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Assessment of different stunning methods and recovery of farmed Atlantic salmon (Salmo salar): isoeugenol, nitrogen and three levels of carbon dioxide

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Abstract

*Isoeugenol (17 mg AQUI-S™ L^{-1}), nitrogen, and three levels of carbon dioxide (low: 70–80, medium: 180–250 and high: > 400 mg CO_2 L^{-1}) were tested as stunning agents for Atlantic salmon (*Salmo salar*) fasted for six days. All methods were tested under optimised conditions (starting with rested fish, and stunning and recovery under good water quality conditions). The fish were assessed in relation to behaviour and stress in terms of blood chemistry and muscle biochemistry. Only isoeugenol fulfilled all of our set criteria related to fish welfare and stress as it: (i) minimised aversive reactions upon exposure and ability to render the fish unconscious; (ii) showed no recovery during a period of 10 min post stunning; and (iii) achieved minimal muscle activity (good muscle quality). The fish treated with nitrogen showed the strongest aversive reactions, produced the most stressed fish, and fish that did not appear to be sedated. Nitrogen stunning cannot therefore be recommended. None of the levels of carbon dioxide fulfilled all criteria. When exposed to high and medium levels, fish exhibited aversive reactions and became considerably stressed. At the low level, changes in behaviour and stress were modest, but in such cases the fish were not sufficiently immobilised to facilitate easy handling in a possible pre-stunning context. No level of carbon dioxide rendered the fish unconscious. Even under optimised stunning conditions, the use of carbon dioxide cannot be recommended in connection with slaughter of Atlantic salmon.*

Keywords: *animal welfare, recovery, rested harvest, salmon, stress, stunning*

Introduction

Fish welfare during harvesting and slaughter has received increasing attention during the last decade (Wall 2001). Particular focus has been placed upon stunning and killing methods, despite the fact that welfare may also be seriously compromised during the preceding steps in a slaughter line. A slaughter method can be considered to be humane if insensibility (unconsciousness) is introduced instantaneously without fear or pain (EFSA 2009). Carbon dioxide narcosis has been identified as a potential cause of poor welfare since the fish exhibit clear aversion reactions and fail to be rendered unconscious by the treatment (Robb & Kestin 2002). Consequently, the fish might experience distress or pain during subsequent processing steps, namely bleeding and gutting. In Norway, the use of carbon dioxide for stunning of fish will be banned and alternative methods of stunning, such as electrical or percussion, are currently being introduced. However, other methods (see later) might be conceivable in the future, either as stunning agents, or in connection with pre-slaughter procedures.

This study evaluates the effect of using gases (carbon dioxide and nitrogen) and an anaesthetic (isoeugenol, administered in liquid form) on Atlantic salmon (*Salmo salar*) welfare and stress during exposure and recovery. The novel points addressed here were the evaluation of all stunning methods on equal terms, ie under optimal conditions with good water quality and without confounding factors, such as crowding stress, pumping or other factors affecting homeostasis of fish before stunning in the industry (Erikson 2008). Moreover, the study was designed to be valid in an industrial context where adequate stunning, and preferably with no subsequent recovery, were considered key factors. For good welfare, the fish should not recover after stunning, remaining unconscious until they eventually die due to loss of blood during exsanguination. Robb *et al* (2000) showed that fish that were not stunned prior to bleeding lost visual-evoked responses (VERs) 2.5 to 7.5 min after exsanguination, whereas fish subjected to carbon dioxide narcosis and exsanguination lost their VERs after 5 and 9 min. This is cause for concern since it takes at least 6 min to lose sensibility under carbon dioxide narcosis (Kestin *et al* 2002). Therefore, to avoid possible pain sensation during bleeding, we set a criterion of no recovery within 10 min post stunning in the present study. The rationale behind selecting the various stunning methods differed to some extent, and they were as follows.

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Carbon dioxide

In the salmon industry, carbon dioxide narcosis is used to immobilise fish prior to bleeding (cutting of gill arches). A concentration of 200–500 mg CO_2 L^{-1} is necessary for complete sedation of large salmon (Bell 1987; Iwama & Ackerman 1994). Typically, the levels of carbon dioxide used in stunning tanks in the salmon industry is 250–460 mg L^{-1} (Erikson 2008) and fish holding time in such tanks is 5–10 min. Under these conditions, it takes 2–4 min before fish are immobilised. Eventually, larger refrigerated seawater (RSW) live chilling tanks were introduced into the industry. Here, carbon dioxide was introduced to the chilled water (0–3°C) at lower levels (80–120 mg L^{-1}). Oxygen gas is also added to ensure sufficient levels of dissolved oxygen (DO > 70% saturation) for adequate respiration (Erikson *et al* 2006). Nevertheless, the fish still struggle for 2–4 min before eventually becoming motionless in such tanks. Normally, the fish are kept in the RSW tank for 20–50 min prior to exsanguination (Erikson 2008). Commercial live chilling of salmon at high CO_2 concentrations for 40 min does produce calm fish, however, they are not rendered unconscious as all demonstrate eye rolling (Roth *et al* 2006).

In the salmon industry, it has been speculated that there may be a level of CO_2 at which fish can be sedated without inducing strong aversive reactions and excessive stress. For instance, to facilitate easy feeding (of calm fish) into stunning machines, it might be conceivable to sedate fish prior to killing them by percussion stunning. Gentle transfer of calm fish to the stunning or killing units is currently regarded as an issue requiring special attention to improve the welfare of the fish, see above (EFSA 2009). Low levels of carbon dioxide to induce avoidance reactions have been suggested as a means for self-transfer of rainbow trout (*Salmo gairdneri*) between tanks. By introducing carbon dioxide to the tank, normal swimming behaviour was altered when CO_2 rose from 35–60 mg CO_2 L^{-1} (pH 7.20–6.95). Fish movement between tanks began to occur at 60 mg CO_2 L^{-1}. The best range to move fish by avoidance response was produced between 60–120 mg CO_2 L^{-1} (pH 6.72–6.41). Almost all fish (mean weight 1.3 kg) lost equilibrium at 150 mg CO_2 L^{-1}, however, only a very low death rate was observed even after 24 h (Clingerman *et al* 2007). CO_2 levels above 155 mg CO_2 L^{-1} induce narcosis in rainbow trout in less than 3 min at 14°C. Above 320 mg CO_2 L^{-1}, 20% deaths occurred, in contrast to none during the 72-h recovery period (Gelwicks *et al* 1998).

In this study we assessed the sedation and recovery of salmon subjected to three levels of carbon dioxide: high (as in traditional carbon dioxide tanks); medium (as in RSW live chilling tanks); and low (as a possible treatment to facilitate easy handling before killing).

Isoeugenol

Although not currently licenced for use in food animals in Norway and the EU, isoeugenol has been identified as a true and effective anaesthetic for salmonids (Keene *et al* 1998; Iversen *et al* 2003) which is associated with good fish welfare. Eugenol also has an analgesic effect resulting from the inhibition of prostaglandin synthase (Keene *et al* 1998), and above 20 mg eugenol L^{-1}, eugenol can eliminate stress-induced increases in plasma cortisol (Iversen *et al* 2003). Isoeugenol was included in our study for two reasons: (i) for a comparison with carbon dioxide and nitrogen under optimal conditions and (ii) to check whether the isoeugenol-treated fish might recover within 10 min, our chosen 'limit' for good fish welfare practices (exsanguination step).

Nitrogen

The use of nitrogen has been suggested as a relatively new and effective stunning method for rainbow trout (Wills *et al* 2006). As we are unaware of any anaesthetic effect of the gas, and since the levels of white muscle ATP and pH in that particular study were relatively low after the nitrogen treatment (suggesting considerable anaerobic muscle work), we felt this subject matter required further investigation. We decided therefore to check the stunning effect of nitrogen on Atlantic salmon.

Evaluation criteria

When comparing the different stunning methods, we selected the following stunning-agent criteria related to fish welfare and product quality: (i) it should minimise aversive reactions upon exposure to the gases or anaesthetic; (ii) it should be able to produce unconsciousness; (iii) result in no recovery during the selected 10 min post stunning; and (iv) should promote 'rested harvest' by minimising muscle activity.

Materials and methods

Study animals

Atlantic salmon, not fasted, weighing 2.39 (± 0.62) kg (Mean [± SD], n = 52) with fork length 56 (± 4) cm were obtained from Aqua Gen AS, Kyrksæterøra, Norway. The fish were netted from a sea cage (Kjønsvika, Norway) on 30th April 2009 and transferred into two 1,000-L tubs. Fresh seawater (SW) was supplied continuously to the tubs during the 30-min boat transport to the quay where fish were netted and transferred to five transport tanks on a truck. The tanks had just been filled with fresh SW. The fish were transported under constant oxygenation for 2 h to our laboratory at a density of approximately 19 kg m^{-3}. After transport, a degree of foaming was observed in all tanks. SW temperature, pH and dissolved oxygen (DO) in the tanks ranged from 8.7–9.2°C, 6.95–7.13, and 225–367% saturation, respectively. The lowered pH indicated elevated levels of carbon dioxide since no water exchange took place during transport. This, together with the fact that the SW was heavily oxygen supersaturated, suggested the fish had developed hypercapnia. The fish were netted individually from the truck to a 1,000-L tub filled with fresh SW. The tub was transferred (< 5 min) to two holding tanks (4,000 L each) in our laboratory into which the fish were divided equally. The procedure was repeated three times resulting in a fish density of approximately 23 kg m^{-3} in both holding tanks. Fresh SW was pumped, sand-filtered and circulated to the tanks at a rate of 5 m^3 h^{-1}. After an hour or so, the fish

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regained normal swimming behaviour and distributed themselves evenly in the water. The SW pH, temperature and DO levels in both tanks were 7.92, 7.2–7.9°C and 84–94% saturation, respectively, during the six days the fish were allowed to recover from stress due to transport. No feed was offered during this period.

Experimental procedure

The fish were subjected to seven different treatments (see below). The number of fish per treatment was eight, with the exception of nitrogen where only four fish were used. For each stunning method (except for the 'isoeugenol stunning' and 'low carbon dioxide' groups), the fish were transferred immediately to 200-L recovery tubs filled with fresh SW after stunning, one fish per tub. The fish were kept there for 10 min for behavioural observation and to check whether consciousness had been regained. The DO levels in the recovery tubs ranged from 79 to 126% saturation. After 10 min, the fish was removed and killed by a sharp blow to the head. The ventral aorta was cut with a scalpel causing a considerable flow of blood into the cavity behind the operculum. Glucose and lactate were immediately measured using test-strips. Blood pH was also measured immediately after cutting the aorta. The fish were then tagged and transferred to an SW tank for bleeding out. After 20 min, muscle pH and twitching were measured before recording of fish length and weight. Subsequently, fish were placed into ice-filled styrofoam boxes for evaluation of time to onset of *rigor mortis*.

Experimental groups and stunning procedures

Control

Since it is known that quick netting of individual salmon followed, within seconds, by a sharp blow to the head, represents a relatively good approximation for sampling of 'rested' fish (Erikson 1997), the method was applied here to create the control group. Fish were netted, individually, from the holding tank and killed by a percussive blow to head within 5–10 s before being placed into the observation tub (10 min).

Isoeugenol stunning

Eight fish were transferred from the holding tank to a 400-L tank with good SW exchange. The tank was covered with a black plastic sheet and fish left undisturbed until the following day. The water supply to the tank was then closed and oxygen gas was distributed to the tank through a diffuser (Point Four Systems Inc, Richmond, Canada). A predetermined volume of concentrated AQUI-S™: 5.8 ml was added to the tank resulting in the recommended concentration of 17 mg L^{-1} for salmonids (AQUI-S Ltd, Lower Hutt, New Zealand). AQUI-S™ contains 540 g L^{-1} isoeugenol which is the active component of the anaesthetic. During the stunning (40 min), DO levels varied between 106–115% saturation.

In order to check for possible differences in blood and muscle stress indicators immediately after stunning, and after recovery, two isoeugenol groups were studied. Single fish (in the stunning group) were sampled from the tank 10, 15, 19, 23, 26, 30, 36 and 40 min after isoeugenol was added to the tank. The fish were killed immediately and not monitored post stunning.

Isoeugenol recovery

The same procedure was repeated as in the stunning group. In this case, however, two fish at a time were transferred from the stunning tank to four observation tubs (two fish per tub) 10, 15, 25 and 30 min after isoeugenol was added. Here, DO levels varied between 95–182% saturation in the stunning tank.

Nitrogen

Nitrogen gas was added from a gas cylinder through a diffuser to a 400-L tank filled with fresh SW. No water exchange took place during the experiment. After approximately 20 h, individual fish were quickly netted from the holding tank and switched to the experimental tank (~5 s). The DO level and water temperature in the stunning tank were 5% saturation and 11°C, respectively. Four fish (one at a time) were monitored constantly for 10 min prior to transfer to the recovery tub.

High levels of carbon dioxide

Carbon dioxide gas was added from a gas cylinder to a 400-L tank using a diffuser, and the pH of SW was used as an indicator of carbon dioxide levels. The tank was filled with fresh SW without water renewal. The gas was added until pH reached the desired level of 5.6. The gas flow was then reduced to maintain this level. As with the nitrogen group, one fish was monitored at a time during stunning and recovery periods. The water temperature in the stunning tank was 7.4°C and the DO level dropped gradually from 93 to 81% saturation during the course of the experiment.

Low levels of carbon dioxide

The experiment was carried out as with the high-level carbon dioxide experiment, with the exception that the acidity of the water was reduced to pH 6.4 to produce a lower concentration of carbon dioxide. Here, the water temperature was 6.7°C and the DO concentration 100% saturation.

Medium levels of carbon dioxide

The experiment was carried out with eight fish. The volume of the water in the tank was halved approximately. To simulate a closed well-boat transport, the water supply was shut-off and oxygen gas added via a diffuser. The fish were kept in the tank for 19 h prior to stunning. At this point, the water acidity had been reduced to pH 7.37 due to excretion of metabolically produced carbon dioxide. The water temperature and DO level were 10.1°C and 144% saturation, respectively. A water sample (500 ml) was withdrawn from the holding tank at this point, for subsequent analysis. For comparison, fresh SW (tank inlet water) was also sampled. Fish were netted, one fish at a time, and transferred to the stunning tank. From this point onwards, the fish were treated as with the two other carbon dioxide experiments. The pH, temperature and DO level in the stunning tank were 6.0 (± 0.2), 9.5°C and 94% saturation, respectively.

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Table 1 Behavioural changes at various stages of anaesthesia (Schoettger & Julin 1967).

Stage	Description	Behaviour
1	Light sedation	Partial loss of reaction to external stimuli
2	Deep sedation	Partial loss of equilibrium, no reaction to external stimuli
3a	Total loss of equilibrium	Fish usually turn over but retain swimming ability
3b	Total loss of equilibrium	Swimming ability stops, but responds to pressure on the caudal peduncle
4	Anaesthesia	Loss of reflex activity, no reaction to strong external stimuli
5	Medullary collapse	Respiratory movement ceases (death)

Table 2 Fish behavioural stages during recovery after stunning.

Stage	Description	Behaviour
I	Normal	Normal swimming behaviour, complete equilibrium
II	Escape behaviour	Excessive struggling (tail flaps) voluntary, or when fish is attempted to be removed from tub
III	Incomplete previous stunning (or secondary signs of recovery III*)	Clearly evident and regular respiration, fish lying on the bottom of the tub, in some cases interrupted by short occasional swimming periods, or attempts to regain equilibrium
IV	Incomplete previous stunning (or first signs of recovery IV*)	Weak response (tail flaps) if fish is attempted to be lifted from tub, or minor sporadic movements, weak or occasional opercular motions, fish is lying on bottom
V	Unconscious or dead	Motionless, no responses whatsoever

Analytical methods

Water quality

The concentration of dissolved oxygen, temperature and pH was measured by an OxyGuard model Handy Polaris Portable DO Meter (Birkerød, Denmark), a Testo 110 thermometer (Lenzkirch, Germany), and a shielded glass electrode (WTW SenTix 41) connected to a portable pH meter (model WTW 315i, WTW, Weilheim, Germany), respectively.

The approximate levels of carbon dioxide used in this study were obtained from the measured SW pH values. The values were extrapolated from a plot of values from previous studies where pH and carbon dioxide were determined simultaneously (Erikson *et al* 2006; Erikson 2008).

The water samples, a clean SW sample (reference) and a sample from the closed system, were analysed with respect to total ammonium (TA-N), alkalinity, colour (water clarity), total organic carbon (TOC) and ferric iron (indices of haem iron, ie loss of blood). A detailed description of the analytical methods has been reported previously (Erikson *et al* 2006).

Fish behaviour

As an index of the speed of recovery from the various stunning methods and behaviour during recovery (10 min), each individual fish was monitored constantly. Indices of fish recovery were swimming activity, opercular movements (signs of respiration), loss of equilibrium, response to stimuli (first touching the lateral line, then, if no response, grabbing the tail) and convulsions. Vestibulo-ocular reflex (VOR or 'eye roll') was also used as suggested by Kestin *et al* (2002). Robb and Roth (2003) established a good correlation between recovery of VER and recovery of opercular movements, suggesting the use of opercular movements as a possible method to determine unconsciousness in salmon after stunning. Where relevant, different stages of anaesthesia were assessed according to Schoettger and Julin (1967), see Table 1. For assessment of behaviour during recovery after stunning, different stages (range: Stages I to V) were devised as shown in Table 2. The stages describe behaviours after incomplete stunning or partial recovery from stunning.

Glucose

The tip of a glucose test strip was dipped briefly in blood immediately after cutting of the aorta. The strip was then inserted into an Ascensia Contour Meter (Bayer HealthCare LLC, Mishawaka, Indiana, USA) and the glucose concentration read in mmol L^{-1}.

Lactate

Whole blood lactate was measured using the Lactate Pro™ Test Strip method (Arkray Inc, Kyoto, Japan). The test strip was dipped briefly in blood, virtually simultaneously with the glucose strip, before being inserted into the test meter. The measuring range is 0.8–23.3 mmol L^{-1}. The method has been tested on fish and is regarded as a reliable method of assessing the welfare of farmed fish (Brown *et al* 2008).

Blood and muscle pH

The blood pH was measured immediately after cutting the aorta whilst muscle pH was recorded in the epaxial white muscle between the lateral line and dorsal fin. During this process, the instrument (described above)

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Table 3 Major behavioural changes during stunning of Atlantic salmon with isoeugenol (n = 8), nitrogen (n = 4), high (n = 8) and low (n = 8) levels of carbon dioxide. Within treatment, the number of fish assigned different behavioural characteristics are shown in brackets.

Time (min)	Isoeugenol stunning[1]	Nitrogen	High carbon dioxide	Medium carbon dioxide	Low carbon dioxide
0–2		SS, FS, EB (4)	SS; FS; EB; G; C (8)	FS → SS (7); SS (1)	FS → SS (7); SS (1)
0–3	SS (8)				
2–3	Stage 1 → Stage 2 (8)	EEB (3/4)		EB (8)	
2–7			Stage 1 → Stage 3b (7) Stage 5 (1)[3]		
3–4		SLE; Stage 3a (4)		Stage 2 → Stage 3a; G (8)	
3–6					G; CUS (8)
4–9	Stage 3a → Stage 3b (8)			Stage 3a → Stage 3b (8) CUS → CBS; G (8)	
5–7		SBU (2); CBS (2)			
6–10					Stage 1 (1); Stage 2 (5); Stage 3a (2); G; CUS (8)
7–10		C, G (4)			
10		NRS (4)	Stage 3b (7/8)	EB (6/8)	EB (7/8)
10–40[1]	Stage 3b (4, 10–23 min) Stage 4 (2, 26–30 min) Stage 5 (2, 36–40 min)				
VOR[2]	0[4]/8	4/4	5/8	8/8	8/8

SS = Slow swimming; FS = Fast swimming; SBU = Swimming with belly up; CBS = Fish lying calm on bottom, interrupted by short bursts of intense swimming activity; CUS = Fish standing calm on bottom in an upright position, occasional swimming to surface; EB = Escape behaviour; EEB = Extreme escape behaviour, the fish try to jump out of the tank; SLE = Sudden loss of equilibrium (belly up); G = Gasping; C = Convulsions; NRS = No reactions to stimuli (touching lateral line, pressure on caudal peduncle, or lifting).

[1] To avoid acute stress during transfer to isoeugenol tank, the anaesthetic was added directly to a tank containing eight fish (batch stunning). Single fish were sampled after 10, 15, 19, 23, 26, 30, 36 and 40 min after isoeugenol was added to the tank; [2] Eye roll observed when fish were removed from tank (number of fish showing VOR/total number of fish per treatment); [3] Stage 5 was reached after 7 min. Thereafter, one fish remained totally motionless, including during subsequent 10 min in recovery tub; [4] Difficult to assess whether five fish actually exhibited very weak VOR or no VOR at all. These fish were exposed to isoeugenol for 10–26 min. Three fish sampled from the isoeugenol tank after 30, 26 and 40 min did not show VOR.

was frequently calibrated using pH 4.01 and 7.00 buffers. Frequent cleaning of electrodes and recalibration was required to obtain consistent results.

Muscle twitches

Early post mortem muscle contractions were determined using a Twitch Tester Quality Assessment Tool (AQUI-S Ltd, New Zealand) an instrument which measures the electrical excitability of muscle tissues. An electrical pulse was generated (9 V DC) by the instrument every 0.6 s. One, or a few (< 4), measurements were performed on one side of each fish. For each measurement, the electrodes were in contact with the fish for approximately 1 s. The following scale was used: (3) strong tail twitch (electrodes placed along the entire lateral line, behind the head and near the caudal fin); (2) weak tail twitch (electrodes placed as above); (1) minor muscle contractions in (small) restricted areas of the fish surface (electrodes placed a few cm apart); and (0) no contractions whatsoever.

Rigor mortis

The time to onset of *rigor mortis* was determined. Early onset of rigor is directly linked to peri mortem muscle activity (muscle pH) and is an important parameter for fish processors since the fish should be processed before the onset of *rigor mortis*. The rigor status was determined as: (0) pre-rigor; (1) rigor onset (first sign of stiffness, for instance in neck or tail region); (2) rigor (a larger area is clearly in rigor); (3) whole fish in rigor; (4) stronger rigor; and (5) very strong rigor (the fish is extremely stiff, rod-like) (Erikson 2001).

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Table 4 Atlantic salmon behaviour stages during recovery for 10 min after stunning with isoeugenol (n = 8), nitrogen (n = 4), high (n = 8) and medium levels of carbon dioxide (n = 8). Within treatment, the number of fish assigned different behavioural characteristics are given in brackets.

Time (min)	Control	Isoeugenol recovery[4]	Nitrogen	High carbon dioxide	Medium carbon dioxide
0[1]	Stage V (8)	Stage IV (2, 10 min)	Stage III (4)	Stage II (2)	Stage II (6)
		Stage V (4; 15, 25 and 30 min)		Stage III (3)	Stage III (2)
				Stage IV (2)	
				Stage V (1)	
0–10	Stage V (8)	Stage V→ Stage IV* (5; 10, 15 and 25 min)	Stage III (4)	Stage III (3)	Stage III (8)
		Stage V (3; 10 and 30 min)		Stage IV → Stage III* (3)	
				Stage IV (1)	
				Stage V (1)	
10[2]	Stage V (8)	Stage IV (3; 10, 15 min)	Stage III (4)	Stage III (3)	Stage II (5)
		Stage V (5; 10, 25, 30 min)		Stage IV (3)	Stage III (3)
				Stage V (2)	
VOR[3]	0/8	0/8[5]	4/4	6/8	8/8

[1] Initial reaction upon transfer to recovery tub; [2] Reaction when the fish were removed from tub; [3] Eye roll when the fish were removed from tub (Number of fish showing VOR/total number of fish per treatment); [4] Batch stunned. Two fish at a time were transferred to recovery tubs after exposure to isoeugenol for 10, 15, 25 and 30 min; [5] Difficult to assess whether four fish actually exhibited very weak VOR or no VOR at all (fish had been exposed to isoeugenol for 10 and 15 min). Two of these fish responded to stimuli (when touching lateral line or tail) after 10 min. Fish exposed to isoeugenol for 25 and 30 min did not show VOR.

Statistics

Blood and muscle stress indicators were compared using a one-way ANOVA. Where significance between treatments was indicated, data were analysed using a Tukey *post hoc* test. The significance level was set at $P < 0.05$.

Ethics

These experiments were conducted in accordance with the Norwegian Animal Welfare Act and the experimental design approved by SINTEF Fisheries and Aquaculture personnel authorised by the Norwegian National Animal Research Authority.

Results

Behaviour during stunning and recovery

Control

No reactions whatsoever were observed during the 10-min period that fish were in the recovery tub. This indicated that the fish had been effectively killed by percussion stunning.

Isoeugenol; stunning tank

Fish behaviour during stunning is shown in Table 3. After isoeugenol was introduced into the tank, no obvious change in behaviour was observed (slow swimming). After 2–3 min, the first signs of sedation were observed, the changes corresponding to changes from Stage 1 to Stage 2, according to Schoettger and Julin (1967) as described in Table 1. Total loss of equilibrium, and, eventually, cessation of swimming (Stage 3a to 3b) was observed between 4–9 min. Individual fish were sampled and subjected to stress assessments after exposure to isoeugenol for 10–40 min. It was clear that behavioural stage depended on exposure time. At the present concentration of isoeugenol (17 mg AQUI-S™ L^{-1}), at least 30 min of exposure was needed to produce fully anaesthetised salmon. Accordingly, eye roll (VOR) was not present in fish sampled after 30 min. Prior to this (Stage 3b), we considered it difficult to assess whether the fish actually exhibited very weak eye roll as opposed to none at all.

Isoeugenol; recovery tub

Also, during recovery, behavioural stage depended on exposure time to isoeugenol (Table 4). Basically, the fish did not recover significantly during the 10 min in the tub containing fresh SW (recovery Stages IV and V). Moreover, very weak, or no eye roll was also observed at this stage.

Nitrogen; stunning tank

During the first 2 min after transfer from the holding tank, all fish changed between slow and fast swimming modes, and exhibited escape behaviour (Table 3). Shortly afterwards, the fish attempted to leap from the tank. After 3–4 min, all fish suddenly lost equilibrium and floated at the water surface, belly up. Two fish eventually started to swim upside down whereas the other two were lying on the bottom, occasionally exhibiting short bursts of intense swimming activity. When removed from the tank, the fish did not respond to stimuli, but all displayed VORs.

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Table 5 Mean (± SEM) changes in blood chemistry and white muscle demonstrating stress due to stunning (and recovery) of Atlantic salmon.

Stunning method	Blood pH	Blood lactate (mM)	Blood glucose (mM)	Muscle pH	Muscle twitch[nsd] (range 0–3)	Rigor status/time post mortem
Percussion stunning (control)	7.54 (± 0.10)[a]	2.2 (± 0.5)[a]	3.3 (± 0.7)[a]	7.31 (± 0.04)[a,b]	n/a	0.6 after 22 h
Isoeugenol (stunning)	7.37 (± 0.05)[a]	4.7 (± 0.8)[a,b]	3.6 (± 0.3)[a,b]	7.50 (± 0.04)[b]	2.5 (± 0.2)	n/a
Isoeugenol (recovery)	7.36 (± 0.05)[a,c]	6.9 (± 0.9)[b]	5.6 (± 0.7)[b]	7.34 (± 0.03)[a,b]	n/a	2.2 after 30 h
Nitrogen	7.17 (± 0.05)[b,c]	7.4 (± 1.5)[b]	4.0 (± 0.6)[a,b]	6.59 (± 0.06)[d]	n/a	1.9 after 2.5 h
Carbon dioxide (high)	7.06 (± 0.05)[b,d]	4.1 (± 0.3)[b,c]	3.8 (± 0.3)[a,b]	6.96 (± 0.06)[c]	2.4 (± 0.3)	2.5 after 7 h
Carbon dioxide (medium)	7.30 (± 0.06)[a,c,d]	5.4 (± 0.9)[b,d]	n/a	7.16 (± 0.07)[a,c]	2.4 (± 0.2)	n/a
Carbon dioxide (low)	7.30 (± 0.02)[a,c,d]	3.0 (± 0.4)[a,c,d]	3.4 (± 0.5)[a,b]	7.28 (± 0.04)[a]	2.6 (± 0.1)	1.3 after 9h

Different superscripts indicate significant difference at $P < 0.05$; n/a = not analysed; nsd = no significant difference.
Rigor scores: 0 = pre-rigor; 1 = rigor onset (first sign of stiffness, for instance in neck or tail region); 2 = rigor (a larger area is clearly in rigor); 3 = whole fish in rigor; 4 = stronger rigor; 5 = very strong rigor (the fish is extremely stiff, rod-like). (n = 8 except for nitrogen group where n = 4).

Nitrogen; recovery tub

Just as the fish entered the recovery tub, each individual exhibited two very strong tail flaps before they lay sideways on the bottom of the tub, gasping. Until the fish were removed from the recovery tub 10 min later, all showed behaviour typical of incomplete stunning (recovery Stage III, Tables 2 and 4), as well as VORs.

High carbon dioxide; stunning tank

The acidity in the tank varied between pH 5.6–5.2, corresponding to 400–1,000 mg CO_2 L^{-1}. Each fish introduced into the tank, behaved differently. Behaviour changed between slow and fast swimming, escape behaviour and gasping. Convulsions were also observed shortly after transfer to the tank (Table 3). Overall, the initial (2–3 min) behaviour of the fish seemed to be less active compared with the fish subjected to the nitrogen treatment. Between 2–7 min after transfer, seven of the fish changed behaviour corresponding to Stages 1 to 3b. One fish showed no responses at all (Stage 5), including during the subsequent 10 min in the recovery tub. Otherwise, Stage 3b behaviour prevailed until the fish were removed from the tank. Five out of eight fish showed VORs.

High carbon dioxide; recovery tub

Upon transfer to the recovery tub, individual fish exhibited recovery Stages II, III, IV and V (Tables 2 and 4). Afterwards (0–10 min), no escape behaviour (Stage II) was observed and there were some transient changes between stages. In three cases, recovery from Stage IV to Stage III* was seen. Immediately prior to netting (10 min), six fish were assigned recovery Stages III and VI. As the fish were netted from the tub, they struggled markedly. Six out of eight of these fish also exhibited VORs.

Low carbon dioxide; stunning tank

The pH varied between 6.40–6.45, corresponding to ~70–80 mg CO_2 L^{-1}. After netting from the holding tank, most fish initially swam fast before they slowed down to a normal swimming mode (Table 3) and, after 3–6 min, all fish were standing at the bottom, gasping, interrupted by occasional swimming to the surface. The fish were partly sedated after 6–10 min (Stages 1, 2 and 3a). Seven out of eight fish displayed strong escape behaviour when they were netted from the tank and all showed VORs. They were killed immediately, therefore, as opposed to being transferred to recovery tubs.

Medium carbon dioxide; overview

After exposure in the closed system for 19 h, the fish exhibited normal behaviour. By this point, the pH had been reduced to 7.37, corresponding to ~5–10 mg CO_2 L^{-1}. The water quality in the closed system had not deteriorated to a great extent, apart from elevated levels of total ammonium (TA-N). Compared with our laboratory SW supply (see brackets), the closed system values were as follows: TA-N: 968 (24) µg L^{-1}; alkalinity: 2.375 (2.346) mmol L^{-1}; colour (water clarity): 4.6 (4.3) mg Pt L^{-1}; TOC: 1.8 (1.1) mg L^{-1}; Fe^{3+} (indices of haem iron): < 0.003 (< 0.003) mg L^{-1}. We considered these changes in water quality to be too small to

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affect the condition of fish prior to stunning. Therefore, closed system issues are discussed no further and the treatment is solely regarded as medium carbon dioxide.

Medium carbon dioxide; stunning tank

The pH varied between 5.8–6.1, corresponding to 180–250 mg CO_2 L^{-1}. For the first two minutes following transfer, the fish clearly changed from initially fast to relatively slow swimming modes. Short bursts of high activity were observed after 2–3 min. Between 3–9 min, fish displayed a gradual loss of equilibrium (Stage 2 to 3b). Gasping was observed for all fish from 3 min onwards and eventually most fish were lying on the bottom of the tank. After 10 min, all fish exhibited VORs and six out eight demonstrated escape behaviour when lifted from the tank.

Medium carbon dioxide; recovery tub

Immediately following transfer to the fresh SW in the recovery tub, all fish responded with a short, but fierce burst of activity before they settled lying on their sides on the bottom of the tub. During the 10 min the fish were kept in the tub, they remained in recovery stages II or III and upon removal, all of the eight fish were judged as having VORs. As all fish were clearly conscious and could not be easily lifted out by hand, quick netting was necessary to avoid imposing unintentional stress before killing and assessment of stress.

Stress during stunning and recovery

If we assume that the fish had recovered after transport stress (see *Materials and methods*), the results presented in Table 5 can be associated with stress as a consequence of stunning and recovery only.

Blood

The highest mean blood pH values were measured in the control group (pH 7.54) followed by the two isoeugenol groups (pH 7.37) but they did not differ significantly (Table 2). The pH of fish exposed to isoeugenol did not decrease during the 10 min in the recovery tub. Exposure to low and medium levels of carbon dioxide resulted in lower blood pH values of 7.30 whereas the blood of fish from the high carbon dioxide level was 7.06. The latter group, along with the fish from the nitrogen treatment (blood pH 7.17), exhibited the lowest mean pH values recorded.

The lowest mean concentration of blood lactate was observed in the control fish (2.2 mmol L^{-1}; Table 2). Lactate increased from 4.7 to 6.9 mmol L^{-1} during recovery of the fish treated with isoeugenol. The highest mean level of lactate (7.4 mmol L^{-1}) was observed in fish treated with nitrogen. The mean lactate concentrations in the blood of fish from the low, medium and high levels of carbon dioxide were 3.0, 5.4 and 4.1 mmol L^{-1}, respectively. Since there were relatively large differences among individuals within each group, significant differences between groups followed a rather complex pattern (Table 2).

The variation in blood glucose levels was less than with lactate (Table 2). With the exception of the highest mean level of 5.6 mmol L^{-1} seen in fish treated with isoeugenol and then sampled from the recovery tub 10 min later, all other treatments exhibited similar glucose concentrations within the narrow range of 3.3 to 4.0 mmol L^{-1} ($P > 0.05$). There was a common trend however, in that mean glucose levels corresponded with lactate levels.

For the isoeugenol group (batch stunning), a serial effect of sampling was observed as an increasing trend in lactate concentration (from 2 to 8 mmol L^{-1}) as the fish were successively sampled during a 30-min period after the set stunning time of 10 min. No serial effects were observed for blood glucose and pH over the same time-period.

White muscle

The degree of muscle activity, or escape behaviour, is shown in Table 2. As expected, when the salmon were anaesthetised using isoeugenol, a muscle pH typical of rested fish was obtained (pH 7.50). A somewhat lower mean value (pH 7.34) was observed when pH was determined after recovery instead of immediately after stunning ($P > 0.05$). At pH 7.31, the control group had a slightly lower mean pH than with the isoeugenol treatment ($P > 0.05$). The lowest mean value (pH 6.59) was recorded in fish subjected to the nitrogen treatment ($P < 0.05$). The mean pH values of fish subjected to low, medium, and high levels of CO_2 were 7.28, 7.16 and 6.96, respectively.

The mean muscle twitch values (2.4–2.6) did not differ significantly (Table 2), ie all fish exhibited significant tail flaps upon electrical stimulation.

Rigor mortis in fish from control and isoeugenol groups started after 22 and 30 h post mortem, respectively (Table 2). In the low and high carbon dioxide groups, rigor started after 9 and 2.5 h, respectively, while in the nitrogen group, rigor had clearly started after just 1.3 h.

Discussion

Blood chemistry

The blood pH values in this study ranged from approximately 7.5 (rested fish) to 7.1 (exhausted fish) (Table 2). By comparison, when rainbow trout are stunned with CO_2, the plasma pH drops from 7.8 to 7.0 due to respiratory acidosis (fish exposed to CO_2 were assessed after cessation of opercular movements as was the case here) (Iwama *et al* 1989). In Atlantic salmon, exhaustive exercise results in a drop in blood pH from the resting value of 7.848 to 7.316 (Tufts *et al* 1991). This means our change in pH (rested vs exhausted fish) of 0.4 units was lower than the published change (pH 0.8–0.5). This discrepancy may be explained by the use of our slightly crude field method for assessment of blood pH, perhaps making it difficult to obtain true blood pH values for rested fish (around pH 7.8). Where the fish were exposed to carbon dioxide, it should be pointed out that low environmental pH (caused by addition of CO_2) *per se* can also cause a significant drop in blood pH (Packer & Dunson 1970). Thus, the reduction in blood pH of our three groups of salmon exposed to carbon dioxide was probably a result of both exercise and low environmental pH.

Our blood lactate values ranged from about 2 to 7 mmol L^{-1} whereas glucose did not vary to any great extent between

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treatments (Table 2). In Atlantic salmon smolts, Iversen *et al* (2003) also reported that plasma glucose did not change during a 30-min exposure to isoeugenol whereas plasma lactate concentrations rose initially from 1.2–2.3 mmol L^{-1} to 3.4 and 3.9 mmol L^{-1} after 10- and 20-min exposure, respectively. For adult salmon, the difference between control fish and fish subjected to stress and exercise was about 3–4 and 5-9 mmol L^{-1} (Thomas *et al* 1999). In adult rainbow trout, the resting levels of whole blood glucose and lactate have been reported as 3.79 and 0.58 mmol L^{-1}, respectively. Following exercise to exhaustion, the glucose levels immediately, and 30 min after exercise were 4.35 and 4.79 mmol L^{-1}, respectively. Corresponding lactate levels were 8.82 and 10.95 mmol L^{-1} (Milligan & Wood 1986).

Muscle biochemistry

As we would expect, a clear relationship was demonstrated between our visual observations of excessive struggling (escape behaviour) and initial white muscle pH (Table 2). Compared with the anaesthetised fish (isoeugenol), it is well established that anaerobic depletion of the glycolytic energy reserves leads to formation of lactate and protons which causes a drop in muscle pH. The muscle pH values in Table 2 cover the whole physiological range possible, from 7.50 (rested fish, isoeugenol group), to an extreme low of 6.59 (exhausted fish, nitrogen group) (Booth *et al* 1995; Wilkie *et al* 1997; Misimi *et al* 2008).

Fish from all treatments showed relatively strong muscle twitches (values between 2 and 3) upon stimulation (Table 2). This showed that ample amounts of ATP, necessary for muscle contractions, were still present in the muscle immediately after killing. A somewhat larger difference between rested and exhausted fish has nevertheless been observed previously (Misimi *et al* 2008).

In line with the assessments of stress, the earliest onset of *rigor mortis* was observed for fish exposed to nitrogen, whereby mortis had already developed considerably 2.5 h post mortem (Table 2). Rigor had clearly started after 7 and 9 h in fish exposed to high and low levels of carbon dioxide, respectively, whereas in the control and isoeugenol-recovery groups, rigor commenced after more than 20 h. In the case of iced, stored Atlantic salmon, the extremes of time to onset of rigor are 1–2 h for exhausted fish and 24–30 h post mortem for anaesthetised fish (Erikson 2001; Misimi *et al* 2008).

Summary of stress assessments

The salmon in this study were kept in holding tanks for 6 days under excellent water conditions to recover from transport-induced stress. For comparison, the blood chemistry (cortisol, chloride, sodium, osmolarity and haematocrit) of salmon subjected to intense handling and confinement for 10 min is restored to resting levels after a recovery period of 12–24 h (Franklin *et al* 1990). When rainbow trout are exercised to exhaustion, up to 2–4 h is necessary for full recovery of muscle phosphocreatine and ATP, whereas at least 8–9 h is required for recovery of muscle glucose, lactate and pH (Milligan 1996). This shows that our fish had ample time to fully recover after transport and prior to the onset of experimentation.

Taken together, the stress measurements differed considerably between treatments (Table 2). The underlying causes of these changes in blood chemistry, in relation to muscle activity, probably differed though. The brief netting of control fish resulted in a moderate decrease in muscle pH whereas the effect on blood chemistry was negligible. Exposure to isoeugenol caused changes in blood chemistry although practically no excessive white muscle activity took place. In this case, reduced, or cessation of, respiration (judged by opercular movement) due to the effects of the anaesthetic, resulted in lower blood pH and higher levels of lactate and glucose compared with control fish. Since the fish did not regain visible signs of respiration, blood chemistry was more severely affected by the additional time in the recovery tub. In contrast, fish exposed to nitrogen exhibited aversive reactions in the stunning tank where the escape behaviour suggested they were stressed to exhaustion. Exposure to the three levels of carbon dioxide showed that increasing levels of the gas resulted in a greater degree of stress and escape behaviour.

Exposure to high and medium levels of carbon dioxide resulted in significant stress effects relative to control fish. With the exception of blood pH from low levels of carbon dioxide (70–80 mg CO_2 L^{-1}), the stress parameters did not differ significantly from the control group.

Isoeugenol

This was the only agent to fulfill all three of our chosen criteria for the stunning of salmon in terms of favourable fish welfare and the ability to produce muscle in rested state (rested harvest). We were not able to distinguish whether the fish were unconscious or dead after stunning and recovery. It could be that over-exposure (exposure time) caused cessation of respiration. In such cases, spasmodic over-extension or flaring of the opercules has been observed. Cardiac arrest and death can then follow within a few minutes (McFarland & Klontz 1969). In the salmon industry, where large volumes of fish are slaughtered, the use of isoeugenol (if legalised) would probably call for batch stunning. This would mean a chance that fish eventually taken from the stunned batch to be subsequently bled might in fact be dead. Consequently, blood drainage from such fish can be poor. If fish actually die from an isoeugenol overdose, appropriate processing routines should be instigated to ensure that all fish in a batch are bled within 30–60 min to avoid blood coagulation and incomplete drainage (Botta *et al* 1986).

Nitrogen

Since nitrogen did not fulfill any of our three criteria, the stunning method cannot be recommended. Moreover, fish displayed even greater signs of stress than those observed with the high levels of carbon dioxide. Based on visual observations of purely aversive reactions, it quickly became obvious that fish welfare was being seriously compromised. If left for longer periods in the stunning tank, the fish would

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probably have died from hypoxaemia. Even with the more hardy channel catfish (*Ictalurus punctatus*), exposure to nitrogen gas was not considered an acceptable method of stunning (Bosworth *et al* 2007).

Carbon dioxide

Our results clearly demonstrated that none of the three levels of carbon dioxide fulfilled all of the three set criteria. Fish exposed to medium and high levels of the gas became stressed, showed aversive reactions and were not rendered unconscious. At low levels of CO_2, fish were undoubtedly less stressed, however they lacked the sufficient calmness to facilitate easy handling (eg feeding of immobilised fish into percussion stunning machines).

It was also clear that optimisation of stunning conditions (good water quality) had little effect in minimising aversive reactions and stress. In the industry, considerable variation has been observed in terms of whether the salmon are calm (easy to handle) after being live chilled at 20–100 mg CO_2 L^{-1} (Erikson 2008). These values correspond to the low levels of CO_2 in the present study. Often, when fish are exposed to carbon dioxide in the industry they can be calmer than those in the present study (low CO_2 level). This can be explained either by the longer exposure times used in the industry (20–40 min), or by a combined effect of carbon dioxide with other accumulated components (for example TOC and TAN) in the re-circulated SW in the commercial tanks. The potential effects of poor water quality on stunning efficacy have been discussed elsewhere (Erikson *et al* 2006). Consequently, medium-to-high levels of carbon dioxide appear to be needed for proper immobilisation of salmon, as well as in cases where the fish are intended to be calmed for easy handling (pre-stunning treatment). Even at high levels, our CO_2 treatment did not render the majority of fish unconscious. This means they would feel pain when subsequently bled (Robb *et al* 2000). Thus, regardless of the concentration of carbon dioxide, this method was always associated with compromised welfare or stress.

Animal welfare implications

Since nitrogen and carbon dioxide (regardless of concentration) stunning imposed stress, compromised welfare and did not render Atlantic salmon unconscious, the methods cannot be recommended for use in the salmon industry. Based on the same evaluation criteria, isoeugenol (AQUI-S™) was regarded as a far better alternative for the stunning of salmon.

Conclusion

Taking into account all of the stunning methods tested, only isoeugenol fulfilled all of our set criteria related to fish welfare and low stress. Fish showed the strongest aversive reactions with nitrogen, suggesting this method was the most stressful. Nitrogen should not be regarded as an option for the stunning of Atlantic salmon. None of the carbon dioxide levels (low, medium or high) fulfilled all three criteria. At medium and high levels, fish exhibited aversive reactions and became considerably stressed whereas at low levels, changes in behaviour and stress may have been modest, but fish did not appear sufficiently immobilised to facilitate easy handling. Even at optimised stunning conditions (without confounding, pre-stunning factors, and at good water quality), the use of carbon dioxide cannot be recommended in connection with slaughter of Atlantic salmon.

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