

Sedation and slaughter of Atlantic salmon (*Salmo salar*, L.) with carbon monoxide, and a possible regulatory role of neuroglobin

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ABBREVIATIONS

ATP	adenosine triphosphate	MAP	modified atmosphere packaging
CO	carbon monoxide	Mb	myoglobin
CO ₂	carbon dioxide	MBP	maltose binding protein
COHb	carboxyhemoglobin	MMb	metmyoglobin
COMb	carboxymyoglobin	MNgb	metneuroglobin
CONgb	carboxyneuroglobin	mNgb	mouse neuroglobin
DMb	deoxymyoglobin	Ngb	neuroglobin
DNgb	deoxyneuroglobin	N ₂	nitrogen
EEG	electroencephalography	NO	nitrogen oxide
GC	gas chromatography	O ₂	oxygen
hNgb	human neuroglobin	OMb	oxymyoglobin
Fe ²⁺	ferrous	ONgb	oxyneuroglobin
Fe ³⁺	ferric	PSE	pale soft exudative
Fe ⁴⁺	ferrylradical	ROS	reactive oxygen species
GRAS	generally recognized as safe	RT-PCR	realtime polymerase chain reaction
GMP	guanosine monophosphate	sNgb	salmon neuroglobin
Hb	hemoglobin	TEV	tobacco etch virus

ABSTRACT

Until recently CO₂ has been utilized as an anesthetic method before gill cutting of Norwegian Atlantic salmon farmed fish. From 1. July 2012 this is no longer permitted in Norway since it causes strong aversive reactions and severe stress to the animal. In the search for alternative methods for sedation and anesthesia carbon monoxide (CO) has gained interest. In our project, studies demonstrate that CO treatment has a positive effect on product quality and animal welfare. The use of CO in slaughtering, processing and packaging of muscle foods has been reviewed.

The present thesis focuses on how sedation of Atlantic salmon with CO affects animal welfare and quality of fish when used as an alternative method prior to anaesthetization. CO is widely used in meat and fish industry for smoking of fish or packaging of meat to maintain product quality. In this study CO was used to sedate salmon by diffusion of the gas in seawater tanks with fish. The level of CO in the seawater was estimated on the basis of oxygen level measured in the seawater and the length of time of diffusion. The methodology to measure CO in water by use of GC-chromatography was not until recently available in our laboratories. Behavior analysis and cortisol levels indicate that CO treatment may result in a more calm fish, not showing increased cortisol values compared to other treatments but rather a tendency of the opposite. However, at high levels of CO a more rapid swimming activity was observed. Another effect was a more rapid onset of *rigor mortis* and a faster decrease in pH observed in CO exposed salmon compared to the control group. Early onset of *rigor mortis* could indicate stressed fish, but in this case a more likely explanation is the anaerobic metabolism as a secondary response of CO treatment. Moreover, fillet from CO

treated salmon showed a higher degree of red color. Mackerel fillets exposed to CO also showed a perceptible more red color. Both fillets of salmon and mackerel are rich in Mb compared to *e.g.* cod fishes.

In the search for factors with a controlling effect on CO we focused on a recently discovered globin located in nerve tissue, neuroglobin (Ngb). Ngb has been shown to protect the brain during stress and hypoxia in fish. Ngb was detected in salmon brain tissue, but only in small amounts, 1-10 $\mu\text{g/g}$, after immune precipitation and western analysis. Thus in order to study salmon neuroglobin (sNgb) in more detail, we cloned and expressed the protein in *E. coli*. Recombinant sNgb showed a strong response using a salmon Ngb specific antibody, raised against the C-terminal peptide of sNgb. Homology modeling of 3D structures of sNgb and mouse neuroglobin indicated that sNgb has a more flexible structure compared to the mammalian neuroglobins that have typical psychrophilic characteristics.

LIST OF PUBLICATIONS

Paper I

Bjørlykke GA, Kvamme BO, Slinde E and Sørheim O. (2012) Use of carbon monoxide in slaughtering, processing and packaging of muscle foods. Ed. Taylor, J. C. In: *Advances in Chemistry Research*, Volume 14. Nova Science Publishers, pp. 159-180.

Paper II

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

Paper III

Bjørlykke GA, Kvamme BO, Raae AJ, Roth B and Slinde E. (2012) Slaughter of Atlantic salmon (*Salmo salar*, L.) in the presence of carbon monoxide and the importance of neuroglobin. Submitted to *Fish Physiology and Biochemistry*.

Paper IV

Bjørlykke GA, Kvamme BO, Slinde E and Raae AJ. (2012) Cloning, expression and purification of Atlantic salmon (*Salmo salar*, L.) neuroglobin. Submitted to *Protein expression and purification* (A revised version is accepted for publication).

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1.GENERAL INTRODUCTION

1.1.BACKGROUND

Atlantic salmon (*Salmo salar*, L.) was introduced in the Norwegian fish farm industry in the early seventies. Commercial salmon farming has grown to large proportions and the annual production of Atlantic salmon in 2011 was 1 059 958 ton (Statistics Norway 2012).

During the latter years there has been a growing focus on animal welfare of farmed fish. This is seen in requirements and regulations on national and regional level as well as in the consumer's opinion and the fish farm industry itself. In the case of the consumer, they are increasingly concerned about the way in which animals are reared for food and how the environment is affected. A sort of general consensus has been formed that fish have an intrinsic value beyond that of food production for human consumption. As a result of this it is seen as a moral responsibility that the farmed fish is raised with the best possible animal welfare. This concern is supported by the producers need to get the best quality of the fish product, and the general opinion that good welfare improves product quality. If fish are severely stressed and display high muscular activity at the time of slaughter, this constitutes a welfare issue that will result in a reduced quality of the product. At present situations with severe stress is frequent in slaughter of farmed fish, when a large number of fish is killed during a short time.

Particularly slaughter of fish raises several ethical issues in fish farming. Methods to sedate and anaesthetize may be crucial to obtain a proper killing and to avoid unnecessary stress to the animal. The Norwegian Food Control Authority has decided that carbon dioxide (CO₂) is

prohibited as an anesthetic method in slaughter of aquaculture fish and this has come into effect from 1. of July 2012. Stunning by CO₂ is now being replaced by electricity or percussion.

1.2. FISH WELFARE

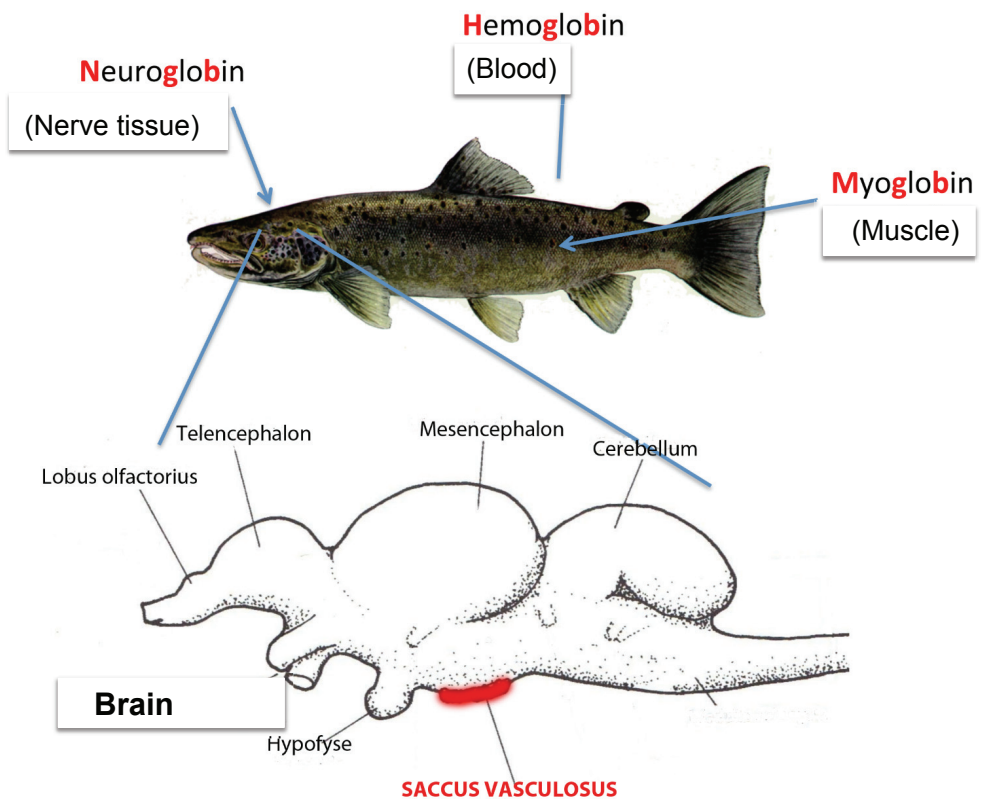


Figure 1. Atlantic salmon and a drawing of the brain with *Saccus vasculosus*, situated on the ventral side of the brain.

However, although both these methods have been improved to a great extent during the latter years (Mejdell et al. 2010) both these methods have drawbacks (EFSA 2009). Further work is required to establish optimal stunning conditions that are satisfactory with regard to both fish welfare and the quality demands of the industry. In order to improve on these matters, introduction of carbon monoxide (CO) in the slaughter process may be an alternative, both from a welfare point of view and regarding the quality of the product.

As the main effect of CO is to inhibit oxygen transport throughout the animal, and the fact that oxygen is vital for brain function, one important welfare aspect of slaughter by using CO is to investigate the possible role of the oxygen-binding neuroglobin (Ngb) (Figure 1). When oxygen (O₂) is available in the brain, or the brain is protected from damage due to hypoxia, the brain activity may be prolonged. A likely result of this is that the fish might feel pain for a longer period. Introduction of CO displaces O₂ and inhibits O₂ replenishment with high effectivity and low reversibility. Hence, CO would annihilate the brain activity and reduce the possibility of prolonged pain.

1.3. ANESTHESIA AND SLAUGHTER OF ATLANTIC SALMON

Before slaughtering it is essential to anesthetize in the fish. The intention of anesthesia prior to killing is to obtain immediate unconsciousness until death in order to avoid excitement, pain or suffering (Anon 2004). To this end the animal is stunned to make it insensible to pain. The time from the animal is stunned until it is killed must be minimized to prevent any recovery of consciousness before death. When fish were being slaughtered it was necessary in our study to observe the behavior of the fish in order to determine the stage of

consciousness. In this connection, according to Roth et al (2003), five different stages of behavioral description based on swimming activity, reactivity to visual and tactile stimuli, equilibrium efforts and ability to ventilate, were used (Paper II) as a guide to assess consciousness (Table 1). Indicating whether the fish were conscious or unconscious at the end of CO exposure was done by evaluating the behavior by giving scores of the responses to stimulation and clinical reflexes (Paper III) described by Kestin et al. (2002).

TABLE 1.—Modified protocol from Burka et al. (1997) to determine, based on behavioral observations of Atlantic salmon, different stages (0–5) of reaction to electrical exposure. Behavioral studies were based on signs of swimming activity, reactivity to visual and tactile stimuli, equilibrium efforts, and ability to ventilate.

Stage	Description	Behavioral signs
0	Normal	Active swimming patterns Normal reactivity to visual and tactile stimuli Normal equilibrium Normal ventilation of operculum
1	Light sedation	Reduced swimming activity Slight loss of reactivity of visual and tactile stimuli Problems with equilibrium Normal ventilation of operculum
2	Light narcosis	Weak swimming activity No reactivity to visual stimuli Weak reactivity of tactile stimuli Slow and long ventilation rate Equilibrium loss with efforts to right
3	Deep narcosis	No swimming activity No reactivity to visual stimuli Barely reactivity to strong tactile stimuli Problems of ventilation of operculum Total loss of equilibrium
4	Surgical anesthesia	No swimming activity No reactivity to visual or strong tactile stimuli Ventilation ceases Total loss of equilibrium
5	Medullary collapse	Death ensues

(Roth et al. 2003)

Gill cut without prior stunning, asphyxiation on ice or air are all regarded as inhuman and unsatisfying slaughtering methods of fish and are not permitted in Norway. In response to CO₂ treatment (Erikson et al. 2006; Roth et al. 2007) flight reactions and aversive behavior have been reported (Robb and Kestin 2002; Roth et al. 2002; Poli et al. 2005) and this is the background for the prohibition of the use of CO₂ in fish slaughtering in Norway. Percussive stunning (Roth et al. 2002; Roth et al. 2007) and electrical stunning (Roth et al. 2003) are the two alternative methods currently used in slaughtering of farmed fish in Norway. When applied correctly both percussive and electrical stunning can induce irreversible and almost immediate insensibility. Percussion and electrical stunning give the fewest challenges regarding welfare compared to other slaughtering methods (Robb and Roth 2003; Roth et al. 2007), but still the anesthetic techniques may raise welfare problems. The challenge with electricity is to avoid carcass damage such as muscle hemorrhages and broken spinal column (Roth et al. 2003). With percussive stunning there is a challenge to hit the skull correctly, and problems may arise *e.g.* when fish are active or of different sizes. It may be necessary to use manual percussion when the automated procedure fails, and hitting the fish twice raises welfare issues (EFSA 2009).

Pre-slaughter handling may be stressful to the animal, and may raise quality and welfare issues (EFSA 2009). Today fish are pumped from the rearing cages to the well boat and transported to the slaughterhouse where the fish are pumped to storing cages. They are pumped again from the storing cages to the slaughter station where they today are stunned by percussion machine or by the use of electricity. The transport and especially the pumping are known to cause severe stress to the fish.

Sedation has a calming effect on the animal and may be useful when the animal is stressed prior to anesthesia, and sedation may have a beneficial effect on the anesthesia itself. Using gas to obtain sedation previous to or as the part of the stunning has several advantages including improvement of electrical or percussive stunning when used prior to these techniques. As an alternative to CO₂, the use of carbon monoxide (CO) is an interesting possibility (Figure 2).

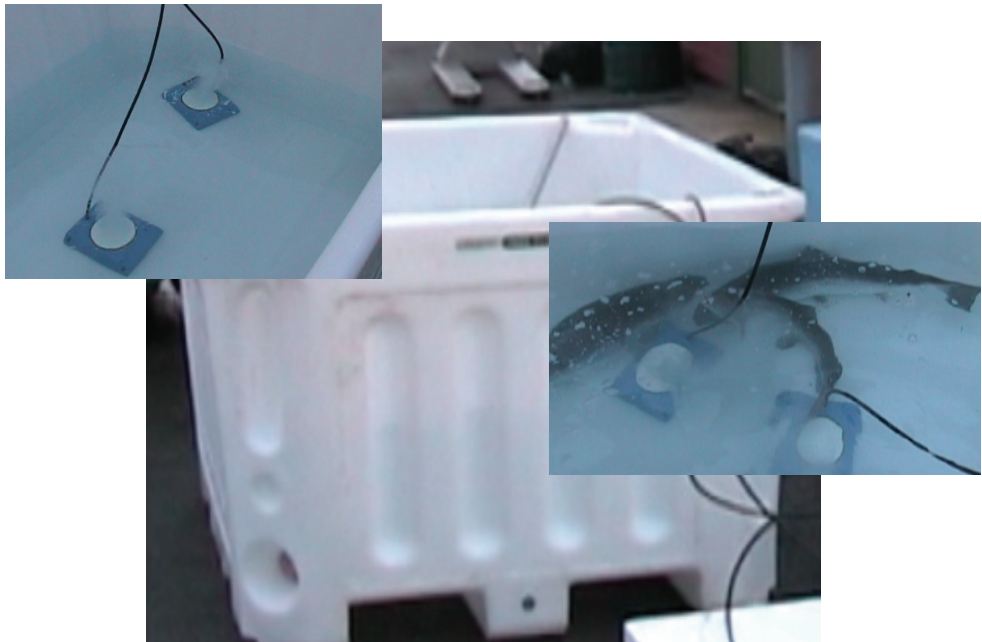


Figure 2. Illustration of ceramic diffusers adding CO to the seawater (left) and fish treated by CO (right) both in experimental tank (middle).

1.4.USING CO EARLY IN THE SLAUGHTERING PROCESS

1.4.1. GENERAL

CO is produced by incompletely combustion of carbon-containing materials and is a colorless, odorless, tasteless and non-irritant gas (Sørheim et al. 1997; Cornforth and Hunt 2008) Paper I). CO is a toxic gas and binds > 200 times stronger to hemoglobin (Hb) than O₂. CO also replaces bound O₂ effectively and prevents uptake of O₂ to Hb. There may be several effects of CO treatment, both known and unknown. Among others CO binds to different heme proteins and electron transport in the respiratory chain of mitochondria is inhibited. Due to this inhibition anaerobic metabolism and early acidification of the muscles occur.

For centuries CO has been naturally produced when practicing traditional wood smoking. CO has for several decades been used for food preservative reasons in the food industry (Paper I). CO for packaging has been extensively studied. Packaging of meat with a low CO concentration gives rise to a more bright red, stable color (El-Badawi et al. 1964; Lanier et al. 1987; Sørheim et al. 1997). Meat packed in low CO, without O₂ shows reduced oxidation (John et al. 2004; 2005) and also reduced microbiological growth (Luño et al. 1998; Sørheim et al. 1999; Nissen et al. 2000). Filtered smoke, naturally contains 15-40 % CO (Kowalski 2006), is a preservative method used on fillets of tuna or other fish species. Filtered smoke is shown to have beneficial effect on quality; gives a more red stable color (Schubring 2007), reduces lipid oxidation (Pivarnik et al. 2011) and most importantly, the amount of histamine producing bacteria are reduced.

CO is used in the meat and fish industry worldwide for purposes like pre-treatment and packaging (Kristinsson et al. 2006b; 2006a; Sørheim et al. 2006). The application of CO in food industry has accelerated the last decade, especially in USA.

CO can also be used as a method to sedate, anaesthetize or kill animals as well, so far on basis of experimental studies. Early exposure of CO to the fish seems to be beneficial by enhancing the meat quality (Paper II and Paper III). Smith (2001) points out that CO might be used on animals for sedation, anaesthesia or even death without aversive reactions. When fish are exposed to CO early in the slaughtering process lipid oxidation may possibly be reduced (Lanier et al. 1987). This would be especially preferable for salmon and mackerel, which are highly vulnerable to lipid oxidation due to the high level of unsaturated fatty acids and the heme containing proteins myoglobin (Mb) and Hb.

The legislation practice for CO in processing, slaughter and treatments of fish and meat varies between world regions or within separate countries. In USA different preservatives listed below were authorized as GRAS (Generally Recognized As Safe): In 2000 “Tasteless smoke” of Hawaii International Seafood (Hawaii International Seafood 1999; USFDA 2000), in 2002 CO (0.4%) in a modified atmosphere packaging (MAP) system for muscle meats and ground meat in master pack (USFDA 2002) and in 2004 four different alternatives of CO as a component of MAP in case-ready packaging (USFDA 2004). The Norwegian meat industry has been applying low CO gas blends for packaging of meat. CO as a food gas was disallowed from 1. July 2004 in EU, as it was not listed as permitted additive. At the same time, on 29. July 2004, CO was approved for retail packaging of meat in USA.

1.4.2. POSSIBLE EFFECTS OF CO

Meat and fish exposed to CO at low levels show desirable bright red, stable color of the muscle (El-Badawi et al. 1964; Lanier et al. 1987; Sørheim et al. 1999; Cornforth and Hunt 2008). Color of fish fillets is also affected when live fish are exposed to CO (Mantilla et al. 2008) (Paper I). CO is shown to enhance the color and quality of fish (Gee and Brown 1981; Chow et al. 1998; Hsieh et al. 1998). Atlantic salmon exposed to CO showed a perceptible more red color of the gills compared to control group, illustrated in figure 3. Also mackerel killed by CO had significantly more reddish gills and fillets compared to control fish (Paper I).



Figure 3: The gills of Atlantic salmon, which are CO treated (left), show a more reddish color than the control group (right), prior to percussive killing.

CO may inhibit metmyoglobin (MMb) formation and promote MMb reduction and thereby it has an anti-oxidative capacity (Lanier et al. 1987). The gas may reduce lipid oxidation and browning; therefore the shelf life of the product is prolonged (Cornforth and Hunt 2008). CO acts as a reducing agent in which it forms complexes with iron or copper in enzymes (White et al. 1973), and therefore the heme-catalysed lipid oxidation is reduced when CO is bound

to the heme. CO may reduce oxidation and microbiological growth in muscle food, however this is less documented (Lanier et al. 1987; Nissen et al. 2000). Using CO in the slaughtering of fish may therefore contribute a more stable product, which is more resistant to oxidation (Paper I).

Several studies have been carried out on the effect of CO during slaughtering of terrestrial animals (Carding 1968; Lambooy and Spanjaard 1980; Blackmore 1993) and fish (Mantilla et al. 2008) (Paper I and II). Still, the use of CO for slaughtering is not well developed and understood. Methods for assessing the effect on brain and heart of fish will lead to further understanding of CO and its effect on fish or terrestrial mammalian. To determine the effect of CO treatment the activity of brain during exposure should be recorded by use of electroencephalography (EEG). EEG provides validated measures of the responses and function of the brain of an animal and indicates whether the animal is conscious or not (Lopes da Silva 1983).

1.5. SLAUGHTERING QUALITY

Slaughter is the endpoint of life of a food animal and the living animal is turned into meat. Many quality characteristics are fixed at the moment when the animal is slaughtered, but further processing is also of importance.

Quality can be defined in different ways and the meaning depends on the experiences of the people defining. Flesh quality, for the purpose of this thesis, is measured by the color, water

holding capacity, muscle pH and the development of rigor mortis. To maintain the quality and safety of muscle foods from slaughter to consumption is highly important in the modern food supply.

1.5.1. QUALITY CRITERIA

Color

The flesh color of salmon is one of the most important quality parameters. The carotenoid astaxanthine contributes to the color and is the most common pigment used for pigmentation of farmed Atlantic salmon (Torrissen et al. 1989; Storebakken and No 1992). Another central color pigment is Mb, which exists in three dominant states, oxymyoglobin (OMb), deoxymyoglobin (DMb) and MMb. Carboxymyoglobin (COMb) is also an important state of the pigment, and the color differences found in paper I and II are caused by CO bound to Mb. The different states are determined by the oxidative status of its single iron molecule, ferrous (Fe^{2+}), ferric (Fe^{3+}) or ferryl radical (Fe^{4+}) (Kanner and Harel 1985), its ligand bound to the iron atom and the total composition of Mb in the respective states. The four different states of Mb and their transitions are illustrated in Figure 4. MMb is reduced to DMb. DMb is converted to OMb in the presence of O_2 . In the absence of oxygen Mb may exist as DMb. In the presence of CO DMb is converted to COMb.

The oxidative states, ligand and color of the four different states of the globin are presented in Table 2.

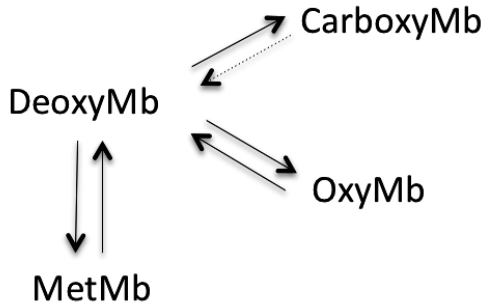


Figure 4. The four different states of Mb and their transitions.

Table 2: Different states of Mb, their oxidative state, ligand bound and color.

Mb State	Oxidative state	Ligand	Color
DMb	Fe ²⁺	None	Purple
OMb	Fe ²⁺	O ₂	Bright red
MMb	Fe ³⁺	Water	Brown
COMb	Fe ²⁺	CO	Cherry red

CO competes with O₂ for binding with Mb but also Hb, and binds to cytochromes as well as Ngb (Dickerson and Geis 1983; Brunori and Vallone 2007). CO binds the deoxy state of the iron atom of the heme and may displace other ligands. This strong binding is the major cause of CO toxicity as Hb bound to CO do not participate in the transport of oxygen, and the slow reversibility of carboxyhemoglobin (COHb) at atmospheric pressure and oxygen saturation.

Muscle pH and waterholding capacity

The muscle structure mainly consists of striated musculature and is made up of bundles of muscle fibers. Each muscle fiber is made up of cylindrical fibrils, called myofibrils and these consist of filaments, consisting of the proteins myosin and actin as the most important.

When the muscle contracts the actin and myosin are pulled in different direction, slide over each other, and make the myofibrils shorter. The muscle fibres in skeletal muscle are mainly white or red muscle cells. The majority of the fish skeletal muscle is composed primarily of white fibers. The white muscle fibers contain higher levels of glycogen and the glycolytic rate is greater in white muscle compared to red muscle. Stress or high activity prior to killing may cause rapid glycogenolysis, and lead to reduction in muscle pH and adenosine triphosphate (ATP). Pale soft exudative (PSE) in slaughtered chicken or pig caused by stress prior to slaughter gives loss of quality, like reduced water-binding capacity (Solomon et al. 1998). This might be the case also in fish stressed before slaughtering. At very high levels of activity white muscles will predominantly be used, resulting in a large amount of anaerobic respiration and high production of lactic acid.

Rigor mortis

The process of *rigor mortis* is not fully understood. Immediately after death the muscle of the fish are soft and limp, and can easily be flexed. When the fish is slaughtered the glycolytic pathway leads to breakdown of the energy reserves ATP and glycogen. The latter is turned into lactate, which gives a pH reduction in muscle cells during rigor. As pH is reduced the energy status becomes poorer. At last the supply of ATP runs out and is not

longer available in the muscle cells. This may be the explanation for the onset of *Rigor mortis*. Actin and myosin are then gradually locked together and the actomyosin filaments are inflexible, tightly bound and make the muscle rigid, and the fish gradually become stiff. High muscle activity or stress before the fish are killed causes anaerobic respiration and causes reduced ATP production and in turn increases the rate of the onset of rigor (Korhonen et al. 1990; Robb 1998). Osmotic changes in the muscle may also be used to explain both development and release of *rigor mortis* (Slinde et al. 2003). Factors such as temperature at slaughter, the temperature at which the fish is kept after death, as well as the physical condition of the fish at death affect the onset and rate of *rigor mortis* development. Hence, all these parameters determine the time it takes to pass through *rigor*.

1.6. STRESS RESPONSES IN TELEOST FISH

Stress is defined as an organism's response to changing conditions. Stress is an important part of the ability to adaption, with the function to re-establish homeostasis. If homeostasis of fish is threatened, biochemical and physiological changes are invoked as a protective mechanism, the neuroendocrine stress response. The response comprises two main acute changes: a rapid release of catecholamines (adrenaline, noradrenaline) into the blood stream (from the chromaffin cells) and slower release of the steroid hormone cortisol into the blood (from the internal tissue) (Sumpter 1997).

In the field of welfare of farmed animals it is generally regarded as a negative consequence of an unsatisfactory regime (Pottinger 2008). Stress and muscle activity at slaughter are detrimental to meat quality, in addition to the ethical issues, which stress implies. Cortisol is the main corticosteroid in teleost fishes. In situations stressful to the animals, secretion of

cortisol in the blood plasma increases to high levels. Cortisol is the major stress hormone in fish (Ellis et al. 2007), and is regarded as an important primary stress response (Mommensen et al. 1999). This hormone has a regulatory effect on the metabolism of proteins, carbohydrates and fats. Cortisol is furthermore involved in the regulatory functions in the immune system, heart, growth and reproduction (Pottinger 2008). If the cortisol level in the blood is elevated, the supply of blood and the activity of the gills are increased. This leads to different physical responses such as uneasy movements. Cortisol has the function of increasing the blood glucose levels and has a promoting effect on the liberation of glycogen in liver. Cortisol also has influence on the hyperosmotic effect, which gives increased values of hematocrit and higher levels of ions, especially sodium (Guyton and Hall 2006). In addition to this, stress may give rapid anaerobic metabolism, resulting in reduction of glycogen which in turn gives a rapid decrease of pH and faster onset of rigor (Van Laack et al. 2000). Plasma cortisol has been measured in the present study as an indicator of stress load (Paper II and III).

1.7.NEUROGLOBIN

1.7.1. GENERAL

Ngb is a recently discovered globin (Burmester et al. 2000; Trent et al. 2001; Burmester et al. 2002; Burmester et al. 2004). Ngb is regarded as an ancestral globin that separated from the cluster containing Mb and Hb for approximately 800 million years ago as illustrated in Figure 5.

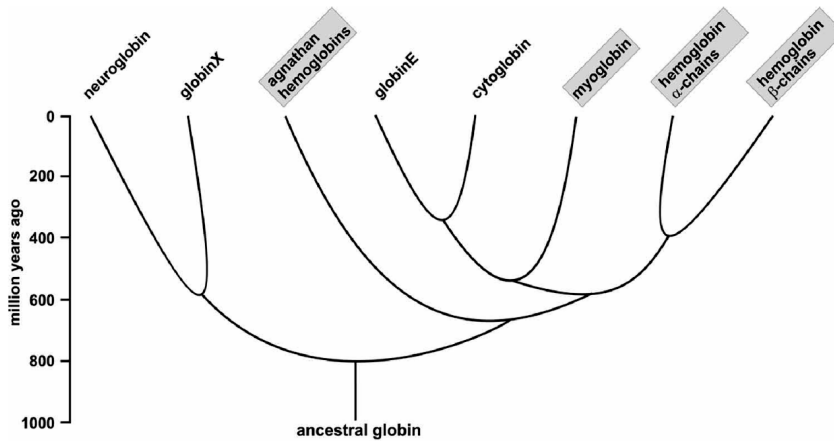


Figure 5. Phylogenetic tree of vertebrate globins. (Burmester et al. 2004)

Ngb is expressed in nervous tissue in both central and peripheral nervous systems (Burmester et al. 2000; Burmester and Hankeln 2009), in addition it is also found in some endocrine tissue (Reuss et al. 2002) and in the gills (Fuchs et al. 2004). Ngb is expressed in all vertebrates, however only in small amounts (Burmester et al. 2000).

Ngb is a monomeric heme-protein containing from 140 (mouse Ngb) and 156 aa (salmon Ngb), constituting about 17-18 kDa in molecular weight. Ngb is a globin with the typical α -helical globin fold. Nevertheless, Ngb has less than 25 % sequence identity with Mb and Hb. Also the heme iron atom in Ngb is hexacoordinated, in contrast to the pentacoordinated iron in Hb and Mb (Dewilde et al. 2001).

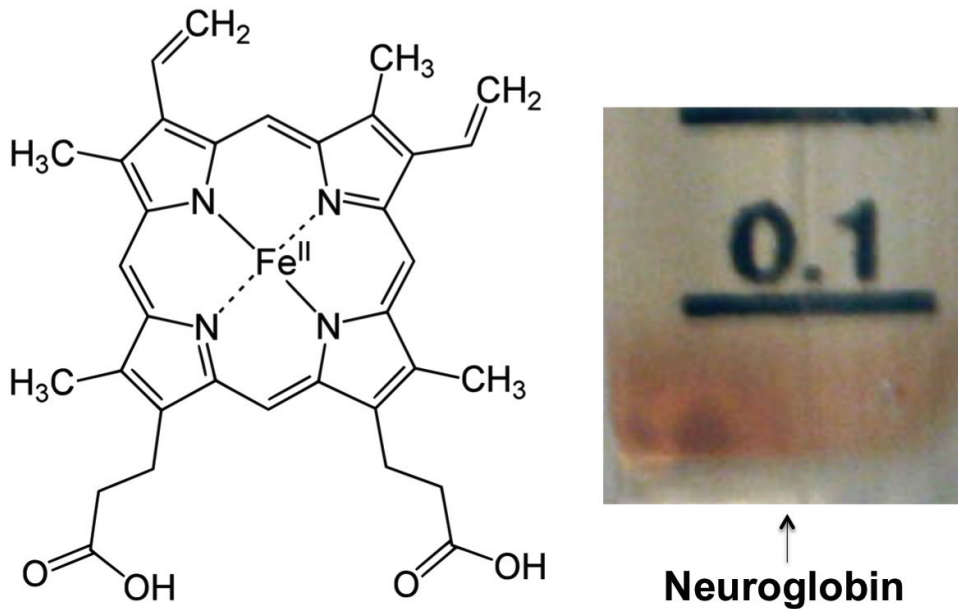


Figure 6: Heme prosthetic group gives the typical red color of the globins, which is also seen in salmon neuroglobin (right).

Different from Hb and Mb, Ngb displaces a hexacoordinated heme structure, seen in both the ferrous deoxygenated (Fe²⁺) and ferric (Fe³⁺) forms. The proximal His F7 and the distal His E7 side chain are occupying the fifth and sixth coordination sites, respectively (Pesce et al. 2002). The heme prosthetic group with iron atom shown in its ferrous state is illustrated in Figure 6. Ngb reversibly binds small ligands like O₂, CO and nitrogen oxide (NO) at the sixth coordination site of the heme, and the distal histidine is displaced by the ligands such as O₂, CO, or NO. Mouse Ngb bound to CO is illustrated in Figure 7.

Ngb binds O₂ with a half saturation pressure (P₅₀) of 1-2 Torr and is similar to that of a typical myoglobin (Burmester et al. 2000; Dewilde et al. 2001).



Figure 7: A 3D-structure of mouse Ngb, a respiratory porphyrin-containing protein with CO (pink colored ball) bound to the sixth coordination of the Fe of the porphyrin group. Proximal and distal histidines are shown in red.

Ngb is present at low concentrations in organisms compared to the pentacoordinated globins like Mb and Hb. Ngb shows an oxygen affinity similar to that of Mb and Hb indicating that O₂ binding is conserved during the course of evolution. Hence, a role for Ngb in oxygen homeostasis is likely (Burmester et al. 2000; Sun et al. 2001; Schmidt et al. 2003; Fuchs et al. 2004).

The physiological functions of Ngb are still uncertain. However, several studies have shown that Ngb has a neuroprotective function (Sun et al. 2001; Sun et al. 2003; Fordel et al. 2004; Fordel et al. 2006; Khan et al. 2006; Liu et al. 2009). Ngb is most likely linked to the oxidative metabolism since Ngb is associated with the presence of mitochondria at the subcellular level.

Most terrestrial mammals will not be exposed to environments with a low oxygen atmosphere and their brains are not adapted to low oxygen levels. In contrast, fish live in an environment with frequent episodes with various or low oxygen saturation. Upregulation of Ngb in accordance with hypoxia is seen in several fish species. Zebra fish and turtles, with naturally different oxygen level in their environment, show increased Ngb expression in the brain at hypoxic condition (Milton et al. 2006; Roesner et al. 2006). Also in hypoxia-tolerant species like goldfish Ngb expression is generally higher compared to hypoxia-sensitive relatives. It is likely that Ngb is involved in oxygen metabolism and has a key role in hypoxia tolerance.

2. AIM OF THE STUDY

The primary goal of this study was to achieve a better understanding of the effect of CO treatment on Atlantic salmon and in its relation to the nerve protein neuroglobin that might be a potent regulator of the effect of CO. To achieve this several secondary aims were set.

- Review the use of CO in meat and fish industry. How does CO affect the color of fish, especially salmon but also mackerel (*Scomber scombrus*)?
- Study the quality and welfare parameters of Atlantic salmon (*Salmo salar*, L.) exposed to CO before stunning and slaughter.
- Clone and heterologously express salmon neuroglobin in *E. coli* and develop an optimized purification procedure to obtain large amounts of sNgb for physiochemical studies with CO.

3. ABSTRACT OF PAPERS

Paper I

Carbon monoxide (CO) is utilized in the fish and meat industry worldwide, mainly for pretreatment and packaging purposes. Furthermore, CO can be used in slaughter of animals, but the latter application is still mostly on an experimental basis with limited commercial access. Methods and equipment for the sedation and killing of fish and terrestrial animals with gas blends containing CO are described, as well as effects of this early CO exposure on subsequent food quality. Animal welfare aspects are discussed by comparisons with existing methods for sedation and killing. Methods for accurately analyzing the concentration of CO in air, water and foods are highlighted. Studies of the penetration, distribution and dissipation of CO in fish and meat tissues are presented. The use of CO for pretreatment of muscle foods, especially fish, is presented, either as filtered smoke or gas blends with CO.

Several packaging systems for CO gas mixtures have been introduced and implemented by the meat industry. An important topic includes the reaction of CO with proteins, in particular heme pigments. CO reacts with the heme protein neuroglobin in the brain and might also take part in biological signaling. The legal aspects involving the use of CO in the slaughtering and processing of muscle foods in different countries are discussed. Aspects from the consumer point of view are described, including both beneficial and undesirable effects of the gas.

PAPER II

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 color 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 pCO₂. Exposure to CO did not increase plasma cortisol, sodium, haematocrit or glucose; however, lactate was high. Exposure to salmon or other fish to CO before slaughter could improve product quality and animal welfare.

PAPER III

The different stunning methods for Atlantic salmon can still be improved with regard to animal welfare. Salmon exposed to carbon monoxide expressed no aversive reactions towards CO as such. CO exposed fish showed an earlier onset of rigor mortis, and a faster decrease in muscle pH due to depletion of oxygen during treatment. Exposure to CO did increase the level of cortisol compared to undisturbed control fish, but the increase was less than in the water only control group. Neuroglobin, a CO binding globin, was found in salmon brain and *Saccus vasculosus*, a richly vascularised sac connected to the fish brain. Binding of CO to neuroglobin during sedation might improve animal welfare.

PAPER IV

Neuroglobin (Ngb) exists only in small amounts in salmon brain. In order to study the protein in more detail salmon neuroglobin (sNgb) was cloned, heterologously expressed in *E.coli* and purified. The protein had red color and showed the characteristic peaks at 411 nm (metNgb), 415 nm (carboxyNgb) and 424 nm (deoxyNgb). Western analysis showed that sNgb reacted weakly against a rabbit anti human neuroglobin (hNgb) and strongly to a sNgb specific antibody. Our 3D- homology model of the sNgb indicated modifications adjacent to and in the O₂/CO binding site. This may correlate to differences in substrate affinities for the sNgb compared to the hNgb. Also sNgb contained shorter helices and longer interhelical loops typical for psychrophilic proteins.

4. GENERAL DISCUSSION

4.1.METHODOLOGICAL ASPECTS

The general methods used in this study are in line with similar studies and experiments. This is the case for both experiments with fish and laboratory work. Here follows a discussion on some aspects of the study.

Regarding parameters, we have in the present study focused on a selection of technological quality parameters. Quality seen from the consumer point of view has not been taken into consideration. Texture, taste and juiciness are examples of parameters that could have been analyzed, and which are important for consumer acceptance or perceptions.

Rigor mortis represents a quality criterion affecting the processing of fish, and we have used this criterion in our experiment. When the fish reach *rigor mortis* and the muscles stiffen, processes like filleting are not appropriate. The early onset of *rigor mortis* when the fish are exposed to CO makes the fish suitable for *post rigor* filleting. *Rigor mortis* as a criterion requires frequent measurements, often at night. However, this is not always convenient, and some resolution in measurements must often be sacrificed.

Different technologies to measure color in meat are available. We chose the Minolta Chroma Meter because we had access to the instrumentation and because many studies are based on this method. But there may be differences in quality and reliability of the measurements due

to the surface properties and texture of the surface of the fillets. Lightness (L), red color (a) and yellow color give numbers that reflect the visual impression of the surface, however it does not give any indication of what kind of pigments that contribute to the visual impression.

The measurement of CO concentration in seawater represented a challenge in our study. We measured the CO concentration in air and the concentration of O₂ in water, but we had no method available to measure CO concentration in seawater. We presumed that the amount of dissolved CO was sufficient for behavioral and fillet quality observations at this stage of the study (Paper II, Paper III). The solubility of CO in seawater and the saturation level are of major importance and are desirable to take into consideration in future studies.

Acclimatization of the fish to the environment in the experimental facilities, as well as elimination (or at least reduction) of stress induced by the experimental procedure is another important aspect. This is a common problem in all fish stress experiments, as unsatisfactory acclimation and/or stress induced by the experimental procedure potentially will confound any of the stress effects the study was designed to investigate. Cortisol, an often used marker of stress, has a response time measured in minutes and reacts strongly to acute stressors such as handling. We used two different experimental procedures in the experiments described in paper II and paper III. In Paper II we transported and hauled the fish into the experimental tank by net. This strongly confounded our test of stress load by cortisol level measurements, and it was not possible to conclude. In the second experiment (Paper III) we tried to alleviate the problems from Paper II by acclimatizing the fish over night in the experimental tank, and exposing the fish to CO in a different manner. Also here the results indicated that the stress

load from the experimental procedure was important. However, the fish treated in water only, showed higher cortisol levels than the CO treated fish. This indicates that the total stress load were reduced by the CO. Future experiments are planned and an experimental unit has been designed where fish may be given longer acclimation periods before the water source is switched without any change in flow or current within the tank. This setup will hopefully reduce the stress from the experimental procedures to very low, and allow a non-confounded analysis of CO induced stress.

To observe precisely whether the fish were stressed or not represented also a challenge. In addition to the behavior analysis we also measured the amount of cortisol as an indicator of stress load in Paper II and III. Additionally, plasma ions and gases from blood gases were also taken into consideration. The latter were analyzed by use of i-STAT, a method designed for use in human medicin. It has been questioned to what extent i-STAT is useful for cold-blooded animals, and it is possible that the results are not describing the fish blood chemistry as good as first believed. However, blood parameters with a significant increase, lactate and pCO₂, is in line with the occurrence of anaerobic metabolism caused by CO as well as with our hypothesis. However, the significant increase in potassium requires further more study.

4.2.DISCUSSION OF THE MAIN FINDINGS

4.2.1. CO AND Ngb

When analysing Ngb in CO treated fish we detected very small concentrations in brain and in *Saccus vasculosus*. Only after immune precipitation detection was possible by using

western blotting analysis by use of an anti human Ngb antigen. The small amount of Ngb constituted a challenge regarding analysis of the protein, as purification of enough protein would be difficult, and in addition it would take a time. Hence, in order to characterize salmon Ngb, we decided to clone, express and purify sNgb. This made the study more extensive than planned, but resulted in a protocol for expression of sNgb with inserted heme-group. This is highly valuable for future studies of the protein, its ligand specificities and binding strengths as well as in determination of its three-dimensional structure. Such studies might add valuable information of the functions of Ngb as well as for controlling its reactivity.

The question of affinity of CO to Ngb is especially interesting and important. It is likely that the affinity of Ngb to CO is higher than to O₂ as is the case for both Mb and Hb. For Hb the affinity to CO is approximately 200 times stronger than to O₂.

Homology modeling of sNgb indicated a more flexible and loose polypeptide-structure of the globin fold compared to mouse neuroglobin (mNgb). This is typical for psychrophilic proteins.

4.2.2. CO AND ANIMAL WELFARE

The main finding in our studies of CO and animal welfare is that the fish had a behavioral response to CO added to seawater. However, the response to CO came first a time after the salmon had come in contact with the gas. In Paper II, the fish actually swam through the CO

bubbles without any reaction. Furthermore, the effect seemed to be dependent on the concentration. At low and gradually increasing levels CO was shown to have a calming effect (Paper II) without any sign of flight reactions. In contrast, at high concentrations of CO the behavior altered into more erratic swimming and a clear escape behavior (Paper III). This strongly indicates that CO as such does not induce an aversive reaction in salmon. However, there are clearly mechanisms that trigger flight reactions at higher concentration. The nature of these mechanisms is not clear, but one hypothesis is that high concentrations of CO rapidly deplete the fish body of oxygen, and that secondary oxygen sensors such as lactic acid or ATP then act as triggers.

Behavior analysis was a part of our study and fish behavior is linked to animal welfare. Behavior analysis has been described on the basis of five different stages of consciousness as shown by Roth et al (2003) (Table 1). Swimming activity, reactivity to visual and tactile stimuli, equilibrium efforts and ability to ventilate were observed on CO treated fish. One main problem is the occurrence of uncontrolled, sporadic convulsions and what these reactions imply. Is this painful or stressful to the fish? In what way can we avoid it? Addition of O₂ to the water may be one possible solution to avoid these reactions. More research on CO is required to answer these questions. Paper III describes fish exposed to higher concentrations of CO and this had a behavioral effect on the fish. Increased activity was seen immediately after exposure, and this might be due to higher concentration of CO, but might also be caused by the water flushing into the tank. The fish exposed to CO also tried to escape from the tank, and most likely the concentration of CO was the main reason for this behavior.

An interesting issue is at what level of CO the calming effect is in action and when it alters into a level with stressing effects. Unfortunately, measurements of CO in water are not straightforward, and we have no exact measures of the CO levels used in Paper II and III. In future studies use of gas chromatography (GC) of the gas atmosphere in the water will give us more knowledge about how CO dissolves in seawater and at what levels the different reactions start. We may also ask to what extent our study gives us the opportunity to discover all the possible behavioral reactions to CO. During evaluation of consciousness only a few of the criteria described by Kestin et al (2002) were used. We might have focused on more parameters within the groups “response to stimuli” and “clinical reflexes”, in addition used a parameter from the group “self initiated behavior”. This would have given us more information and more reliable results. According to Roth et al (2003) different behavioral stages in connection with levels of consciousness are described. If we to a greater extent had based our observations on these descriptions we might have drawn more knowledge from our experiments.

The moment when unconsciousness occurs is also an issue worth discussing. According to Kestin et al (2002) and Roth et al (2003) we evaluated the CO treatment by observing the behavior indicating stages of consciousness. A more accurate evaluation of unconsciousness is to measure brain wave activity, but recording EEGs is invasive, technically demanding and time consuming. The technique is widely used on mammals to assess slaughter method, but it requires more improvements and studies to make it suitable for fish.

A main problem connected to this behavior analysis is the dependence on human observations, which differ from person to person. In addition there are also different

definitions and standards in use in different research groups. This subjectivity may be reduced by common standards and technology.

For future studies it will also be important to design experiments that will answer whether or not the stress effects seen are an effect of the CO itself, or whether is it secondary effects that triggers these. One example might be to investigate if the behavior seen is due to CO or if it is a reaction to a secondary effect of CO displacing O₂ and then causing anaerobic metabolism followed by rapidly declining energy levels and a dramatic buildup of lactic acid. How will the fish react if we add more O₂? As we diffused a pure CO gas into the water, other gasses would be washed out inducing amongst other things a lower level of O₂ at higher CO concentrations. Furthermore, in Paper II, where a gradual increase of CO concentration was induced, the fish would experience a high and only weakly reduced O₂ level before it was rendered unconscious. Hence, a mixture of CO and O₂ may be an alternative strategy, and future experiments should be designed to investigate the effects of different CO/ O₂ atmospheres.

The connection between observed behavior reaction and the feeling of pain or stress to the salmon is also an important issue. We cannot be sure that the calming effect seen is unambiguous to good welfare for the fish. On the other hand erratic swimming behavior and panic reactions indicate poor welfare. When the fish try to escape from the experimental tank seen at high levels of CO, it is very likely that the fish try to avoid a stressor (Schreck et al. 1997). The connection between CO and fish behavior requires even more comments. It may be likely that the erratic swimming behavior is caused from the secondary effect of CO exposure and not that CO itself caused the difference in reaction. Or it might be possible that

CO bound to sNgb causes a specific reaction in the brain. As long as there is oxygen available in the brain it will function perfectly and the fish may be able to feel pain. Therefore, neuroglobin may be a key factor in the CO-induced hypoxia in salmon. The toxic effect of CO is due to its strong affinity to oxygen binding proteins, including the heme-containing globins. We hypothesize that replacement of oxyneuroglobin (ONgb) with carboxyneuroglobin (CONgb) in salmon using CO would inhibit the protective role that Ngb has in hypoxia, and render the salmon more unresponsive and calm compared to untreated salmon, improving the welfare of the fish during slaughter.

4.2.3. EFFECT OF CO ON QUALITY

In our study we have determined quality on the basis of different parameters. The following discussion focuses on color and *rigor mortis*.

Color analysis showed two main findings. A significantly increased redness of prerigor fillets was measured by Minolta Chroma Meter, on fish that had been exposed to ten minutes CO treatment before slaughtering. An extended exposure to CO, with increasing levels of CO in the seawater, for 20 min or 30 min, did not show any more significantly increased redness of the fillets. The explanation of this may be that the level of COMb in the muscle may have been saturated after ten min, or that the time from slaughter to measurement is enough for Mb to be completely saturated with CO due to a higher binding affinity (Paper II). The other finding was that CO exposure gave a significantly more red color in the fillets and gills 10 days post-mortem, measured by DigiEye and also visually seen. Hb is possibly the main contributor to the color effects seen at this time since blood may have reached the

surface of the fillets. The general explanation of the increased redness for both findings immediately after slaughtering and ten days postmortem may be the CO concentration and length of exposure, but factors like the amount of Mb and Hb and the redox potential also affects the color.

Rigor mortis also has product quality implications. Paper II and III have shown that CO treatment caused an earlier onset of *rigor mortis* and faster decrease of pH. This is most likely due to the high affinity of CO to all heme proteins and consequently O₂ is excluded and causes anaerobic metabolism and lactate production. The concentration of CO in the treatment may also have a great effect as high CO level (Paper III) showed an even faster onset of *rigor mortis* compared to a medium or low level of CO (Paper II, III). A fast decrease of pH was shown at both high and medium CO levels compared to low levels of CO. But the final pH of 6.3-6.4 was similar for salmon exposed to CO and the control group in both the experiments described in Paper II and Paper III. In this connection we have to take into consideration that CO treatment has an effect on product quality and animal welfare. Rapid glycolysis may be associated with stressed animals. Early onset of *rigor mortis* affects the processing and may influence the product quality.

4.2.4. Ngb – CLONING, PURIFICATION AND CHARACTERIZATION

As mentioned before, due to very low concentrations of sNgb in brain tissue from Atlantic salmon we had to clone and purify the protein in order to study it. Thus, sNgb was PCR cloned into a pETM41 vector, so that it was fused to the maltose binding protein (MBP) containing a tobacco etch virus (TEV) - protease cleavage site and a histidine-tag and

expressed in *E. coli*.

One challenge in this process was to find the optimal expression temperature. Our results show that expression at 15 °C overnight gave the best yield of high quality monodisperse sNgb. Expression temperatures at 30 °C and 37 °C resulted in less yield of monodisperse sNgb. As Atlantic salmon is a low temperature adapted organism it was not surprising that the 3D model of sNgb showed structural traits that were typical for psychrophilic proteins. Therefore expression at low temperatures should slow down the protein synthesis process that should ensure improved folding for flexible structured proteins.

Aminolevulinic acid was added to the growth medium during expression, in order to produce sufficient amounts of heme. The heme group is of major importance for structural and functional properties of the globins. MBP is thought to function as a chaperone and improves proper folding (Kapust and Waugh 1999; Fox et al. 2001; Nomine et al. 2001; Sati et al. 2002; Bjørndal et al. 2003; Nallamsetty and Waugh 2006). These factors were crucial in order to express high amounts of correctly folded sNgb.

Analysis of spectra was carried out on recombinant sNgb on metneuroglobin (MNgb), deoxyneuroglobin (DNgb) and CONgb. The purification procedure of sNgb provides the protein in its ferric form, since ONgb is unstable *in vitro* and rapidly autoxidizes to MNgb (Dewilde et al. 2001). Reduced Ngb was obtained by adding sodium dithionite, while addition of CO gave CONgb.

5. CONCLUSIONS AND FUTURE RESEARCH

It has been shown that CO has a potential as a sedative agent that will calm down Atlantic salmon before electricity is used as an anesthetic tool followed by gill cutting and bleeding. Sedation of fish before crowding and transport will reduce stress and thereby improve animal welfare. CO binds to Hb and Mb and these compounds give a redder color of fish generally regarded as favorable in the market. The finding of Ngb in brain and nervous tissue is a challenge when it comes to welfare since the protein might store O₂. Availability of O₂ in the brain might prolong the existence of brain waves and the fish might feel pain for a prolonged period. CO displaces O₂, and might therefore possibly shorten the life span by annihilation of the brain waves. The present thesis point to a number of research activities that is now possible to solve.

- 1) Effect of different concentration of CO in seawater. For practical use of CO we need to understand how easy CO dissolves in seawater and how the concentration of other gasses are influenced. There is also a need for understanding how the solubility is affected by temperature and salinity. Our present use of GC (Paper I) shows that a tool is available for finding the optimal conditions. It is of course possible that CO should be diffused into the seawater in a mixture with other gasses.
- 2) Color of different fishes when exposed to CO. As shown for mackerel (Paper I) the color of pelagic fish becomes redder when CO is used. For tuna it is known that CO enriched smoke give a more market favorable color due to the formation of COMb. A study on the effect of CO on different fish species with regard to fillet color should be conducted.

- 3) Effect of CO on different fish species welfare. We have seen that the effect of CO on salmon and mackerel are very different. This should be further studied on different species since it might improve welfare as well as quality. In this regard water holding capacity is of importance, since pH reduction is fast due to lactate formation and this is a problem that needs further investigation. A market study has also to be conducted when it comes to red color.
- 4) Rancidity and CO i.e. quality. It is known that CO binds to the iron in the porphyrin group when it is in the reduced form. The ability for this iron to catalyze oxidation is impaired. This is very favorable for fat species like salmon, herring and mackerel and the effect of CO on storage should be studied.
- 5) Screening of other components that have potential to bind in the active site in sNgb. It is for instance known that eugenol sedates and anesthetizes salmon at higher concentrations. Our initial docking analysis has shown that this compound can bind in the active site of sNgb. Modeling of other compounds might give us insight in how sNgb might be controlled. Furthermore, sufficient amount of high quality sNgb makes it possible to determine the binding and kinetic constants of the different actual compounds.
- 6) Quantification of sNgb in situ by real time polymerase chain reaction (RT-PCR) at different CO-conditions. Our antibody specific towards the C-terminal tail of the sNgb makes it possible to histologically study the location of sNgb in brain and nervous tissue. In addition to this, study of the effect of CO on Atlantic salmon in slaughter and finding correlation to the amount of sNgb in the brain.
- 7) At present we do not know if Ngb is a constitutive or inducible protein. Since it is localized in the brain it is likely that it is an inducible protein. This has implication for animal welfare since fish might suffer for a long time after gill cut if there is

available oxygen in the brain. Brain wave measurements have to be performed. In future perspective we would like to do quantification of neuroglobin from different tissues in Atlantic salmon, in response to different treatments. By use of eugenol as a method to sedate or anaesthetize fish during slaughter, eugenol will work as an inhibitor as it blocks the binding site of the active site.

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