Measuring methods for fish welfare during slaughter based on electrical impedance, EEG, ECG and blood parameters

Endre Grimsbø

Dissertation for the degree of Philosophiae Doctor (PhD)

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Scientific environment

The work in this PhD thesis was conducted at the Department of Biology (BiO), University of Bergen (UiB) in Norway under the research project "Welfare of Farmed Fish from Harvest to Killing- Meeting the Future Challenge (Farewell)". The Farewell project was funded by the Norwegian Research Council (NFR project no. 178938), Fiskeri - og Havbruksnæringens Forskningsfond (FHF project no. 542042) and a cluster of industry partners (Grieg seafood A/S, Lerøy seafood ASA, Bremnes seashore A/S, Scanvacc A/S, Seaside A/S and Marine Harvest Norway AS). The aim of the project was to obtain knowledge on how different lairage and stunning methods influence the welfare and quality of farmed coldwater species (Atlantic salmon, cod and halibut) and seek solutions that will promote the welfare of fish and quality of the products. Besides University of Bergen, the project included research partners from Institute of Marine Research (IMR), Akvaplan Niva AS, Nofima AS and Wageningen Institute for Marine Resources & Ecosystem Studies (IMARES) in The Netherlands. The PhD thesis focused in particular on both the biology and physics behind the measuring methods for fish welfare during slaughter on electrical impedance, EEG, ECG and blood parameters. There work was therefore in cooperation with several research partners in the project accordingly. Some part of the PhD work was spent at IMR, Austevoll Research Station, in the field at various slaughtering facilities, and at IMARES.

Acknowledgements

I will hereby thank the University of Bergen (UiB), the Norwegian Research Council (NFR), and FHF for funding my PhD candidate position in industrial biology.

I am especially grateful for guidance and support from my supervisors Professor Ragnar Nortvedt, BIO, UiB, Professor Erling Hammer, Department of Physics and Technology (IFT), UiB and Senior Scientist Dr. Anders Mangor-Jensen, Austevoll Research Station, IMR. As a research fellow and PhD student the cooperation and guidance from supervisors and experienced researchers in the Farewell project have been of crucial importance to my professional development as a researcher.

I also want to thank all the joint venture partners in the Farewell project and participants in the four scientific publications. I especially want to thank to Dr. Bjørn Roth at Nofima A/S for introducing me to electro stunning of fish and the challenges related to this subject. I am also grateful for his co-operation and guidance in the scientific work related to electro stunning which is a part of this thesis.

I am also thankful for the co-operation with Dr. Atle Foss from Akvaplan-niva A/S during the chill experiment which gave an important contribution to the understanding of cooling of fish. I am also grateful to Dr. Hans van de Vis (Wageningen IMARES), Dr. Bert Lambooij (Wageningen UR Life stock Research), and Henny G. N. Reimert (Wageningen UR Life stock Research) for co-operation in the electro stunning experiment and for introducing me to use of EEG and ECG measurements in fish welfare. I am also grateful for their hospitality during my visit to The Netherlands.

I especially want to thank Associate Professor Dr. Anne Sverdrup for her lecture about muscles and nerve signals, which represented an important turning point in my PhD study. I also want to thank Dr. Erik Slinde (IMR) and Dr. Lars Helge Stien (IMR) for co-operation in both the Farewell project and in the related experiments. I am grateful to Frode Håkan Kjølås (Seaside A/S) which provided electro stunning equipment for the experiments. An especial thank goes to Grieg Seafood A/S at Stjernarøy who provided fish and facilities for the experiments that led to two of the publications in this thesis.

I am also grateful to the Norwegian National Insurance Fund (Folketrygda) and the Norwegian governments labor market agency (Aetat) which gave me the opportunity to start an academic career.

Finally, I want to thank my colleague Gavin J. Macaulay for his improvement of the English language in this thesis. I am thankful that family and friends have kept up with me and given me support and have motivated me during my studies.

Preface

This dissertation was submitted in fulfilment of the requirements for the degree Doctor of Philosophiae (PhD) at the Department of Biology (BIO), University of Bergen (UiB). The research presented in this thesis has an industrial multidisciplinary character including both biology and physics, with a focus on fish welfare. To accomplish a PhD of such a multidisciplinary character has been challenging and has involved a heavy workload.

The apparent increase in my knowledge is documented through four included peer reviewed and published papers (see List of papers) where I am 2nd author in the two first and 1st author in the two last papers. Moreover, several additional experiments were performed during my PhD study, both published and accepted publications (Roth *et al.* 2010, Grimsbø *et al.* 2011, Roth *et al.* 2012, Roth and Grimsbø 2016). The knowledge gained from the Farwell project and my PhD study was important input in a report (Slinde *et al.* 2013) where I was one of the contributors, especially regarding electrical stunning. Knowledge from his report was later implemented in the Norwegian Food Safety Authorities' recommendation for slaughter of fish (Mattilsynet 2014).

During my PhD study I have also contributed to unpublished experiments related to measurements of stress in fish, not included in this thesis, where oxygen consumption (cod and haddock), electrical impedance of swimming cod, and blood parameters were measured.
I have participated in EEG and ECG experiments on eels and swimming cod in addition to a large scale slaughtering experiment on rainbow trout, unpublished, and not presented in this thesis which also have been important for my scientific knowledge development.

The experiments described in paper I were planned within the Farewell project research group, which included myself. In addition to the planning of the experiment, I also contributed in carrying out the experiments', especially the cooling procedure of the second experiment described in the paper and finally the writing process. In paper II my main contribution was the increased understanding of the stunning signals' electrical characteristics and electrical measurements. In this publication I also have participated in the planning and writing process. The experiment in paper II has given me an increased knowledge about EEG and ECG, which was one of the goals for my research fellow position in the Farewell project.

Since I am the first author in paper III, I am also the main contributor from planning to writing, including the experimental setup. As being the first author in paper IV, the responsibility for the analyzing and writing process is mine. My main contribution to the experimental setup was the electrical arrangement including the filter unit. It is, however, important to note that the idea of the filtered signal concept occurred as a result of a unpublished large-scale rainbow trout slaughtering experiment I participated in, organized within the Farewell project.

Abstract

Improved animal welfare during industrial slaughtering of fish is the aim of the scientific work presented in this thesis. The thesis is based on four publications that cover different stages of an automated industrial slaughtering line for fish. The publications are presented in a similar order to those on a slaughtering line.

The results from paper I are relevant for all types of pre-chilling of fish before slaughtering and reveal the physiological effects of live chilling in Atlantic salmon (*Salmo salar*). Chilling of fish is commonly used in the industry, both during transportation and processing of the fish in the slaughtering house.

The publication is based on two experiments where the first experiment included fish (mean weight 840 g) acclimatized to a water temperature of either 16, 8, or 4°C and which were directly transferred horizontally or vertically (9 combinations) to temperatures of 16, 8, 4, or 0°C using a dip net. In the second experiment, fish (mean weight 916 g) acclimatized to 16°C were exposed to four temperature-drop regimes (no physical handling): 16–4°C (over 5 h), 16–4°C (over 1 h), 16–0°C (over 5 h), and 16–0°C (over 1 h). Physical transfers in the first trial, *i.e.*, temperature drops, resulted in immediate (1 h) increases in blood lactate concentrations at all three temperatures, but levels were significantly reduced and close to pretransfer levels after 6 h. Horizontal transfers, *i.e.* 16–16°C, 8–8°C, and 4–4°C, resulted in similar increases and were not significantly different from the groups exposed to temperature drops. The most severe vertical transfer (16-0°C) resulted in a swift loss of equilibrium and eventually death. In experiment No.2, temperature drops from 16 to 4°C and from 16 to 0°C over a period of one or 5 h, without physically handling the fish, resulted in no significant increases in any of the measured parameters 1 h post-transfer, except in the 16–0°C (1 h) group. The latter experienced a significant increase in blood sodium, glucose, lactate and cortisol levels compared to all other groups. The results suggest that salmon are capable of tolerating relatively steep temperature drops without any significant negative effects on blood stress parameters and that physical stress from gentle handling overrides the effect of thermal insults, which is important for the slaughtering procedure.

The overall objective of the study in paper II was to find the optimal configurations for industrial percussive and electrical stunning by evaluating the methods under laboratory conditions. In an automated slaughtering line electrical and percussive stunning are common methods used to ensure unconsciousness, which is critical for fish welfare, before bleed out. The work described in this publication defines the settings, especially voltage and air pressure, needed for efficiently rendering the fish unconscious and also to verify the effect of the stunning machines.

Evidence of unconsciousness and insensibility of Atlantic salmon was provided on the electroencephalogram (EEG) by the appearance of slow waves and spikes, followed by a strong depression in electrical activity. This phenomenon was observed in 17 salmon after percussive stunning using an air pressure of 8.1 to 10 bars, whilst 8 fish were considered conscious at pressures below 8.1 bars, although some were seemingly unconscious in behavior. Consequences were a haemorrhage in the brain cavity in 15 out of 17 fish, broken upper or lower jaws in 9 fish and eye burst in 8 fish.

A general epileptiform insult (unconscious and insensible) was obtained by delivering a voltage, consisting of a direct current (DC) coupled with 100 Hz alternating current (AC) with a peak value of ≈112 volt (V), head to body, for approximately 0.5 s. The total duration of the insult was 62±44 s (mean±SD; n=25) which was followed by minimal brain activity in 19 fish. The heart rate was 20±7 beats/min prior to stunning. After stunning, the electrocardiogram (ECG) revealed fibrillation for 22±15 s and became irregular and showed extrasystolae (ventrical contraction) afterwards. Exposing the salmon for 5 s with electricity followed by a gill cut resulted in 1 out of 3 fish temporarily recovering after 3 min. Haemorrhages were not observed in the fillets. Average current for head to body electrical dry stunning was 668 milliampere (mA) root mean square (RMS) with an average stunning voltage of 107.9 V_{rms}. Electrical head to body stunning can be recommended when using coupled AC and DC current of 668 mA_{rms} and ≈107 V_{rms}. The salmon can be stunned in approximately 0.5 s. However, the experiment concluded that a correct bleeding procedure should be developed. For percussive stunning it was concluded that if sufficient force is used the fish will be rendered unconscious and insensible, however this resulted in damage to the carcass, whereas a combined AC and DC signal is recommended for dry electrical head to body stunning.

The objective of paper III was to verify the optimal AC frequency range to be used during industrial electro stunning, *i.e.* electronarcosis, of Atlantic salmon by investigating the electrical impedance spectra of the combined fish and electro stunning device entity. This is an important task since the frequency of the electrical signals is crucial to the electro stunner's effect.

The electrical impedance and associated phase shift was measured in the frequency range 40 Hz to 1.0 MHz for individual fish (n = 11) placed in a regular electrical stunner. The results of the experiment showed that the average overall impedance of the combined fish and electrical stunning device increases with frequency from 40 to 60 Hz before leveling out in the range from 60 to 800 Hz. Thereafter the impedance decreases to a negligible value at 1 MHz. Measurements on impedance and phase angle showed that the highest average electrical impedance appeared at 100 Hz. Furthermore, there were individual peak impedance variations between 70 and 100 Hz. In all fish measured, the impedance at 900 Hz was observed to be lower than that at adjacent frequencies.

Due to average measured impedance values and the expected influence of the alpha dispersions on the cell surface, as reported in previous research, it was concluded that the optimal AC frequency range for electro stunning of the Atlantic salmon brain is 70 to 100 Hz.

The aim of paper IV was to understand the importance of electrical signal frequency spectrum on stunning, recovery and inflicting injuries. Hemorrhaging in the filet, caused by broken backbones, has been a quality problem for the industry when electro stunning is used to render the fish unconsciousness. The paper also shows the effect of chilling during bleed out.

In this article Atlantic salmon were exposed for 5 seconds to either 217 V_{rms}, 50 Hz, AC or 107 V_{rms} coupled AC+DC at 200Hz, with and without a high frequency spectrum. Post stun the fish were placed back into water, either at ambient seawater temperature (10.4 °C) or cold water (-1.3 °C), to investigate recovery or mortality. The results showed that a high frequency spectrum, but low amplitude prevented the muscles from contracting and causing spinal injuries and hemorrhaging, for all individuals. Injury rates of 14 and 18% was observed when using electrical signals containing only low frequencies of 200 Hz AC+DC and 50 Hz, AC. The high frequency spectrum also reduced the stimulation of the brain as fish recovered faster

with no mortality. Adding a cold shock post stunning delayed or prevented recovery of all groups within the time span required to kill the fish by exsanguination.

Papers III and IV will potentially have relevance for other disciplines, such as medicine, where electroshock and electronarcosis are used.

Samandrag (Norwegian)

Temaet i denne avhandlinga er å forbetre dei automatiserte slakteprosedyrane av oppdrettsfisk brukt i industrien med omsyn på dyrevelferd og matkvalitet. Dette var også det overordna målet for det NFR finansierte «Farewell» prosjektet, som stipendiatstillinga mi i industriell biologi var ein del av.

Denne PhD-avhandlinga er basert på detaljerte studiar der ein bruker instrumentering og måleteknikk for å optimalisere dyrevelferd. Arbeidet avhandlinga byggjer på kombinerer difor både biologi og fysikk, med hovudfokus på industriell biologi. Moderne industriell slakting av fisk er ein svært automatisert prosess med høg produksjonskapasitet. Ei slik produksjonslinje for slakting er vist i figur 1.

Publikasjonane denne avhandlinga bygger på fyljer som vedlegg, merka som I, II, III og IV. Dei tek føre seg ulike delar av slakteprosessen ut frå ei vinkling mot både biologi og fysikk, der målemetodar for dyrevelferd er i fokus. Sjølve avhandlinga knyt saman desse fire publikasjonane og gir ei samla oppsummering og konklusjon av arbeidet. Rekkefylja publikasjonane er presentert i tilsvarer rekkefylja prosessane førekjem i ei automatisert slaktelinje. Difor er publikasjon I, som omhandlar levandekjøling av fisk, presentert først. Denne publikasjonen er relevant både for kjøling under transport og i samband med prosessering av fisken, då særleg bruk av RSV tankar før slakting. Konklusjonen til denne publikasjonen er at laksen toler vesentleg raskare nedkjøling enn tidlegare antatt, noko som er eit særs viktig resultat for at industrien.

Den andre publikasjonen (paper II) tek føre seg elektrobedøving og bruk av slagmaskin, det vart her brukt måling av hjerneaktivitet (EEG) og hjerteaktivitet (EKG) for å verifisere og

optimalisere dei ulike metodane. Publikasjonen konkluderer med kva som er optimale parameter å gjere fisken bevisstlaus både ved bruk av elektrobedøving og slagmaskin. Det er viktig å merke seg at denne publikasjonen også viser at berre bruk av visuelle observasjonar av fisken sin bevisstheit ikkje er tilstrekkeleg, men at det er òg naudsynt å bruke EEG.

I publikasjon III vert meir den teoretiske bakgrunnen vurdert og bekrefta for kva som er den optimale frekvens ved elektrobedøving ut frå måling av elektrisk impedans av fisken plassert i eit vanleg oppsett for elektrobedøving. I forsøket som publikasjonen byggjer på viste det seg at det er samsvar mellom målt maksimal impedans og optimal frekvens for elektrobedøving av fisk.

Den siste publikasjonen (paper IV) presenterer løysinga på skadeproblemet med påfyljande kvalitetsfeil på grunn av kraftige muskelkontraksjonar som kan oppstå ved bruk av elektrobedøving. Det vart i denne publikasjonen konkludert med at overharmoniske frekvenskomponentar, vist som eit frekvensspekter, i det elektriske signalet brukt til elektrobedøving forhindrar skade som følje av muskelkontraksjonar. Noko overraskande viste det seg òg at ved å fjerne dei overharmoniske frekvenskomponentane i det elektriske signalet frå elektrobedøvaren vakna ikkje fisken opp att, men døde. Publikasjonen viser også at bruk av kjøling under utbløding vil sikre at fisken ikkje vaknar opp att etter elektrobedøvinga.

Resultata vist i publikasjon III og IV vil kunne ha relevans også for andre fagfelt der elektrobedøving eller elektrosjokk vert brukt.

List of publications

Paper I

Foss, A., Grimsbø, E., Vikingstad, E., Nortvedt, R., Slinde, E., Roth, B. (2012). Live chilling of Atlantic salmon: physiological response to handling and temperature decrease on welfare. Fish Physiology and Biochemistry, 38, 565-571.

Paper II

Lambooij, E., Grimsbø, E., van de Vis, J.W., Reimert, H. G. N., Nortvedt, R., Roth, B. (2010). Percussion and electrical stunning of Atlantic salmon (*Salmo salar*) after dewatering and subsequent effect on brain and heart activities. Aquaculture, 300, 107–112.

Paper III

Grimsbø, E., Nortvedt, R., Hjertaker, B. T., Hammer, E., Roth, B. (2016). Optimal AC frequency range for electro stunning of Atlantic salmon (*Salmo salar*). Aquaculture, 451, 283-288.

Paper IV

Grimsbø, E., Nortvedt, R., Hammer, E., Roth, B. (2014). Preventing injuries and recovery for electrically stunned Atlantic salmon (*Salmo salar*) using high frequency spectrum combined with a thermal shock. Aquaculture, 434, 277–281.

Table of contents

List of abbreviations

°C – Degree Celsius

A – Ampere (Electrical current I)

A/D – analog-to-digital

AC – Alternating current

ACT –Clotting time

ADC – Analog-to-digital

AEPs – Auditory evoked potentials

AV node – Atrioventricular node

BIO – Department of Biology

C – Electrical capacitance (Measured in microfarad mF)

C_A – Capacitance pr. unit area

Ca_2^+–Calcium ions

Cl^- – Chloride ions

CO_2 – Carbon dioxide

d – Distance

dB – Decibel

DC – Direct current

DFT – Discrete Fourier transform

ECG – Electrocardiogram

ECoG – Electrocorticography

ECT – Electroconvulsive therapy

EEG – Electroencephalogram

EKG – Electrocardiogram

ERPs – Event-related potentials

EST – Electroshock therapy

et al. – et alii (Latin)/and others

f – Frequency

Farewell – Welfare of Farmed Fish from Harvest to Killing- Meeting the Future Challenge

FFT – Fast Fourier transform

FHF – Fiskeri - og havbruksnæringens forskningsfond

FNS – Functional neuromuscular stimulation

f_S – Sampling frequency

G – Electrical conductance

H – Henry (Electrical inductance L)

HCO_3 – Bicarbonate

Hct – Hematocrit

Hz – Hertz

I – Electrical current (Measured in Ampere A)

i.e. – id est (latin)/that is

IFT – Department of Physics and Technology

IMARES– Institute for Marine Resources & Ecosystem Studies

IMR – Institute of Marine Research

K^+ – Potassium ion

L – Electrical inductance (measured in henry H)

mA – Milliampere

mF – Microfarad (Electrical capacitance C)

MHz – Megahertz

mS/m – Millisiemens pr. Meter

n – Number

N – Part of the ERPs - event-related potentials

Na^+ – Sodium ion

NFR – Norges Forskningsråd

OKR – the optokinetic response

P – Part of the ERPs – Event-related potentials

P – Power

P deflection – Part of the P-QRS-T complex

pCO_2 – Partial pressure carbon dioxide

pDC – Pulsed DC

per se– (Latin) in itself

PhD – Philosophiae Doctor

pO_2 – Partial pressure oxygen

P – QRS-T complex – A complete heart beat

QRS complex – Main part of the P-QRS –T complex

R – Resistance (measured in Ohm Ω)

R deflection – Part of the P-QRS-T complex

RMS – Root mean square

RSW – Refrigerated sea water

s – Second

S – Siemens

S/m – Siemens pr. Meter

SA node – Sinoatrial node

Sec – Second

SI – The International System of Units

T deflection – Part of the P-QRS-T complex

T depletion – Part of the P-QRS-T complex

UiB – University of Bergen

V – Volt

VEPs – Visually evoked potential

VOR – Vestibulo-ocular reflex

X – Reactance (The imaginary part of the electrical impedance Z)

Y – Electrical admittance

Z – Electrical impedance

ε – Electrical permittivity

θ – Phase angle (of the electrical impedance)

ρ – Electrical resistivity

σ – Electrical conductivity

Φ – Heat flux

Ω – Ohm (Electrical resistance R)

1. Introduction

The overall aim of the Norwegian Research Council (NFR) and FHF financed Farewell project, which my research fellow position has been a part of, is to improve the slaughter procedures of farmed fish with respect to fish welfare and food quality. The aim of my PhD study was therefore to contribute to and establish gentle and efficient procedures to put fish to death, based on detailed studies on stress responses and on physical instrumentation, thus combining scientific evidence from both biology and physics, with a primary focus on biological significance.

Modern industrial slaughtering of fish is a highly automated process with a high production capacity. The different machinery in a typical slaughtering production line is shown in Figure 1.

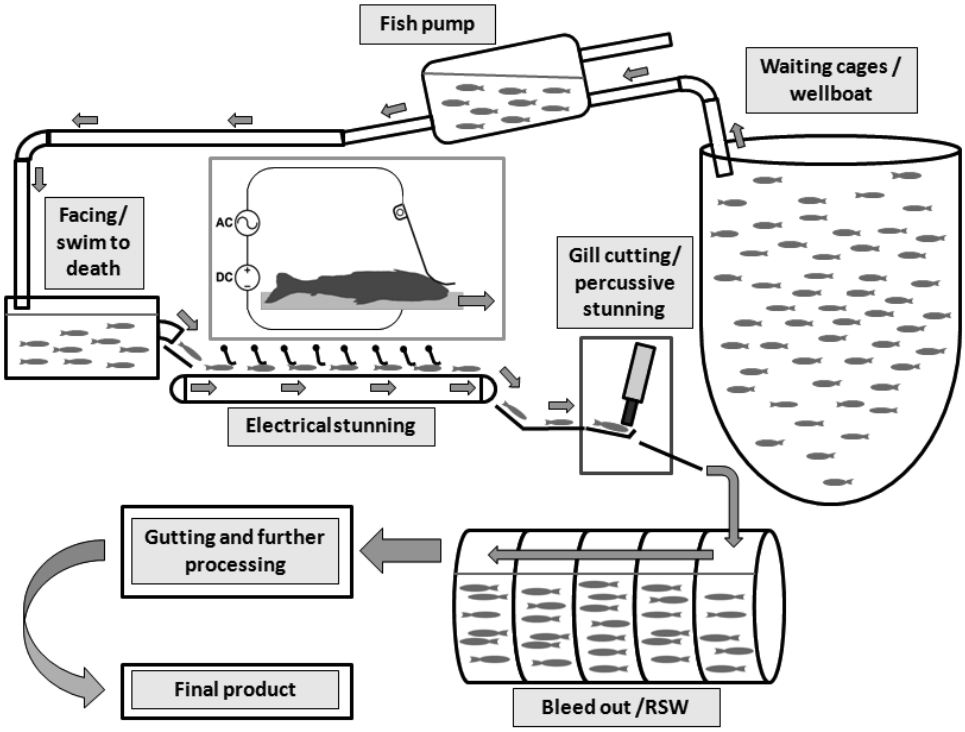

Figure 1. *Automated industrial slaughtering line for fish, schematic presentation. The separate frame shows the principle of electro stunning of fish.*

Well boats are commonly used for transportation of salmonids between aquaculture farms and the slaughtering facility. The fish will be stored in waiting cages or pumped directly from the well boat into the factory (Merkin *et al.* 2010). If the fish are pumped directly into the factory, it is common that the well boat has a capacity for refrigerated sea water (RSW) so that the fish are cooled during transportation. If waiting cages are used it is common to chill the fish before slaughtering either in a separate RSW tank or by using cold water in the facing/swim to death apparatus. Paper I has a focus on live chilling of salmon and will be relevant to any procedures where the fish body temperature is decreased prior to slaughtering. However, some slaughter houses do not practicing live chilling of the fish prior to slaughter.

Prior to gill cutting and bleed out the fish are electrically or percussively stunned, or in some cases both. The electrical stunning procedure is done by placing the fish between a steel conveyer belt and an electrode, also called a shoe or finger, with an electrical potential difference that makes the fish unconscious. Percussive stunning is done by machine where a piston driven by compressed air hits the fish head so that the fish becomes unconscious. After the fish are unconscious knifes cut the gills so that bleed out starts. After gill cut the fish are be transferred to the RSW tank where bleed out occurs. It is crucial for the animal welfare that the fish are unconscious during bleed out, and it is unacceptable that the fish are bleeding out in a conscious state. The fish will then be processed when bleed out is completed and it is crucial that the fish are dead and not processed alive.

1.1. Legislation for commercial slaughter of farmed fish

The Norwegian law of animal protection (Anon 1974), which defines animal protection at slaughterhouses, is the basis for the Norwegian directive for animal welfare. In the Norwegian directive for animal welfare that concerns slaughter of mammals and poultry (Anon 1995) it is stated that: “Animals shall be spared any avoidable stress, pain and suffering during unloading, herding, positioning, restraining, stunning, and killing.” In the EC directive for protection of animals at slaughter (Council Directive no 93/119, 1993) a similar expression occurs: “Animals shall be spared any avoidable excitement, pain or suffering during movement, lairaging, restraint, stunning, slaughter or killing”.
The regulations for slaughtering of farmed fish in Norway are defined by the Norwegian directives on slaughter of farmed fish (Anon 2001). It is stated in the directive that: “Farmed

fish shall, if it is necessary to prevent injuries, be stunned prior to exsanguination. Stunning is allowed only with the use of methods approved by the Director of Fisheries." However, the directive for slaughter of fish expresses greater concern about physical injuries, related to the struggle that might occur which affect quality rather than fish welfare. These approaches to the fish slaughtering process are not in accordance with the future demand for fish welfare. However, new recommendations and directives for welfare and slaughter of farmed species, fish included, are coming up in Europe. In Norway new recommendations and directives for fish slaughter also demand changes in slaughtering procedures. There are many indications that legislation for slaughtering of fish in the future will become equal to regulations for poultry and mammals. A stunning procedure equivalent to poultry and mammals requires that the fish must be stunned unconscious immediately, *i.e.* within < 1sec., and remain unconscious until death occurs (EFSA 2009).

1.2. Fish welfare challenges under application of different sedation, stunning and killing methods

With respect to both animal welfare and quality it is important to avoid a stressful situation for fish during slaughtering (Digre 2011). Therefore, a proper stunning procedure is required to render the fish unconscious before slaughtering. This will also be recognized as a human slathering procedure (van de Vis 2003) and also in accordance with best practice. However, the ongoing debate regarding whether the fish are able to feel pain (Nordgreen 2009, Braithwaite 2010, Rose 2014) is out of scope for this thesis.

For stunning of fish, especially Atlantic salmon, several stunning methods have been used commercially in the last decades. The most common method of stunning Atlantic salmon prior to exsanguination has been live chilling with or without use of CO_2, or additional stunning methods (Erikson *et al.* 2006, Roth *et al.* 2006, Erikson 2008). However. use of CO_2, even at small concentrations generates stress responses in salmon (Erikson 2011, Foss *et al.* 2015). Live chilling is a common procedure via pre-chilling of the fish in bins with ice slurry and water prior to gill cutting. The live cooling of fish normally takes place in large RSW tanks. Such tanks can have different structures, but normally the fish are brought forward through the tank automatically. RSW tanks were originally used as bleed out tanks before it turned out that it was efficient to cool the fish before bleeding.

By cooling the fish live the time it takes from killing the fish until it goes into rigor mortis is extended (Skjervold *et al.* 2001), which is beneficial for processing and product quality in the market. Live cooling has also been used as a procedure to calm or sedate the fish before slaughtering.
Within the live chilling procedure it is common practice not to expose the live fish to temperatures lower than 0.5 º C. It is common to use ice slurry to kill aquarium fish adapted to higher water temperatures, and this is also common practice for commercial species in southern Europe (Lines and Spence 2012). However, little information exists on how hypothermia affects the fish prior to stunning, which therefore became the main focus of paper I.

The relevant literature and articles on the subject of cooling of fish often has little focus on the basic physical principle in relation to cooling and heat transfer. It is reasonable to assume that a basic understanding of heat transfer is important in order to evaluate the different details of the various methods. It is, however not possible within the framework of this thesis to provide a complete model for heat transport in fish, but I will try to present some important principles related to the topic.

To simplify the understanding of heat transfer, consider the fish as a solid physical object or body. The heat transfer or cooling occurs at the surface of the fish. A larger fish, due to its larger surface, has a higher total heat flux (Φ) than a small fish. Since volume and weight are dependent on each other, a larger fish has a smaller surface to weight ratio than a smaller fish and thereby a smaller Φ per unit weight. The weight is therefore crucial to the fish's heat capacity or latent heat per unit surface. Since the surface determines the heat transfer and the latent heat is dependent on the weight it is reasonable to assume that a small fish will cool down faster in cold water than a big one, which gives the smaller one a shorter time for adaptation and acclimatization. The expected difference in time for cooling of a small fish versus a large is a reasonable explanation for the difference in mortality versus fish size described by Skuladottir *et al.* (1990) and mentioned in paper I.

In this context it is important to take into consideration that a living animal has a blood system which also contributes to the temperature exchange. The influence of the blood flow on heat exchange is included in Pennes (1948) analyses of the relationship between arterial and body temperature via a model that describes the actual heat in a living animal. The gill

and blood system's influence on the temperature exchange are conspicuously shown by Stevens and Sutterlin (1976). Since the gill area is dependent on the fish mass, it can be described as an exponential function of the fish weight (Graham 2006).

Because most teleosts, except tuna, do not have a mechanism to maintain an independent body temperature they are described as poikilotherms. Poikilotherm fish, including salmonoids, are dependent on the surrounding water temperature and the physical principle of heat transfer. These are the main temperature regulation mechanisms, and not metabolism. Rapid changes in environmental temperature cause physiological responses, alter the behavior, and can even cause death (Donaldson *et al.* 2008). A release of primary stress hormones in fish are shown to be a response to cold shock (Tanck *et al.* 2000. Muscle stiffening (cold shortening) prevents the fish from expressing behavioral signs such as panic during chilling. It is unclear whether arctic species lose consciousness during live chilling.

Some experiments show that live chilling in combination with CO_2 are insufficient in order to obtain unconsciousness in the animal (Roth *et al.* 2006; Erikson *et al.* 2006). However, initial experiments done early in the Farewell project showed that by slowly increasing the content of CO_2 in the water it was possibly to use CO_2 to sedate and even make Atlantic cod (*Gadus morhua*) unconscious without any observable panic reaction, but the same effect was not observed for salmon.

Other stunning methods used in the fish slaughtering industry today are percussive and electrical stunning, which are investigated in paper II. In both the fish slaughtering industry and in regular fisheries, percussive stunning is a well established method. Electrical stunning procedures in the fishery industry have become more common and the principle is equivalent to electronarcosis or electroshock, also named electroconvulsive therapy (ECT), that is used in human medicine. The effect of electro stunning is related to the electrical impedance in the fish, as shown in paper III. A major side effect of electrical stunning as used in the slaughtering industry is internal bleeding and carcass damage, and is especially evident in salmon (Roth *et al*., 2003, 2004). This is discussed in paper IV. Other species, such as Atlantic cod and rainbow trout (*Oncorhynchus mykiss*), are less susceptible to injuries compared to salmon (Olsen 2006). Both methods are proven to be efficient and have the ability to almost immediately render the animal (Robb *et al*., 2000; Robb and Roth, 2003, Lambooij *et al*. 2002a,b, 2004 and 2006a,b, Roth *et al.* 2007). For salmon it was, prior to the

Farewell Project, unclear if permanent loss of unconsciousness was caused by efficient stunning in combination with bleed out (Roth 2006). Previous experiments (Lambooij *et al.* 2006a,b) showed that exposing the fish to a thermal insult during bleed out is a suitable method to secure permanent insensibility in electric stunned African catfish. For gill cutting machines, which are highly dependent on the fish position in the machine, the error in the procedures appears to be unacceptable (Roth 2006).

At the starting point for the Farewell project and thereby also my PhD work the aquaculture industry was still operating on a non-evidential background in several fields, such as slaughtering of fish. The intention of my work was to improve the slaughtering procedures, clarify and hopefully contribute to solving some of the challenges related to acceptable fish welfare during slaughtering, in the context of the biology and physics fields.

1.3. Stress in fish and blood parameters

The word stress has a wide and diffuse use in everyday language, but in a scientific context there is a need for a clearer definition. A possible definition of stress is a condition which extends the animals adaptive responses or disturbs the normal functioning and is a result of the environment, such as density and feeding, or is produced by other factors (Engelmann *et al.* 2004), also described by Selye (1936) as the *"general adaptation syndrome"*. However, both in artificial conditions and in a natural environment fish are exposed to stressors but the responses are often impossible to generalize. The most obvious responses to a stressor are changes in the animal's behavior. Depending on the stressors, the return to a pre-stress condition takes minutes to weeks and tends to occur fastest for behavior that is important to the animals' survival (Iwama *et al*. 2006). Behavioral stress responses might be due to pain perception, aversion or fear, which prove the fishes awareness of itself in the environment (Braithwaite and Boulcott 2007).

The fish physiological stress responses are normally categorized into primary, secondary and tertiary responses. Metabolic, hematological, hydromineral, and structural changes related to stress are categorized as primary and secondary responses (Barton and Iwama 1991). The tertiary stress response is more a long term response occurring when the fish is not able to adapt or acclimate to the stressor and is of less interest in a slaughter setup.

The primary stress response is the immediate or initial response to a stressor with release of stress hormones such as catecholamine, cortisol and adrenocorticotrophic hormone (ACTH). It is often difficult to measure the hormones released by a primary stress response because there is a risk that the sampling procedure itself triggers a stress response. Primary physiological stress responses are therefore often determined by measuring blood plasma cortisol, which has a slower response than, for instance, adrenaline (Iwama *et al.* 2006).

Metabolic, hematological, and hydromineral changes in the fish as a result of stressors are important for the secondary stress responses. The changes that occur as secondary stress responses are detectable by measuring alterations in the blood chemistry. Alterations in the blood chemistry that can be observed include changed levels of glucose, lactate, sodium (Na^+), potassium (K^+), chloride (Cl^-), calcium ions (Ca_2^+), bicarbonate (HCO_3), oxygen partial pressure (pO_2), carbon dioxide partial pressure (pCO_2), lactic acid, Hematocrit (Hct), and clotting time (ACT) (Barton and Iwama 1991, Ashley 2007). It is also possible to observe changes in oxygen consumption during the acclimatization to stressors. The fish will also increase its blood flow and an increase in heart rate will be detectable as a response to stressors (Schreck 1982).

The physiological responses from rapid changes in temperature such as cold shock affect the membranes in the gill directly or indirectly by the release of stress hormones and are detectable by blood analyses (Tanck *et al.* 2000, Rørvik *et al.* 2001), and are described in paper I.

1.4. Heart rate in fish

Fish have a simplified heart and a circulatory system compared to mammals, where the blood is pumped to the gills for oxygenation and purification, then directly out to the body. Compared to the human heart, which has four chambers, a fish heart has only one atrium and one ventricle. However, like all other vertebrates the fish heart consists of special muscle tissue called cardiac muscle tissue. Since a muscle, like the heart, contracts as a result of a depolarization caused by an electrical signal it is possible to measure the muscle activity electrically, which is described as Electrocardiography (ECG). The cardiac muscle tissues

contract as a result of depolarization in the tissue itself, often called pacemaker cells. Factors that affect the regulation of the heart rate are primarily the autonomic nervous system and the temperature (Evans and Claiborne 2006). For human hearts it is possible to investigate the electrical depolarization signal using a standardized ECG setup consisting of several (normally 12) electrodes. For fish the same standardized setup does not exist but it is common to use a two electrode setup. Depending on electrode placement the ECG signal of a fish heart is not unlike a human heart, even though the heart has a simpler anatomy. An example of ECG signal from salmon is shown on Figure 2.

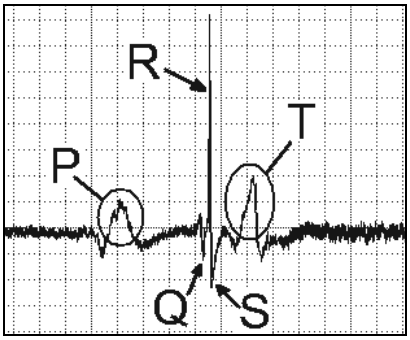

Figure 2. *ECG example from experiment with percussion stunning of Atlantic salmon (paper II) shows a P-QRS-T complex similar to what found in humans.*

For teleosts, such as salmon and cod, the P deflection represents the atrial depolarization, the QRS complex shows the ventricular depolarization and T deflection the ventricular and atrial repolarization. This P-QRS-T complex is similar to that found in humans. There are differences, however, especially since the T deflection has different characteristics. Compared to what is normal for humans the T deflection on fish is abnormal. The ECG recordings in paper II often had a similar shape to the QRS complex, but was smaller. The P deflection in fish has a tendency to be less marked than common in ECG of the human heart, which is expected due to the anatomical atrium design of the fish. In fish the atrium has a smaller muscle mass than the ventricle and lacks a cardiac valve to prevent blood from flowing back into the terminal veins (Evans and Claiborne 2006). The differences in the fishes P-QRS-T complex indicate a possible different depolarization of the heart muscle compared to humans, which is not unexpected due to the anatomical differences. If a P-QRS-T complex measured on a human had the same characteristics as a normal fish, it would have indicated an error in signal transfer between the sinoatrial node (SA node) and the rest of the heart, like the

atrioventricular node (AV node). For instance, a different frequency (Hz) in the P deflection compared with the QRS complex (Figure 3), indicates a blockade in the signal from the SA node to the rest of the cardiac muscle tissue (Hampton 2009). If the depolarization signals from the SA node are blocked, other tissue in the heart will generate its own depolarization signal, but often with a lower frequency, which contracts the ventricular chamber.

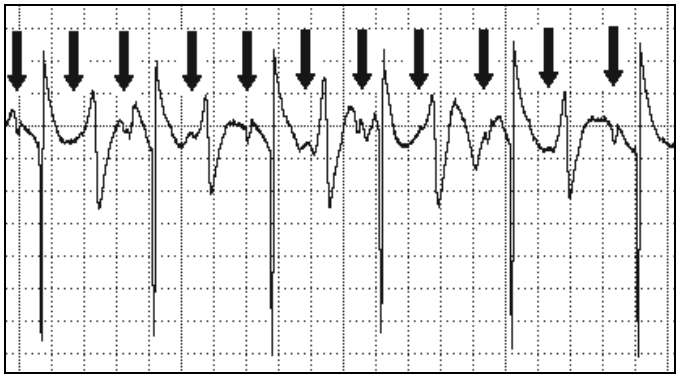

Figure 3. *ECG from swimming cod with abnormal T deflections and small P deflections (arrows) with an independent frequency of the QRS complex, which indicates a signal blockade in the depolarization sequence*

In the recorded ECG signal reported in paper II and in an unpublished experiment done on cod the T deflection, which is the repolarization, often had an abnormal form compared to a human ECG. Since the ventricle is a relatively large part of the fish heart it is possible to explain the abnormal T deflection in fish ECG, compared with human ECG, as a result of the repolarization of a big muscle mass. However, as Figure 3 shows there is within the T deflection (which normally is a repolarization) a new depolarization. Since the initial atrial depolarization in Figure 3 is blocked, it is reasonable to assume that the abnormal T deflection is caused by the influence of a change in the hearts' depolarization sequence on the repolarization of the ventricles muscular tissue. Since the abnormal T deflection occurs frequently it is possible that fish has a major difference in the tissues that generate the depolarization signal compared to those in humans, which is also in accordance wih earlier research (Evans and Claiborne 2006). An increase in the T deflection is also observed, which is assumed to be controlled by the parasympathetic nerve system (Makiguchi 2009) and which makes it difficult to determine the origin of the abnormal T depletion. The lack of a standard protocol for electrode placement in fish makes it difficult to compare ECG in fish and human.

The detection of the R deflection depends on the method used for measurements of the ECG signals. Traditionally, the ECG signal is measured by using electrodes and a bioamplifier connected to an analog-to-digital (ADC) converter which makes the signal available to a computer. Electrode arrangements where the electrodes are wired to a bioamplifier easily disturb the animals' behavior. Maintaining natural behavior is especially difficult for swimming fish when attaching electrodes. An alternative to a regular bioamplifier and electrodes is to use an acoustic transmitter constructed for registration of the R deflection and transmission of a ping signal as shown in Figure 4.

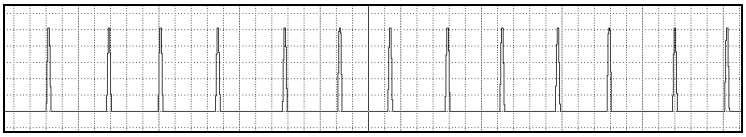

Figure 4. *Example of ECG measurements of swimming cod with acoustic ECG transducer shows a "ping" for each R deflection.*

It is relatively easy to detect the heart rhythm since the R deflection is distinctive also in the fish P-QRS-T complex. By using the time difference between each R deflection it is possible to calculate heart rate. It turns out that the fish heart rhythm has an irregular tendency (Figure 4). Compared to the human heart this tendency for an irregular heartbeat also corresponds with previous mentioned differences in the P-QRS-T, especially the shape of the T deflection. Even with the evident differences in anatomy and possibly also the depolarization of the heart between fish and human, it is expected that the factors that control the heartbeat are the same. It is therefore expected that a change in heartbeat will be a result of an acute stress situation but also at different level of unconsciousness (Zahl 2011).

Since cardiac tissue has the ability to generate depolarization, the detection of ECG in a slaughtering procedure does not necessarily prove if the fish is conscious or not – the heartbeat is often detectable even after the fish is brain dead. Regardless, if the fish brain does receive sufficient oxygen supply it will cause unconsciousness and eventually brain death, therefore the blood supply is crucial. Electro stunning often causes disturbance in the heart rhythm such as fibrillations (Figure 4 and paper II), and even cardiac arrest. A cutoff of the blood supply to the brain after an initial stunning will ensure that the fish remain unconscious until death has occurred, which makes monitoring of ECG an important tool for fish welfare during slaughtering.

1.5. Consciousness and brain activity

As a consequence of the regulation earlier described (Anon 1995 and Council Directive no. 93/119, 1993), it is important to ensure that animal suffering, pain and unpleasant excitement is avoided during slaughtering. The key to a humane slaughtering procedure is therefore to ensure that the animal does not have the ability to feel or have any awareness during slaughtering, which is the purpose of stunning. In the stunning procedure before slaughtering, the fish will lose awareness of its surroundings and of its own body and in the state of unconsciousness be unaroused and unresponsive. Determining if the fish is in a state of unconsciousness is crucial for evaluation of slaughtering methods and fish welfare. The most common way in the fish industry for evaluation of consciousness is the eye roll method (Kestin *et al.* 2002). The eye roll protocol is based on behavioral observation and describes the consciousness in a value from 0-2. Since the effect of anaesthetization is loss of consciousness it is also relevant to compare the effectiveness of stunning in fish slaughtering with protocols for evaluation of stages in anesthesia. Adapted from previous work, Zahl (2011) presents four stages and planes (that is, sublevels), of anesthesia in fish and thereby describes the different levels of consciousness. The different stages are described as follows:

I. Light sedation
II. Excitatory stage
III.
    1. Light anaesthesia
    2. Surgical anaesthesia
    3. Deep anaesthesia
IV. Impending death

Each stage is defined by both visually observable and other parameters. Since electro stunning is equivalent to electronarcosis the comparison between levels of anaesthesia and consciousness in stunned animals is highly relevant. It is not necessarily possible to describe other stunning methods, such as percussive stunning, as anaesthesia but it is still possible to use the same evaluation method for consciousness.

1.5.1. Visual observations of consciousness

The easiest way to determine fish consciousness is to observe its behavior. If the behavioral responses to stimuli is considered to be normal, there is no question about whether the fish is fully conscious. The parameters that are visually observable in the first of the previous described stages, light sedation, is defined as disoriented behavior, reduced activity and slightly reduced responsiveness. In the excitatory stage the observable behavior of the fish is excited, has increased activity, normal or exaggerated responsiveness, irregular or increased respiration and struggles to maintain balance. It is not possible to describe the fish as unconscious in any of these two first stages. Compared to the eye roll method these two first stages will be described as "0". However, methods that cause light sedation will increase animal welfare if used as pre sedation before stunning.

In stage III Zahl (2011) describes three planes or sublevels named light, surgical, and deep anaesthesia, which may all be described as different levels of stunning. The first sublevel, light anaesthesia, is characterized by normal or decreased respiration, although the fish reacts to strong tactile stimuli which possibly make this an insufficient stunning level. In both the second and third sublevel, surgical and deep anaesthesia, the fish has no responsiveness and thereby has a sufficient stunning level. The difference between the two last subgroups is respiration, which in surgical anaesthesia is described as shallow and for deep anaesthesia is nearly absent.

The behavior in all the three planes or sublevels of stage III is described as anaesthetized. In contrast to the levels in stage III, stage IV, impending death, the behavior is described as moribund, stopped respiration, and the fish has no responsiveness.

The activity for all the sublevels of stage III together with stage IV is described as arrested and the fish has lost equilibrium, which is compatible with value 1 and 2 in the eye roll method (Kestin *et al.* 2002). Therefore, both swimming activity and equilibrium are important parameters in evaluation of consciousness. The eye roll method for evaluation of consciousness involves the vestibulo-ocular reflex (VOR) which use the heads velocity information to stabilize the vision (Raphan and Cohen 2002). Since the eye roll method is normally used in an enlightened environment and actually is an evaluation of gaze and image stability there will be an influence from other eye movement responses such as the optokinetic

response (OKR) (Ebenholtz 2001) which supplements the fishes VOR (Huang and Neuhauss 2008). However, all visual observation methods involving a motor response, like the eye roll method and which depend on the response from the oculomotor system, do not necessarily represent the fishes awareness of the surroundings and of its own body. The only way to ensure that the fish is unconscious is to measure the electrical activity of the brain. However, accurate electrical measurements of brain activity are difficult without working in a laboratory, so visual observations methods, like the eye roll method are still an important tool for field evaluation of fish consciousness.

1.5.2. EEG

Electroencephalography (EEG) is commonly used to measure the electrical activity in the brain. The registration of the brains electrical activity, so called brain waves, are measurements of electrical potential differences and frequencies. Different electrical potentials in the brain are well documented (Empson 1986) and led to recordings of EEG signals.

The concept of EEG is based not only on analyses of frequency spectra and amplitude of the signals, but also the shape and behavior of the signal. Main classification of the electrical activity frequency spectra recorded on the EEG is defined into delta (0 -4 Hz), theta (4-7 Hz), alpha (8-13 Hz) and beta (> 13 Hz) frequency bands. The beta frequency bands are normally defined as up to 30 Hz, although it is possibly to classify the $beta_2$ frequency band as high as 50 Hz (Rampil 1998). There are also other classifications in addition to the main classification of the frequency components in the EEG signal but they have little relevance for consciousness in fish, so frequencies below 30 Hz are the main frequencies of interest. By evaluating the alpha and beta rhythms it is possibly to determine the animal's consciousness (Kooi *et al.* 1978). However, it is assumed that during a general epileptiform insult, such as described in paper II, the animal is unconscious (Bilgili 1999).

For analyses of the different frequency components in the EEG signal it is possible to use either hardware or software solutions. A common way of analyzing the strength of different frequency components in a signal is the Fast Fourier transform (FFT), which transforms the EEG signal from the time domain into the frequency domain (Figure 5).

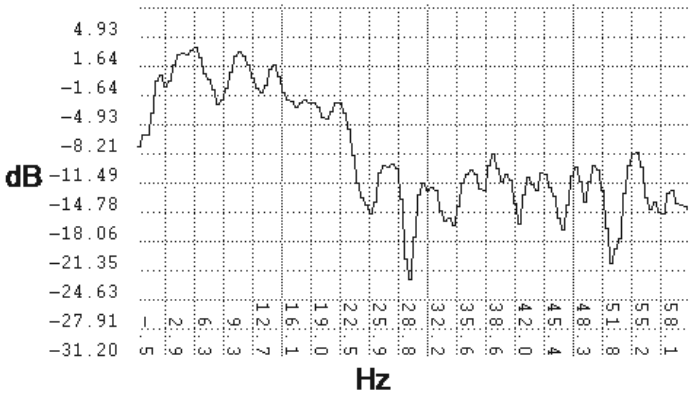

Figure 5. *Section of FFT analyses (Hanning window) where the analyzed EEG signals' stronger part corresponds with what are common frequencies. From the experiment shown in paper 2.*

The FFT analysis normally gives the relative signal strength of the different signal components in decibels (dB). In Figure 5 it is important to notice the peak at frequency ranges representing alpha and beta waves. For lower frequencies it is difficult to determine the different frequency components since parts of the spectrum probably have some origin in noise. For recording of EEG signals it is common to use a bio-amplifier with both a high and a low-pass filter which are adjustable and thereby make it possible to eliminate irrelevant frequencies and noise.

Noise is a common problem since the EEG signal is generally weak and since the bio-amplifier will amplify the whole signal including the noise. Muscle activity is often the origin of the noise in the recorded EEG signal. As shown in Figure 6, ECG is often a source of noise in the fish's EEG signals.

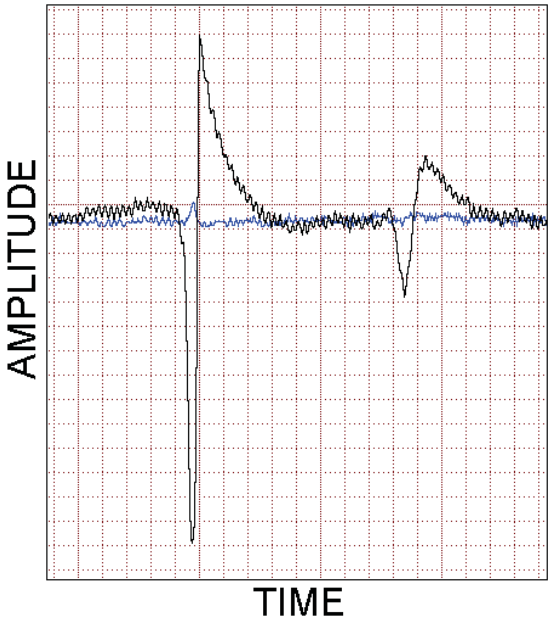

Figure 6. *The EEC signal (blue) is influenced by the ECG (grey) signals Q-deflection. From the experiment in paper II where the EEG signal was measured on cerebral cortex.*

Muscle activity from the movement of the gills is a particular noise source in measurement of brain activity since the muscles that move the gill covers are situated close to the brain, which makes the placement of the electrodes crucial.

In humans, electrical differences in the brain are normally measured through the skull by electrodes mounted on the skin. For registration of EEG in humans it is a common procedure to use a multiple electrode setup (Jasper 1958) that allows measurements of electrical activity in different parts of the brain. A multiple electrode setup it is not necessarily easy to use on animals because the brain often is smaller than in humans. A simplified electrode setup is therefore used in EEG where the animal's total brain electrical potential difference is measured. It is also possible to perform cortical EEG measurements where the electrical potentials are measured directly on the cerebral cortex, also called electrocorticography (ECoG). For registration of electrical brain activity in animals the method of measurement directly on cerebral cortex is a common procedure, especially where it is necessary to detect signals from brains of smaller sizes (Dezaa and Eidelberg 1967). The EEG signal recorded by measuring directly on the brain surface will also have difference characteristics compared to those recorded on the skull (Empson 1986). A small animal brain, such as in fish, will give a

weak signal and thereby an unfavourable signal to noise ratio. Direct measurement of the EEG signal on the cerebral cortex will obviously give a stronger signal than measurement outside the skull and thereby increase the signal to noise ratio.

Modern EEG recording are normally based on digitalization of the signal. It is therefore crucial to use an analog-to-digital (A/D) converter with sufficient resolution within the actual range of the amplified EEG signal as delivered by the bio-amplifier. To avoid drifting of the signal the bio-amplifier has a function that keeps the amplified signal centered and thereby prevents cutoff of the signal in both the amplifier and the A/D converter.

The accuracy of the FFT analyses is dependent on the quality of the digitized recordings of the EEG signal. Since a digitized signal consists of separated measurements placed in the time domain it is not only the resolution, range, and noise that define the signals quality but also the sampling rate. To recreate the EEG signal from the recorded measurements a sufficient sampling rate, called the sampling frequency (f_S), is needed. A recorded signal that is sampled with too low a frequency will possibly have a different frequency than the original signals frequency (f), which is called aliasing (McClellan *et al.* 2003). To avoid aliasing in the digitalized signal the minimum f_S is defined by the Nyquist sampling theorem (Bentley 1995), which says that the sampling frequency should be at least twice the original signal bandwidth.

The earlier described frequency range suitable for EEG measurements indicates that a relatively moderate sampling rate is sufficient to avoid aliasing in the recorded signal. Even though the EEG signals themselves have a relatively low frequency content there is a possibility of high frequency noise. With aliasing, the noise will easily end up in the same frequency range as the EEG signal and hence alters the dB strength of the different EEG frequency components. By avoiding aliasing it is possible to identify the noise from the EEG signal by a FFT analyses. Because of the possibility for aliasing it is recommended to use a f_S that is significantly higher than two times the highest component in the EEG signal of interest. Since all the papers presented in this thesis are about fish welfare related to evaluation of the slaughter process, consciousness in fish is important. Besides the frequency spectrum the EEG measurement characterization also contributes to the definitions of the consciousness in fish. In a state of unconsciousness, the EEG signals will always have an abnormal or an epileptic form (Bilgili 1999). Especially after electro stunning these abnormalities are caused by a rapid and intense depolarization of the nerve cells in the brain, which again are causing a

general epileptiform, as shown in paper II, Figure 5. These abnormal and epileptic EEG signals are defined as a tonic phase followed by a clonic phase. In the tonic phase the EEG signal increases in amplitude and goes into a decrease in frequency in the clonic phase. The abnormal EEG activity is then followed by an exhaustion phase (Lambooij, 2004) or a strong depression of electrical activity which possibly ends up in an isoelectric line. Both abnormal and lack of electric activity indicate unconsciousness.

When the animal is out of the tonic/clonic phase it is important to evaluate the level of consciousness, for instance by evaluation of event-related potentials (ERPs) caused by noxious stimuli. Since evaluation of EEG is necessary because of the possible insufficiency in visual observations, such as the eye roll method, it is important to use a relevant method for evaluation of ERPs. An auditory event exposure for evaluation of consciousness in fish is not an accurate solution because auditory evoked potentials (AEPs) are generated in the same anatomic structures as the VOR. In a situation where the fish has lost VOR the probability for occurrence of AEPs is small. Likewise, loss of eye reflexes, such as pupilla light reflex or OKR/VOR, will reduce the probability for a visually evoked potential (VEPs). The most relevant sign for evaluation of consciousness and pain during slaughtering of fish are ERPs induced by noxious stimuli. Figure 7 shows an example on a sampled EEG signal with ERPs.

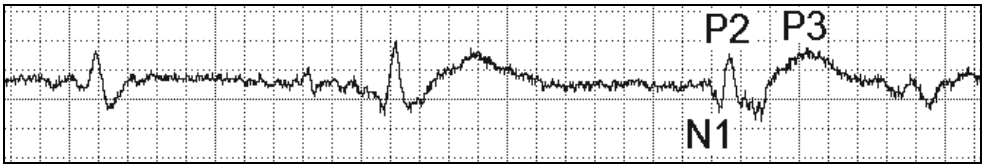

Figure 7. *Recorded EEG after electro stunning with three noxious stimuli induced event-related potentials, 160ms/div. x-axis and voltage on y-axis. The fish has an increasing tendency of consciousness during the insults and the last event-related potential has a similar shape as found in humans where the N1-P2(N100-P200) complex and P3(P300) are possible to recognize.*

The event that causes ERPs like the one shown in Figure 7 may be an expected painful stimulus, such as the one used in paper II. The different parts of the ERP are normally denoted with N or P, depending on the electrical potential, and are named with the time tag in millisecond (ms) from when the event occurred. The N1 and P2 following each other are often denoted as the N1/P2 complex. Together with the N1/P2 complex, the P3 (also called P300), is an important part of the ERP. The P3 component in the ERP signal is especially

important in evaluation of consciousness (Heinke and Koelsch 2005, Kotchoubey 2005). It is important to notice that the different ERP components have wide time intervals even though they are named with a time tag related to the origin of the event. Hence an ERP component such as P300 will still carry the same name even if the components occurrence in time are different from what the time tag indicates. Since this thesis is about fish it is the ERP signals interpretation that is important, not the exact concurrence time. The important interpretations of the EEG signal are, like the ERPs shown in Figure 7, to determine whether the fish recover from unconsciousness with increasing and normalized ERPs, or remain unconscious. If the fish remains unconscious, it is expected to observe decreased electric activity, that is little, none, or irregular ERPs, which ends up in an isoelectric line. (Blume *et al.* 2011).

1.6. Nervous system

As previously mentioned the purpose of electro stunning is to stimulate the whole brain by electric current. The concept of electro stunning uses the same principle as electronarcosis or electroshocks (EST) which are based on electrical stimulation of nerve cells and has a rather long history of use in both animals and humans (Geddes 1965, Kneeland and Warren 2002). For example, the frog experiment Galvani carried out in 1791 is one of the best known experiments of electrical stimulations of nerves (Kipnis 1987, Piccolino 1997). Electrical stimulation of nerves related to muscles is also known as functional neuromuscular stimulation (FNS) where nerve membranes depolarize and generate an action potential (Peckham 1981, Solomonow 1984a, b). A nerve cell or neuron consists of dendrites, axon and the cell body called soma. Figure 8 shows a schematic drawing of a typical nerve cell, not unlike the one described by Retzius in 1892.

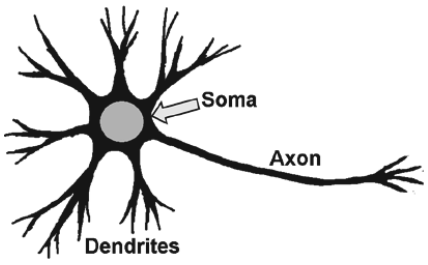

Figure 8. *Schematic drawing of a nerve cell with cell body (soma) surrounded with outgrowths, which are the dendrites and axon.*

The depolarization, causing the action potential, has its origin in the dendrites and the cell membrane of the soma. When the depolarization causes a sufficient voltage difference between the outside and the inside of the cell membrane an action potential will be generated in the axon hillock, which is located in the passage from the soma to the axon. The action potential will then propagate along the axon by depolarization and end up in the axon terminal where it will be transmitted over the synapse to the dendrites in the next neuron or to a muscle. The propagation velocity of the action potential is dependent on the myelination (Schwann cells) around the axon (Shrager 1988). In a myelinated axon the fibers only have active membranes at the nodes of Ranvier, which are exposed and influence the propagation velocity (Rattay and Aberham 1993).

Even though electrical synapses exist, the most common transmission mechanism is by chemical substances first discovered by Otto Loewi and called neurotransmitters (Friedman 1971, Zigmond 1999). These substances are released into the gap between two adjacent cells, and will then cause a depolarization of the receiving cell membrane. If the receiving membrane is a muscle cell, the depolarization will cause the muscle to contract. Chemical substances also influence the interaction and responses of the different parts of the nerve system (Hökfelt *et al.* 2000). However, electrical signals are important for normal neurological activities and are highly dependent on the ion channels in the nerve cell membrane. In a polarized state the ion channels or pumps maintain the ion balance and the electrical potential difference, which ranges from approximately -50mV to -70mV, between the inside and outside of the cell. By depolarization this changes to approximately +40mV as a peak value of the action potential (Rattay 1990). The Hodgkin–Huxley (HH) model describes the initiation and propagation of action potentials including the underlying ionic mechanisms in a cell membrane in unmyelinated squid giant axon (Hodgkin *et al*. 1952, Hodgkin and Huxley 1952a-d, Rattay 1990, Martinek *et al.* 2008). Models of the action potential propagation along the axon are possible by using a network with a basis in models for electric telegraphy, like the one developed by Lord Kelvin (Thomson 1855, Rattay 1990). By using the network model it is possible to simplify the cell membrane into circuit elements where the ion channels are described as voltage sources (Cole 1962). By using an extremely simplified model where the ion channels are eliminated it is possible to describe the cell membrane as an electric impedance network, which is relevant for electro stunning.

Dependent on the animals' acclimatization temperature, reduction in the environmental temperature may influence the nerve system. In extreme situations the nerve transmission may fail and are therefore called cold block (Boyd and Harold 1933). Not only cold block but also cold death, as described in paper I, is an optional result of an extreme temperature drop. Other factors may also influence the temperature tolerance, such as anaesthetics, oxygen and exhaustion (Fryer and Ogilvie 1973). Changes in temperature will also influence the action potentials propagation along the nerve fiber (Hodgkin 1937a, b, Rattay 1990). The alterations in the nerves electrical resistance caused by changes in temperature influence the simplified nerve cell model described in paper III (Hummon and Boyd 1935). Changes in temperature also have the potential to affect the myelination of the nerves, which will alter the actions potentials propagation (Blaurock *et al*.1985).

1.7. Electricity and electrical parameters

The well-known basis for Ohm's Law (Ohm, 1827) is the assumption of the linear electrical relationship between the potential difference measured in volt (V), electrical current (I) and electrical resistance (R), which is valid for metal but not for biological materials. By changing the potential difference applied to biological materials the electrical resistance will also change; this is valid for both alternating current (AC) and direct current (DC).

However, electrical impedance (Z) represents the electrical resistance under alternating current conditions, measured in Ohm (Ω), and is a function of both R and reactance (X). The impedance is a complex number, which is possible to describe in either rectangular or polar form, where R is a real number and X represents the imaginary part (Boylestad 2003). Like the impedance, R is also measured in Ω and this real numbers represents the electrical resistance during DC conditions. The frequency-dependent reactance can possibly be either a capacitance (C) or an inductance (L), depending on its placement on the imaginary axis, which is defined by the impedance phase angle (θ). In the International System of Units (SI unit) the inductances are normally circuit elements like coils and are measured in Henry (H), commonly expressed in milliHenry (mH). The SI unit for measurement of capacitance is farad (BIPM 2006) or microfarad (μF). The name of a circuit element with a capacitive characteristic is a capacitor and is in principle constructed as shown in Figure 9.

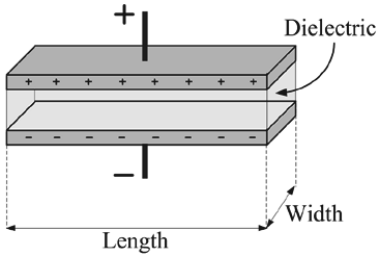

Figure 9. *Schematic drawing of a plate capacitor where the capacitance is defined by the size and distance between the electrodes, including the material (dielectric) between them.*

The measured value of capacitance depends on the distance, and permittivity (ε) of the material between the electrodes and the area of them, as defined by equation 1. For a parallel-plate capacitor the capacitance per unit area (C_A) is described as:

$$C_A = \frac{\varepsilon\varepsilon_0}{d}, \qquad \textbf{Eq. 1}$$

where $\boldsymbol{\varepsilon}$ represents the relative dielectric constant of the medium, which is temperature-dependent. The permittivity of free space is represented by ε_0 (8.85 x 10^{-12} farad) and the distance between the parallel-plates is represented by d.

The concept for definition and measurements of capacitance is also true for biological impedances (Schwan 1963, Foster and Lukaski 1996). The relevance of capacitance to the impedance cell membrane model, described in paper III, identifies the capacitive part of the cell membrane to be the dielectric shown in Figure 9. The cell membrane contribution to the capacitance will then be determined by the area and thickness of the membranes and the membrane materials electrical properties (Rattay 1990).

A capacitor will under DC conditions act as a non-conductor, provided that the applied voltages are not causing a dielectric breakdown, as described in paper III. During switching on and off of the DC current, the capacitor will act as a current source that gets loaded and unloaded at switch on and off, respectively. Especially the contact surface between the electrode and the fish has a capacitive characteristic, which is in accordance with the Helmholtz model (Ohm 1982, Brad and Faulkner, 2001). Therefore the will be a capacitance between the fish surface and electrode like shown in Figure 1, paper III. It is important to notice that in AC conditions a phase shift will occur between the voltage and current during the influence of capacitances and opposite phase shift are caused by inductances (Floyd 2001).

The principles for electrical resistance are similar to those of capacitance but are dependent on the material's resistivity (ρ). In biological materials, electrical resistance (including the capacitive part) is normally highly dependent on the voltage (Šel *et al.* 2005). According to this voltage dependency the impedance will be lower at high voltage, but still keeping a similar developing trend curve for the impedance spectrum at low and high voltages.

Conductance (G) is the inverse of the electrical resistance and the inverse of impedance is admittance (Y), which both are measured in Siemens (S). The term Siemens per meter (S/m) or millisiemens per centimeter (mS/cm) is used in definitions of a materials specific electrical resistance or conductivity (σ). During AC conditions the conductivity will decrease with increasing frequency and it is possible to make computerized models over the electrical currents distribution in the different body tissues (Nadeem *et al.* 2003, Seoane *et al.* 2007). Both conductance and admittance are given by multiplying the conductivity by the conductors' area and dividing on its length. A fish may be regarded as a conductor (Grimsbø 2007), but due to geometry and different body tissues that vary in conductivity, a calculation of its overall conductance or admittance is difficult. It is however important to realize that a fish placed in an electro stunning machine is in principal a conductor where it is possible to regard the different body or tissue parts as elements of an electrical circuit with specific electrical properties.

Electrical current is measured in ampere (A) and is used for both DC and AC. For AC conditions it is possibly to use different terms for both voltage and current measurements depending on which part of the signal characteristic of interest. The expression peak-to-peak value and peak-value describe, respectively, the voltage or current alternating signals value from peak to peak or the absolute value of the peak value. The commonly used expression for voltage and current measurements related to electro stunning is root mean square (RMS), which gives a uniform measurement independent of the signal form. By using RMS values, which represents an integral of the signal, it is easier to compare stunning signals of different types. It is however important to notice that not all measurement equipment that claim to measure RMS has the capacity to do so, especially if the measured signal is different from a 50 Hz sinusoidal. Measurement equipment that handles RMS measurements of non-sinusoidal signals with frequencies different from 50 Hz are normally described as "true RMS" and such equipment is needed for measurement of the stunning signal. Since many electro stunning

machines do not deliver a sinusoidal stunning signal but, like the Stansas machine used in paper II and IV, consists of both a DC and AC component it is important to use measurement equipment with an adequate design. It has been observed that measurement of stunning current consisting of both AC and DC represents a challenge for ordinary measurement equipment since they often are constructed for either AC or DC measurements, not for combined or coupled signals. In measurements of electro stunning signals, it is important to recognize and define the stunning signal. For instance, the commonly used expression pulsed DC (pDC) (Roth 2003, Nordgreen 2008) is actually an AC signal with a square wave form. Only when the pDC signal consists of only one pulse is it possible to measure it with the instrument in DC mode.

For comparison of signal strength the dimensionless logarithmic unit decibel (dB) is often used, with there is a defined reference level. For easily comprehension of the concept of dB it is convenient to start with the formulae used for comparison between signal power (P):

$$dB = 10\log_{10}\frac{P}{P_{ref}} \qquad \textbf{Eq.2}$$

Since the power is dependent on voltage and electrical resistance it is obvious that by using RMS values it is possibly to rearrange equation 2 into (Boylestad 2003):

$$dB = 20\log_{10}\frac{V_{RMS}}{V_{RMS_{ref}}} - 10\log_{10}\frac{Z}{Z_{ref}} \qquad \textbf{Eq.3}$$

Evaluation of signal strength related to impedance and frequency are relevant for electro stunning since the voltage level during stunning of fish is approximately constant.

It has turned out that there is a limited frequency range, approximately 50Hz to 1kHz, that is optimal for electro stunning (Roth 2003). Therefore, frequency is a key parameter for obtaining efficient brain stimulation during electro stunning. The electro stunning dependency of electric frequency is caused by its influence on the nerves at cellular level, which are also known from other scientific fields (Gilad 2007). It turns out that, dependent on the nerve diameter, higher frequencies have a blocking effect on the action potentials (Solomonow 1984a, Rattay 1990). It is also shown that lower frequencies of about 100 Hz, as described in paper III, give the optimum stunning effect (Roth 2003). A correlation between increasing effect of electro stunning and the electrical stimulation frequency is in accordance with

general nerve stimulation where the effect increases up to a certain level (Solomonow 1984a, b, Rattay 1990). The relationship between effect of electro stunning and electrical nerve stimulation has been a source for quality trouble in the fishery industry since a stunning signal that gives an efficient stun also gives heavy muscle contractions thereby causing quality problems such asbroken backbone and blood spots in the filet (Robb *et al.* 2003). Optimization of electro stunning equipment will therefore often be a compromise between the effect of electro stunning and quality issues. However, it is crucial to identify the stunning frequency spectrum as described in paper IV.

Amplitude versus time is a common graphical presentation of a measured electrical signal, which is a presentation of the signal in the time domain. A graphical presentation of an electrical signal in the time domain gives a clear description of the signal form but not the frequency. It is however possibly to calculate the frequency of a clean and simple signal, like those of a sinusoidal form, by counting the cycles pr. second but that is hardly an option if the signal consists of a frequency spectrum as in Figure 10.

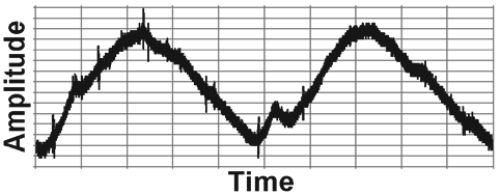

Figure 10. *Time-domain plot AC part of stunning signal from a Stansas stunning machine. The signal consists of different frequencies including spikes, which also contributes to the frequency spectrum.*

By using the FFT it is possible to transform the measured signal from the time-domain to the frequency-domain (Bergland 1969). The FFT is a computerized algorithm to easily calculate the discrete Fourier transform (DFT) of the non-continuous digitalized sampled signal that the measurements represent. By using the FFT it is possible to get the frequency spectrum of the sampled signal.

As part of the FFT analysis it is common to use an additional algorithm called “time-domain window” or just “window”. This window algorithm works as a window into the sampled measurements where the FFT is used and is crucial for the accuracy, often described as frequency leakage (Schoukens *et al*. 1991, Zhang *et al*. 2001). It is therefore important to note

that not all measurement equipment with a built in option for frequency spectrum analysis and does not give a frequency spectrum of sufficient quality, especially with irregular or noisy signals. There are several different algorithms used as time-domain windows, which have different characteristics and influences on the calculated frequency spectrum (Harris 1978). However, the Hann window algorithm is commonly used and is also the default option in the FFT software package delivered with the programming language used in paper IV. By using a filter arrangement of capacitors and eventually coils, as in Figure 1 in paper IV, it is possible to alter a frequency spectrum's characteristic, as shown in Figures 2 and 3 in paper IV.

2. Objectives

The aims of this thesis and the published papers I –IV were to increase the knowledge about fish welfare during industrial slaughtering, as well as to quantify welfare parameters by different measurement methods. The specific aims were to:

- Investigate the maximum acceptable temperature drop for salmon before slaughtering without compromising fish welfare, by measuring the physiological responses of live chilling
- Evaluate and verify the effects of percussive and electrical stunning on the fish's consciousness by measuring its EEG and ECG responses
- Verify the optimal AC frequency for electrical stunning by measuring electrical impedance
- Increase the understanding of the impact of electrical frequency on quality issues such as broken backbones and hemorrhages.
- Quantify the welfare related effects of chilling as a method for preventing recovery during bleed out of slaughtered fish

3. Results and discussion

The experiments described in paper I – IV investigate fish welfare during commercial slaughtering of salmon. In general, the results in paper I are relevant for live chilling as often used before stunning and slaughtering. The results in paper II-IV are about the stunning procedures, where both paper II and IV include the recovery of consciousness in fish.

3.1. Sedation by temperature drops

One goal of live chilling prior to slaughtering is to calm down (sedate) the fish before stunning and bleeding. Perhaps the most important effect for slaughterhouses practicing live chilling is to achieve better quality, provided by less activity, and less impact damage, lower lactate production, smaller pH drop, longer durability, less fillet gaping, and to gain more time to fillet the fish before *rigor mortis* (Skjervold *et al.* 2002). However, cold shock or rapid temperature drops close to the fish tolerance limits can trigger strong stress responses and mortality (Donaldson *et al.* 2008). Therefore, the results from the experiments with live cooling of salmon in paper I are important and show that transfer from high to low temperature does not increase the level of stress *per se* in relation to crowding and handling stress itself, unless the temperature jump is too large or the end temperature is too low.

The experiments described by paper I show the physiological responses in fish to temperature drop, but do not clarify the state of consciousness during cooling. However, the results from paper IV show the importance of low temperature for prevention of recovery from an unconscious state caused by electro stunning. Since it is considered unclear whether arctic species lose consciousness during live chilling, the findings in paper IV are important and prove the ability of low temperature to maintain fish unconscious. The effect of cooling shown in paper IV is beyond what is possible to explain by cold stiffening of the musculature. The effect of the ice-slurry proves that the temperature drop clearly has an effect on the state of consciousness, which is also reasonable as related to the temperatures' general influence on conduction of nerve signals (Boyd and Harold 1933, Rattay 1990).

In the process of chilling prior to slaughtering the cooling represented by the gills and blood flow gives a rapid decrease in temperature and an increase in quality and possibly welfare (Skjervold *et al.* 2001, 1999). However, Skjervold *et al.* (2002) also described that the heat flux over the fish surface is a main contributor to the heat transfer from fish to water.

It is however interesting to notice that the temperature model for fish, developed by Kitagawa and Kimura (2006), describe three factors that determine the temperature as a function of time, that is, exchange with the ambient water temperature, blood flow, and metabolic heat production. Therefore it seems adequate to regard the fish as a control system as shown in Figure 11.

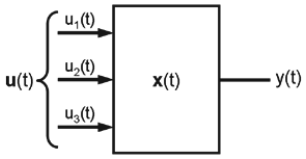

Figure 11. *Simplified schematically drawing of a control system representing the processes which control the fish temperature. In a control system there will be a control matrix* ***u(t)****, a transfer function* ***x(t)*** *and y(t) will then represent the output variable (Balchen et al. 2004).*

The heat flux over the fish surface is represented by control variable $u_1(t)$ (Figure 11). The control variable $u_2(t)$ represents the heat transfer over the gills and internal heat transport caused by the blood flow. Finally, the fish metabolism is represented by control variable $u_3(t)$, but has only theoretical interest since the salmon is a poikilotherm.

The control system model is also useful in a fish welfare perspective since it clearly shows the input variables which determine the fish's temperature. Under normal slaughtering procedures the fish will be rendered unconscious before the gill cut is be done, which rules out $u_2(t)$. After the gill cut, $u_1(t)$ will represent the dominant factor in **u(t)** when the fish ends up in the cold water in the bleed out tanks. In the cooling experiment in paper IV no gill cut was performed, which represents an eventual worst case scenario when a failure has occurred during the slaughtering and the fish will not bleed out before processing starts. The lack of bleed out will, on the other hand cause an efficient cooling, since both $u_1(t)$ and $u_2(t)$ are intact and gives an efficient contribution to **u(t)**, and ensures, as shown in paper IV, that the fish do not end up being processed in a conscious state.

The results from paper I are important for fish welfare during slaughtering, since live chilling of the fish prior to stunning is a common procedure in the fishery industry. In a situation where the fish are pre-chilled before slaughtering both $u_1(t)$ and $u_2(t)$ will contribute to the change in the fish body temperature. The results from paper I show that the temperature slope, which is the result of **u(t)**, is an important parameter causing the physiological responses in the fish. The results in Figure 1, paper I, shows that it is not necessarily the change in temperature that causes a fatal stress situation for the fish but the temperature change versus time. For both temperature drops from 16 to 0°C over five hours and from 16 to 4°C over one hour the measured physiological stress responses were acceptable. Since the temperature flux over the surface is the main contributor to the temperature change (Skjervold *et al*. 2002), a small fish will have a steeper temperature slope than a bigger one. The fact that the fish used in the experiment, described in paper I, were salmon of smaller than average size at slaughtering ensured that the actual body temperature drop more quickly that fish with similar temperature changes at a commercial fishery slaughterhouse. However, the results in Table 1 in paper I show that physical strain overrides the potential stress caused by temperature drops.

3.2. Electro and percussive stunning

The aim of the stunning procedure is to render the animal unconscious before slaughtering. The concept for percussive stunning investigated in paper II is a piston, driven by compressed air, that hits the fish head as described in Figure 1, paper II. The results from the percussive stunning experiment show the importance of using sufficient air pressure. In the part of the experiment where lower air pressure was applied, the fish where not exposed to a sufficient percussive stunning. The use of higher pressure ensured the quality of the perceived stun, but gave unacceptable damages to the visual impression of food quality. Good animal welfare during slaughtering will give a high quality score for the final product and it is therefore interesting to observe that a failure in the visual impression of food quality can be regarded as a quality score for animal welfare during percussive stunning, but which is not acceptable for the market.

Different aspects of electro stunning are investigated in paper II-IV, where different power sources and voltage levels were used. The description of the electrical stunning signal, presented in paper I, is important for the understanding of the signal characteristics which

include both an AC and a DC component. The characterization of the stunning signal in paper II contributed, together with the results from other unpublished experiments , to the premises for the experiment described in paper IV.

3.2.1. ECG

After both percussive and electro stunning fibrillation were observed in the ECG signal post stun, but not for all of the fish. For both stunning procedures the heart rate showed a decreasing trend versus time post stun. It is however interesting to observe the difference in heart rate post stun for the two different stunning methods, that is, percussive and electro stunning (paper II). For percussive stunning the heart rate seems to be higher post stun than for electro stunning, even though the heart rate before stunning was lower for percussive stunning. The difference in heart rate post stun was probably caused by the fact that head to body electrical stunning not only exposes the brain to electrical current but also the pacemaker tissue in the hart and the nervous system. The percussive stunning will only influence the brain and render the heart muscle and its pacemaker tissue unharmed and a possible release of catecholamine increases the heartbeat. However, a cardiac arrest will terminate the oxygen supply to the brain but also prevent efficient bleed out after gill cut.

3.2.2. EEG

The observation of VOR absence but still simultaneous EEG response to noxious stimuli in paper II clearly proves the importance of EEG for verification of consciousness. The VOR procedure for determination of consciousness in paper IV is however regarded as sufficient when the experimental setup earlier has been verified by EEG in paper II.

The measured EEG and response to noxious stimuli reveals clearly the different effects between high and low air pressure used in the percussive stunning procedure. The measured EEG results from paper II proves that a 107V stunning signal delivered by the Stansas 01# machine gives a sufficient stunning result, which again gives a decreased power consumption compared to higher voltages. However, the evaluation of stunning duration found

approximately 0.5 sec. to be sufficient. This is an important result from paper II, even though there is a need for a correct bleeding procedure.

3.2.3. Impedance versus effect of electro stunning

The results in paper III focus on electrical impedance measurements in fish and their impact on the optimal frequency for electro stunning. It is important to note the relatively flat area of the impedance curve with a top in the average value at 100Hz (Figure 2 in paper III), which corresponds to frequencies recommended for use in electro stunning (see references in paper III).

Different tissue types in the fish have different electrical properties, which make it difficult to calculate the electrical currents flowing through the fish in general and through the specific parts of the head and the brain. The measurements done in paper III give an impedance spectrum which represents the total impedance of tissue and stunning system. By doing an extreme simplification of the fish electrical properties it is possible to describe the whole system as in Figure 12.

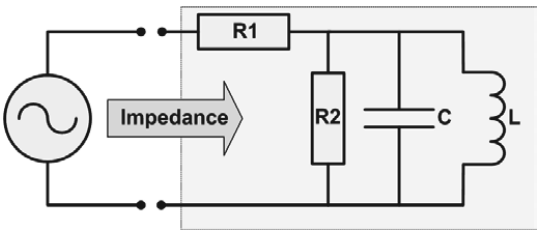

Figure 12. *Principle drawing of an electrical stunning system. The fish, including the stunning machine, simplified into two electrical resistors, R1 and R2, one capacitor C and an inductance L.*

Paper III describes an increasing tendency of the electrical impedance in the fish from low (40Hz) frequency towards higher frequencies (100Hz). An increase in electrical impedance with increasing frequency will normally be described as an inductance, like L in the simplified system in Figure 12. However, the phase shift shown in paper III does not indicate any inductance in the system. The sources of the inductance are, according to Cole (1962), expected to be the ion channels in the cell membranes. The capacitance (C in Figure 12) represents the origin of the decrease in impedance from 100Hz towards higher frequencies

(paper III) which is common for biological tissue. Figure 12 also includes two resistors, R1 and R2 placed in series and parallel with C and L, which represent the linear electrical resistance.

Since the action potential in a nerve cell is trigged by the voltage difference over the cell membrane, it is assumed that the maximum electrical resistance of the system will give the most efficient stun. According to the results shown in paper III the optimal frequency for electro stunning is within the relatively flat top area of the impedance spectra, with the optimum at 100Hz. It is also interesting to observe that the fundamental frequency used in paper II is within the frequency range where the relatively flat area in the impedance spectrum occurs, as shown in paper III.

To regard the fish and the stunning apparatus as one electrical system is a new approach to electrical stunning. This new approach gives a better understanding and an opportunity to optimize the electro stunning procedure in a different way than previously.

3.2.4. Effect of frequency spectrum

In addition to the previously mentioned effects that cooling has on awakening, the results in paper IV contribute to a clarification of cause and effect relationship between damages related to electro stunning.

When the commercial Stansas electro stunning machine entered the marked it quickly received a reputation for giving less damage than other electro stunning apparatus. The assumption was that the combination of AC and DC electrical current used in the Stansas machine prevented injuries. Therefore, a comprehensive unpublished experiment was performed where the aim was to clarify the optimal combination between AC and DC electrical current for electro stunning. In this experiment different settings with different combinations between AC and DC were tested by using a Stansas stunning machine, but none of the settings gave a significant increase in damages. Since there was no increase in damages, regardless of the different AC/DC combinations used, it was suspected that the stunning signal from the Stansas machine contained an additional electrical component that prevented damages.

An attempt to clarify the content of the electrical stunning signal from the Stansas machine was performed by using high frequency sampling of the signal and analysis of its frequency spectrum, using Fast Fourier transformation which showed that the signal contained a high frequency component that possibly prevented damages.

The concept of using high frequency signals for blocking the action potential (Figure 13) is earlier described by both using simulations and practical experiments (Rattay 1990).

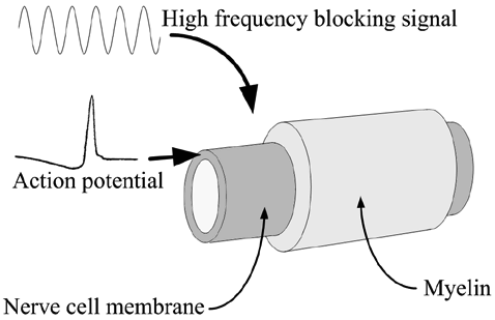

Figure 13. *Principle drawing of a myelinated nerve where high frequencies signal is used for blocking the action potential.*

An action potential in a nerve will propagate along the call membrane of the nerve and finally trigger a muscle contraction. By using a high frequency signal it is possible to block the propagation of the action potential (Solomonow 1984a, b). The myelin works as insulation of the nerve, but will also increase the capacitance between the extracellular and intracellular fluids. The minimum frequency for blocking the action potential depend on the capacitance between intracellular and extracellular fluids. The capacitance will again depend on the diameter of the nerve, that is, the area of the surface and the electrical property of the cell membrane (Rattay 1990). In fish the nerves are more myelinated than in mammals and will influence the propagation of the action potentials, including the minimum blocking frequency.

Based on this knowledge a rather simple experiment, described in paper IV, was carried out to verify if it was the higher frequency component in the electro stunning signal from the Stansas machine that prevented damage. In this experiment a filter was used to remove the high frequency part in the signal from the Stansas machine. It was then possible to compare fillet damages between fish stunned with and without high frequency components in the electrical

stunning signal and thereby verify the effect. As described in paper IV fillet damages occurred when the high frequency part of the signal were removed.

However, it also turned out that the filtered signal gave longer recovery time and higher mortality rate than the unfiltered signal. It is possible that the filtered signal caused permanent damages on the fish nerve system compared to the signal that contained a high frequency component. This finding might be relevant even within human medicine where electroshock or ECT is used since many of the negative side effects (Weiner 1984, Dybedal *et al.* 2014) are similar to those related to electro stunning of fish. It is possible that an electrical signal with a high frequency spectrum will reduce the negative side effects in ECT.

4. Conclusions

The findings in paper I are particularly important for fish welfare prior to slaughtering and shows that salmon are capable of tolerating relatively steep temperature drops. The findings are relevant both for live chilling under transportation and prior to slaughtering. Fish welfare is not affect by short duration exposures to temperatures drops from 16° to 4°C over 1 hour or from 16° to 0°C over 5 hours. Paper I shows that negative effects on blood stress parameters caused by physical stress from handling overrides the effect of thermal insults.

It is important to note that in the experiment described in paper II the fish has no response on VOR but still has detectable brain activity in the measured EEG signal, which means that the animal is possibly only paralyzed. For verification of consciousness it is therefore necessary to use EEG to determine the fish's brain activity. For percussive stunning the conclusion is that if sufficient air pressure (8.1 to 10 bar) is used to operate the percussive stunner the energy in the impact will render the fish unconscious and insensible and eventually die of cerebral hemorrhage.

The conclusion for electrical stunning is that a combined AC and DC supply for dry electrical head to body stunning for a minimum of 0.5 sececonds using a current of 668 mA_{rms} and voltage of approximately 107 V_{rms} will lead to all fish being stunned unconscious. Upon exsanguination the fish did not die unconsciously after a 5 seccond electro stun pulse and

methods to prolong the unconscious conditions until death occurs should be sought or alternatively percussive stunning used, as is commercially practiced.

Paper III shows the importance of electrical impedances in electro stunning and is an interesting tool to find the optimal stunning frequency. The results from paper III demonstrate the importance of using AC frequencies in the range below that including apparent α-dispersions, which in turn cause high electrical impedance during electro stunning of fish. This ensures efficient release of action potentials in the central nervous system, which is essential for rendering the fish unconscious. The measured electrical impedance indicated that the frequency range from 50Hz to approximately 1kHz is acceptable for electro stunning. The highest impedance measurements with low variance are found in the range from 60 to 800Hz, which cause electrical signals within the range well suited for electro stunning. However, in order to generate an electrical current flow giving sufficient stimulation to the brain of the Atlantic salmon, it is concluded that the optimal frequency range for electro stunning of Atlantic salmon is 70-100 Hz, with an average peak optimum at 100 Hz.

For the work presented in paper IV the conclusion is that a high-frequency spectrum at low dB reduces the risk for spinal injuries, while the fundamental frequency of the signal is within a frequency range where stunning is efficient. The proportion of fish that fully recovered or died was significantly influenced by frequency spectrum and temperature. A thermal shock post stunning can ensure that the fish remains unconscious until killed.

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6. Papers I - IV