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The effects of carbon monoxide on Atlantic salmon (*Salmo salar* L.)

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ABSTRACT

Atlantic salmon were exposed to carbon monoxide (CO) before the fish were percussively killed and gill cut. The fish were compared against a control group treated identically, without CO. Salmon exposed to CO expressed no adverse reactions and were easily stunned by percussion. CO-treated salmon had an earlier onset of rigor mortis and a faster decrease in muscle pH than the control group. No significant difference in drip loss was found between salmon treated with CO and the control. A significantly deeper red colour of both gills and fillets of CO-treated salmon was observed 10 days post mortem. Significantly higher levels of plasma lactate and potassium were found in CO-treated salmon compared to control, as well as a lower level of pCO_2. Exposure to CO did not increase plasma cortisol, sodium, haematocrit or glucose; however, lactate was high. Exposure of salmon or other fish to CO could improve quality and welfare when slaughtered.

1. Introduction

The present methods for harvest and slaughter of salmon are not optimal from a quality and welfare point of view. The Norwegian Food Control Authority has decided that carbon dioxide (CO_2) should be avoided for anaesthesia of aquaculture fish. However, at present CO_2 is used as well as electrical and percussive stunning. Although electrical and percussive stunning kills or stuns the animal almost instantaneously, quality and welfare problems can often be associated with the pre-slaughter procedure including handling and loading into the stunning machines (EFSA, 2009).

Carbon monoxide (CO) has been used to kill fish such as tilapia (Mantilla et al., 2008). It is well known that CO binds to haem proteins such as haemoglobin, myoglobin (Dickerson & Geis, 1983) and neuroglobin, found in brain and neural tissue (Brunori & Vallone, 2007). CO is a colourless, odourless and tasteless gas and is produced by carbon-containing materials which are incompletely combusted (Cornforth & Hunt, 2008; Sørheim, Aune, & Nesbakken, 1997). A small quantity of CO is naturally produced endogenously in humans (Ishiwata et al., 1996), regulating blood flow and blood fluidity (Durante & Schafer, 1998). In the USA, modified atmospheres with low levels of CO, up to 0.4%, are used commercially for packaging of meat while filtered smoke containing 30–40% CO is permitted for pre-treatment of fish (Kristinsson, Balaban, & Otwell, 2006a, 2006b; Sørheim et al., 2006). CO may act as an antioxidant in muscle foods, as it has the ability to inhibit metmyoglobin formation and promote metmyoglobin reduction, even when oxygen is present (Lanier, Carpenter, Toledo, & Reagan, 1978). Lipid oxidation and browning effect are reduced and the shelf life of the product is prolonged (Cornforth & Hunt, 2008). CO bound to haem inhibits the haem-catalysed reaction between lipids and oxygen leading to rancidity (Warriss, 2000). For all processes involving the use of CO for meat and fish, it is crucial that the gas is added as early as possible, preferably before stunning and slaughter, in order to avoid undesirable oxidations caused by exposure to oxygen or light. The high degree of unsaturated fatty acids in salmon makes it highly susceptible to lipid oxidation and off-flavour development.

Stress is a natural response to changing conditions and an important part of an organism's adaptive repertoire, used to re-establish homeostasis. In the context of welfare of farmed animals it is in general regarded as a negative consequence of an unsatisfactory regime (Pottinger, 2008). Secretion of cortisol in the blood plasma increases greatly in situations stressful to the animal. Cortisol is the major stress hormone in fish (Ellis et al., 2007), and is an important primary stress response. This hormone has a regulatory effect on the metabolism of proteins, carbohydrates and fats and also has regulatory functions in the immune system, heart, growth and reproduction (Pottinger, 2008). The supply of blood and the activity of the gills are increased if the cortisol level in the blood is elevated. This leads to physical responses including uneasy movements. Cortisol has the function of

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increasing the blood glucose levels and promotes liberation of glycogen in liver. Cortisol also influences the hyperosmotic effect, which in turn would give increased values of haematocrit and also increases the concentration of ions, especially sodium (Guyton & Hall, 2006). Furthermore, stress may result in a more anaerobic metabolism, which in turn results in reduction of glycogen content giving a fast pH decrease and faster onset of rigor (Van Laack, Liu, Smith, & Loveday, 2000).

The aim of the present study was to analyse how exposure of Atlantic salmon (*Salmo salar* L.) to CO before stunning and slaughter affected quality and welfare parameters, when compared to salmon that was only percussively stunned before exsanguination by gill cutting.

2. Materials and Methods

2.1. Experimental set-up

To simulate an industrial slaughter situation a total of 52 Atlantic salmon (*Salmo salar* L.), with mean weight 3.4 ± 1.4 kg were taken in batches from the production cage and transferred into a lidded holding tank with a capacity of 1000 L seawater. This tank was further transported approximately 50 metres by a truck to the area where the experiments were carried out. During the experiment the salmon were hauled in and out of the tanks with 400 L seawater. The temperature of the seawater in both the holding tank and the experimental tanks was constant at 5.8 ± 0.5 °C. The air temperature during the experiment was between 0 °C and 2.6 °C. The experiment was approved according to "The Regulations on Animal Experimentation" in Norway and conducted by certified personnel.

One tank was flushed with 100% food grade CO (Yara Praxair, Oslo, Norway) using two ceramic diffusers (wedge lock base unit, Point Four Systems Inc., Richmond, Canada), at the bottom of the tank for ten minutes at 2–3 bar, and CO was continuously added throughout the experimental period. The control salmon were transferred into an identical tank, keeping the same volume of sea water but with no carbon monoxide added. Both groups were manually percussively stunned followed by gill cut. CO concentrations in air were monitored and measured during the experiment by use of portable gas detectors (GasBadge Pro, Oakdale, PA).

Exposure to CO was carried out in three different experiments, with different length of treatment. In the first experiment, a total of 10 salmon were taken from the holding tank, one fish at a time and exposed to CO for 20 min in the experimental tank. As control 10 salmon were taken from the holding tank, transferred into the experimental tank and kept for 20 min, 1 fish at a time.

In the second experiment, 10 salmon were hauled into the experimental tank and simultaneously CO-treated for 20 min. For the control 10 salmon were hauled into an experimental tank and kept for 20 min.

In the third experiment, a total of 9 salmon were taken from the holding tank into the experimental tank and 3 fish were hauled out after 10, 20 and 30 min, respectively. As a control, 3 salmon were placed in a sea water tank for 20 min.

2.2. Behavioural analysis

During exposure to CO the behaviour of salmon was observed and recorded with a video camera. The behaviour of the salmon, including indicators of consciousness, was assessed according to Roth, Imsland, Moeller, and Slinde (2003). Behavioural indicators at five different stages of consciousness have been described, based on swimming activity, reactivity to visual and tactile stimuli, equilibrium efforts and ability to ventilate (Roth et al., 2003).

2.3. Rigor index, pH, colour and drip loss

The salmon were slaughtered and individually tagged, gutted and stored in bins of slurry ice. After slaughter, muscle pH and rigor mortis were measured. The rigor mortis was measured as tail drop and the rigor index (L_r) was calculated using the following formula:

L_r (%) = $[(L_0-L_t)/L_0] \times 100$ (Bito, Yamada, Mikumo, & Amano, 1983).

L_0 (cm) represents the tail drop at the first measurement and L_t (cm) represents the rigor level at the actual time throughout the experiment.

Measurement of pH was carried out in the neck region of the salmon using a Mettler Toledo SevenGo pro™ pH meter (Mettler-Toledo Ltd, Leicester, UK) equipped with Inlab puncture electrode (Mettler-Toledo Ltd).

Colourimetric measurements, *L*, *a* and *b*, (Hunter & Harold, 1987), were performed at 3 locations along the loin in the Norwegian Quality Cut (NQC) region (Shearer, 2001), after slaughter and after resolution of rigor (48 h), using a Minolta Chroma Meter CR-200 (Minolta, Osaka, Japan). Three measurements were made at each point, and the measuring head was rotated 90° counter-clockwise between each measurement. *L* describes the lightness of the sample, *a* redness and *b* yellowness. Furthermore, chroma (*C*) representing the colour saturation was calculated by

$C = \sqrt{(a^2 + b^2)}$ (Hunter & Harold, 1987).

In addition to colourimetric reading using the Minolta, pre-rigor fillets from the control ($n = 4$) and CO group ($n = 7$) were wrapped in plastic and aluminium foil, packed into Styrofoam boxes with ice and stored in a 0.5 °C storage room until colour measurements using computer vision, 10 days post mortem. Also the heads of 3 fish, from each of the two groups, CO and control, were stored for 10 days before the operculum was cut off and colour was measured on the gills. For computer vision, fillets were placed into a DigiEye, (Verivide Ltd, Leicester, UK) light box exposing the fillets to 6400 K daylight. The fillets and gills were photographed with a Nikon D80 digital camera (Nikon, Japan) through a 35-mm lens. The picture was downloaded into a computer and colour (*L*, *a*, *b*) of the gills and fillets were analysed with software (Digipix, Verivide Ltd). The colour was calibrated with a DigiTizer (Verivide Ltd,) calibration chart, and corrected for the uniformity of the light. The average colour of the fillets was measured along the dorsal loin posterior to the pectoral fins and anterior to the anal fin.

Measurement of drip loss (%) of the fillets was carried out by weighing the fillets at Day 0 and Day 10 and the difference calculated by the following formula:

Drip loss index (t) = $((D_o-D_{10})/D_o) \times 100$.

D_o represent the pre-rigor fillet weight, while D_{10} corresponds to the fillet weight after 10 days of storage.

2.4. Blood parameters and cortisol

Blood samples were collected from the caudal vessel immediately after percussive stunning and gill cut. The blood parameters Na^+, K^+, pH, haematocrit, glucose, lactate, pCO_2 and plasma cortisol were analysed. Plasma ions and gases of unheparinised whole blood were measured on-site using i-STAT® 300 Portable Clinical Analyser. The analyser was used in conjunction with *Chem8* + and *CG4* + disposable cartridges (Abbott Point of Care Inc., Princeton, NJ). CHEM8 + cartridge analysed sodium (Na^+), potassium (K^+), glucose (Glu), and haematocrit (Hct). CG4 + cartridge measured pH and lactate (Lac). Prior to analysis air in the disposable needle was removed. The blood was automatically heated to 37 °C and analysed using i-STAT for plasma ions and gases.

For cortisol analysis plasma was prepared by centrifuging (5000 rpm for 4 min) 1 mL heparinised blood. Plasma was

transferred to fresh tubes, stored on ice for approx 5 h and frozen at −80 °C until analysed. Plasma cortisol was analysed using IBL's Cortisol ELISA kit (IBL International, Hamburg, Germany), according to the manufacturer's instructions.

2.5. Statistical Methods

Statistical analyses were performed with STATISTICA 8.0 (StatSoft Inc., Tulsa, OK). For testing continuous variables, such as drip loss, cortisol, muscle pH, colour, blood gases and chemistry, against the categorical variables, such as CO-treated fish and control, a two-way *t*-test was used. Level of significant differences was set to $p < 0.05$.

3. Results

3.1. Behavioural analysis

The salmon had a normal swimming behaviour immediately after transfer to the experimental tank with CO. The fish were circling around in the tank and seemed calm and peaceful in their behaviour. Moreover, the salmon were swimming through the CO gas from the diffusers, which indicate that salmon did not take any notice of the gas as such. After 3–4 min of exposure the salmon showed reduced swimming activity, slightly loss of reactivity to visual and tactile stimuli and had problems with equilibrium and normal ventilation of operculum, which refers to stage 1 of consciousness (Roth et al., 2003). At about 5–6 min of CO exposure the behaviour of salmon reached stage 2 "light narcosis" expressing weak swimming activity, no reactivity to visual stimuli, weak reactivity of tactile stimuli, slow and long ventilation rate and loss of equilibrium with attempts to right. After 7–8 min of CO treatment the salmon probably reached deep narcosis, stage 3, referring to no swimming activity, no reactivity to visual stimuli, problems of ventilation of operculum and total loss of equilibrium (Roth et al., 2003). After approximately 12 min of CO exposure, abnormal erratic swimming behaviour occurred. The abnormal behaviour involved uncontrolled sporadic convulsions. The salmon convulsed in circles near the surface of the water with the abdomen up to the surface and came up into the air for a short period of time. This reaction lasted three to eight seconds. The convulsions were repeated after some time, although they were not observed for all salmon when individually treated with CO. The fish were hauled from the tank and killed by percussion.

3.2. Rigor index, pH, colour and drip loss

Salmon exposed to CO had an earlier onset of rigor mortis and an earlier reduction of muscle pH post mortem than the control (Fig. 1). Compared to CO-treated salmon which had a rapid pH decline, the control held a constant muscle pH of 7.1–7.2 until about 15 h post mortem, before a sharp decrease in pH was observed. Salmon exposed to CO had increasing rigor index from 3 to 23.5 h (Fig. 1D), while the control showed increasing rigor index from 10 to 30 h (Fig. 1B). Rigor mortis was more intense in the CO fish than in the control, but the final pH of 6.3 was similar for salmon treated by CO and the control (Fig. 1).

In pre-rigor fillets, there were small differences between the colour of salmon exposed to CO and the control. Salmon exposure to CO for ten minutes gave a significant increase in redness (*a*) of pre-rigor fillets compared to the control (Fig. 2), but the colour difference was hardly visible to the naked eye. Extended length of exposure to CO did not give any significant difference in the red colour in pre rigor fillets (Fig. 2). Lightness (*L*), yellowness (*b*) and chroma (*C*) were also equal in both groups of salmon (Fig. 2). After resolution of rigor mortis, approximately four days after slaughter, there were no differences in colour parameters (not shown).

Colour analysis measured by computer vision of fillets 10 days post mortem indicate a significantly more intense redness (a) and showed also a significantly higher value of chroma (*C*) in CO-treated salmon compared to the control (Table 1), but no significant differences were shown in lightness (*L*) and yellowness (*b*). Significantly increased levels of both redness (*a*) and chroma (*C*) were observed in the gills treated with CO compared to the control (Fig. 3). The differences in redness, in the fillets and the gills 10 days post mortem, between CO-treated salmon and control, were clearly visible to the eye. The gills and fillets from CO-treated salmon had a bright reddish colour, while the gills and fillets of the control had a brown–red appearance.

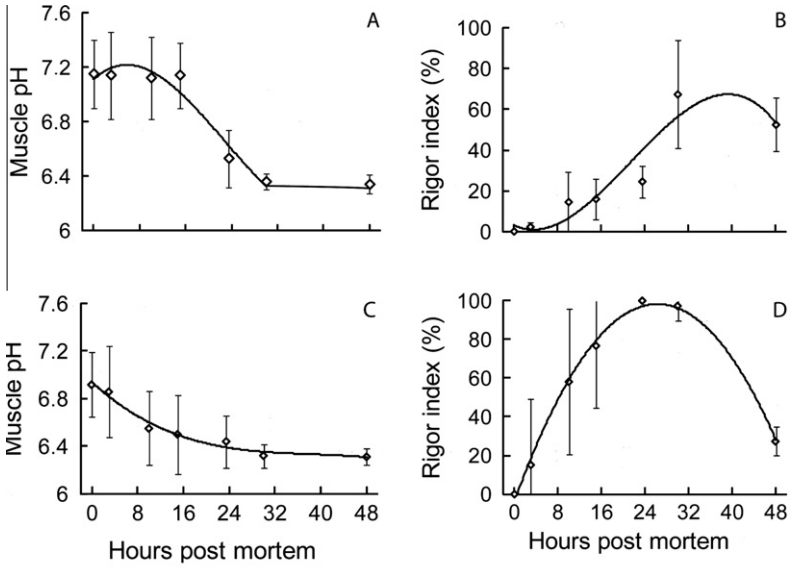

Fig. 1. Comparison of mean pH ± SD and rigor index ± SD for control salmon (A, B) and salmon exposed to CO (C, D). Both groups of salmon were finally percussively stunned followed by gill cut and placed in bins of ice slurry, $n = 10$ for each group.

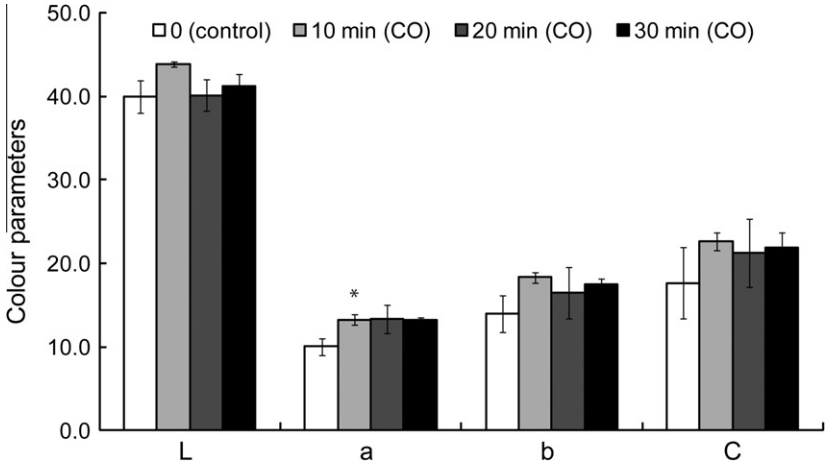

Fig. 2. Mean colour ± S.E measured by Minolta Chroma Meter as *L* (lightness), *a* (redness), *b* (yellowness) and chroma (colour saturation) in salmon pre-rigor fillets, at Day 0 compared to control and CO treatment carried out for different lengths of time; 10, 20 and 30 min ($n = 3$). 10 min of CO treatment gave significant difference in redness (*a*).

Table 1
Mean colour ± SD, measured by colour vision as *L*, *a*, *b*, and calculated chroma (*C*) in salmon fillets sedated and anaesthetised by CO, and control. Pre-rigor fillets were stored for 10 days *post mortem* until colour assessments were carried out. For CO-treated salmon $n = 8$, while for control $n = 4$.

	CO		Control	
	Mean	SD	Mean	SD
L	52.3	1.3	51.8	2.9
a	41.6**	1.5	38.2	1.2
b	35.9	2.2	33.9	1.3
C	54.9*	2.6	51.1	1.6

Asterisks indicate the level of significance.
* $p < 0.05$.
** $p < 0.005$.

No significant difference in drip loss was found between fish treated with CO and control (Table 2).

3.3. Blood parameters and cortisol

A significant difference between the CO group and the control group was observed for the parameters potassium, lactate and pCO_2 (Table 2). No significant differences in sodium, glucose, plasma cortisol, haematocrit or pH were seen in salmon treated by CO and the control group (Table 2). There was no significant difference

Table 2
Mean values ± SD of sodium, potassium, glucose, haematochrit, lactate, pH, carbon dioxide, and cortisol of blood collected from salmon sedated by CO and control immediately after killing. Mean ± SD of drip loss (%) calculated by weighing the fillets after slaughter and 10 days post mortem.

	CO		Control, no CO	
	Mean	SD	Mean	SD
Sodium (mM)	169	3.9	173	9.3
Potassium (mM)	7.4***	1.4	3.9	0.5
Glucose (mM)	6.4	1.8	5.8	1.5
Haematocrit (%)	27.6	6.8	31.4	5.7
Lactate (mM)	5.5***	2.1	2.0	1.4
pH	7.16	0.1	7.12	0.12
*p*CO2 (mmHg)	12.8**	1.2	23.7	2.1
Plasma cortisol (ng/ml)	185	75	245	92
Drip loss (%)	1.3	0.4	1.0	0.2

Asterisks indicate the level of significance.
** $p < 0.005$.
*** $p < 0.000.5$.

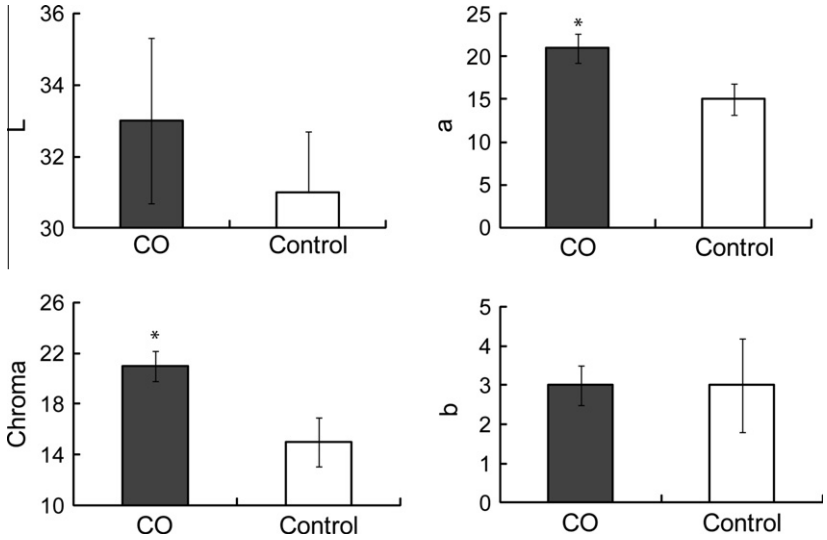

Fig. 3. Colour assessments of the gills from slaughtered salmon kept on ice for 10 days post mortem. The colour parameters *L*, *a*, *b* and chroma (mean ± SD) were analysed with $n = 3$ in each of the two groups. There were significant differences in both redness (*a*) and chroma (*C*).

in plasma cortisol value between the CO-treated salmon and the control. However, both values were high.

4. Discussion

The Norwegian Food Authority demands that loss of consciousness is required prior to gill cutting in slaughter of salmon. According to Roth et al. (2003) the present experiments showed that CO gives light sedation (stage 1) followed by light and deep narcosis (stage 2 and 3) and at least some of the fish died (stage 5). However, it is difficult to visually judge stage 4, surgical anaesthesia, and stage 5 medullary collapse, characterised by "no swimming activity, no reactivity to visual or strong tactile stimuli, ventilation ceases and total loss of equilibrium and death ensues". Discrepancies between behavioural observations in the water and what were observed when analysing the fish directly suggests that the O_2 deficiency associated with CO exposure is also fatiguing the muscles, although the animal at this point had reached states 3–5. More thorough investigations are required to estimate time and concentration combinations for ensuring surgical anaesthesia and death, and electroencephalography (EEG) has to be measured. Monitoring the level of CO dissolved in the water could also be of importance, but the amount dissolved was regarded as sufficient for behavioural and fillet quality observations (Mantilla et al., 2008).

The present method of exposure to CO may achieve its effect through the displacement of O_2 from neuroglobin (Brunori & Vallone, 2007). Neuroglobin is thought to act as a reservoir for oxygen and in that way prolongs activity of the nervous system despite the reduction of oxygen transportation by haemoglobin. Studies on the reaction between neuroglobin and CO will be performed in the future. The data of our study show that exposure to CO prior to slaughtering had no negative effects with regard to increasing stress response behaviour or decreasing meat quality of salmon. Preliminary studies have indicated that temperature has an effect on how fast CO acts.

Since CO has a higher affinity towards haem proteins than oxygen (Dickerson & Geis, 1983), the oxygen transportation by haemoglobin is reduced or excluded and the metabolism changes from aerobic to anaerobic. This involves rapid glycolysis and lactic acid production (Fig. 1). The production of lactate resulted in acidification of the muscle due to the rapid decrease of pH and, as a consequence, onset of rigor mortis was accelerated. However, glucose was not mobilised (Table 2), indicating that the CO-treated salmon were not more stressed than the control, and the stress parameter cortisol was also similar between treatments with or without CO (Table 2).

Exposure to CO led to a significant increase in redness compared to control. The significant difference in redness of both the fillets and gills, observed 10 days post mortem, is mainly due to the cherry-red colour of carboxyhaemoglobin, and the brownish colour seen, especially in the gills of control salmon, is mainly due to methaemoglobin. The amount of blood and haemoglobin is probably much higher than the amount of myoglobin in the muscle and especially the gills, and this is likely to be a contributor to the colour differences. The observed difference in redness in the post-rigor fillets (Table 1) is possibly due to transfer of blood to the surface of the fillets. However, in the fillets of salmon astaxanthin gives the characteristic red to orange colour and may minimise the difference in colour between the two groups.

The study on exposure of Atlantic salmon (*Salmo salar*, L.) to CO indicated that the amount of carboxyhaemoglobin and carboxymyoglobin increases when the duration of CO exposure increased from 10 to 20–30 min, as indicated by the significant increase in redness (a) values (Fig. 2). The amount of myoglobin and haemoglobin, the redox potential of the muscle, the CO concentration and the duration of CO exposure are all factors that influence the amount of carboxypigments.

The significant increase in redness (a) in CO-treated fillets and gills ten days post mortem compared to the control (Table 1 and Fig. 3) is important to consider when the salmon reaches the market for sale. As the differences in redness were visible to the naked eye the customers' impression of the colour of the product at the moment of choice might influence their purchase.

The rapid pH decrease and the intense rigor mortis shown in the salmon exposed to CO (Fig. 1) would have been expected to result in a higher drip loss in the case of CO treatment compared to no CO treatment, but this was not the case. The result of drip loss may be explained on the basis of the ultimate pH value, which showed that the salmon exposed to CO and the control reached a similar final pH value.

The salmon used in this investigation were exposed to stress, through hauling, transport and handling, explaining the similar plasma cortisol, blood glucose and Na^+ values observed in all animals. This certainly reflects some of the challenges of conducting experiments in the field, but one could however expect that an aversive reaction to CO would cause dramatic increase or decrease of the blood parameters. The behavioural investigation showed a lack of stress responses within the CO group. The blood parameters were also changed, as could be expected from the influence of CO on metabolism, and the results indicate that CO may have a relaxing effect on salmon. Apparently CO is not adverse to the fish, nor does the following hypoxia cause release of cortisol. Also the acid–base balance within the fish remained within a normal range (Table 2) with no signs of acidosis, nor were there changes in metabolism associated with a low pCO_2 value (Table 2).

The different behavioural stages (Roth et al., 2003) that can be observed when salmon are exposed to CO, are a result of CO binding to the oxygen storage proteins and neuroglobin. When using current slaughtering methods, such as electricity and CO_2, neuroglobin, which has a high affinity for oxygen, makes the brain able to function for an extended period even under full anoxia. This raises important welfare questions. In our opinion neuroglobin acts either as an O_2 storage reservoir, or alternatively, as a strong protector against hypoxia-induced damage in the brain of fish, explaining why fish have been so difficult to kill before exsanguination. CO could increase animal welfare when used to slaughter salmon or other fish.

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