, Mecatti M, Lupi P, Zampacavallo G, Parisi G, Michelotti D (2007) Two stages electrical stunning/killing method effects on stress and quality indicators in rainbow trout (*Oncorhynchus mykiss*) of different size as compared to asphyxia. Proceeding of the 37th WEFTA Annual Meeting "Seafood source of health and consumer well-being", Lisbon, Portugal, 24-27 October 2007, p 151

Pottinger TG (2001) Effects of husbandry stress on flesh quality indicators in fish. In: Kestin SC, Warriss PD (eds) Farmed fish quality. Fishing News Books, Oxford, pp 145–160

Ribas L, Flos R, Reig L, MacKenzie S, Barton BA, Tort L (2007) Comparison of methods for anaesthetising Senegal sole (*Solea senegalensis*) before slaughter: Stress responses and final product quality. Aquaculture 269:250–258

Robb DHF (2001) The relationship between killing methods and quality. In: Kestin SC, Warriss PD (eds) Farmed fish quality. Fishing News Books, Oxford, pp 220–233

Robb DHF, Kestin SC (2002) Methods used to kill fish: field observations and literature reviewed. Anim Welfare 11:269–282

Robb DHF, Roth B (2003) Brain activity of Atlantic salmon (*Salmo salar*) following electrical stunning using various field strengths and pulse duration. Aquaculture 216:363–369

Robb DHF, Warriss PD (1997) How killing methods affect salmonid quality. Fish Farmer Nov/Dec:48-49

Robb DHF, O'Callaghan MO, Lines JA, Kestin SC (2002) Electrical stunning of rainbow trout (*Oncorhynchus mykiss*): factors that affect stun duration. Aquaculture 205:359–371

Roth B, Imseland S, Moeller D, Slinde E (2003) Effect of electric stunning and current duration on stunning and injuries in marked sized Atlantic salmon held in sea water. N Am J Aquacult 65:8–13

Roth B, Moeller D, Slinde E (2004) Ability of electric field strength, frequency, and current duration to stun farmed Atlantic salmon and pollock and relations to observed injuries using sinusoidal and square wave alternating current. N Am J Aquacult 66:208–216

Roth B, Birkeland S, Oyarzun F (2009) Stunning, pre slaughter and filleting conditions of Atlantic salmon and subsequent effect on flesh quality on fresh and smoked fillets. Aquaculture 289:350–356

Roth B, Grimsbø E, Slinde E, Foss A, Stien LH, Nortvedt R (2012) Crowding, pumping and stunning of Atlantic salmon, the subsequent effect on pH and rigor mortis. Aquaculture 326–329:178–180

SAS Institute (2003) SAS Institute, SAS/STAT® User's Guide. SAS Institute, Cary

Sattari A, Lambooij E, Sharifi H, Abbink W, Reimert H, Van de Vis H (2010) Industrial dry electro-stunning followed by chilling and decapitation as a slaughter method in Claresse® (*Heteroclarias* sp.) and African catfish (*Clarias gariepinus*). Aquaculture 302: 100–105

Sebastio P, Ambroggi F, Baldrati G (1996) Influenza del sistema di sacrificazione su trote iridee di allevamento. I. Considerazioni biochimiche. Industria Conserve 71:37–49

Sigholt T, Erikson U, Rustad T, Johansen S, Nordtvedt TS, Seland A (1997) Handling stress and storage temperature affect meat quality of farmed-raised Atlantic salmon (*Salmo salar*). J Food Sci 62(4): 898–905

Sikorski ZE, Kolakoswa A, Burt JR (1990) Postharvest biochemical and microbial changes. In: Sikorski ZE (ed) Seafood: resources, nutritional composition and preservation. CRC Press Incorporated, Boca Raton, pp 55–75

Simmons NJ, Daly CC, Cummings TL, Morgan SK, Johnson NV, Lombard A (2008) Reassessing the principles of electrical stimulation. Meat Sci 80(1):110–122

Tejada M, Huidobro A, Pastor A (2001) Slaughter methods affecting adenosine triphosphate and derivates in chilled stored gilthead seabream (*Sparus aurata*). In: Kestin SC, Warriss PD (eds) Farmed fish quality. Fishing News Book, Oxford, p 408

Tornberg E, Walgren M, Brøndum J, Engelsen SB (2000) Pre-rigor conditions in beef under varying temperature and pH falls studied with rigormeter NMR and NIR. Food Chem 69: 407–418

Tort L, Gómez E, Montero D, Sunyer O (1998) Stress and immunosuppression in fish. Trends Comp Biochem Physiol 5:17–29

Van de Vis H, Oehlenschlager J, Kuhlmann H, Munkner W, Robb DHF, Schelvis-Smit AAM (2001) Effect of the commercial and experimental slaughter of eels (*Anguilla Anguilla* L.) on quality and welfare. In: Kestin SC, Warriss PD (eds) Farmed fish quality. Fishing News Book, Oxford, pp 234–248

Van de Vis H, Kestin S, Robb D, Oehlenschlager J, Lambooij B, Munkner W, Kuhlmann H, Kloosterboer K, Tejada M, Huidobro

A, Ottera H, Roth B, Sørensen NK, Akse L, Byrne H, Nesvadba P (2003) Is humane slaughter of fish possible for industry? Aquac Res 34:211–220

VKM (2010) Risikovurdering knyttet til bruk av gass, slag mot hode og strøm til bedøving av fisk. Norvegian Scientific Committee for the Food Safety. Pp 1–21

Warris PD, Brown SN, Knowles TG (2003) Measurement of the degree of development of *rigor mortis* as an indicator of stress in slaughtered pigs. Vet Rec 153:739–742

Wills CC, Zampacavallo G, Poli BM, Proctor MRM, Henehan GTM (2006) Nitrogen stunning of rainbow trout. Int J Food Sci Tech 41: 395–398