Masterthesis

Stress and flesh quality in char-fish (*Salvelinus alpinus x S. fontinalis*, Linneaus 1758 & Mitchill 1814) using percussive and electrical stunning methods, combined with blue light and water temperature reduction

by

Katharina Fraunbaum (1140720)

Master programme: Applied Limnology (H066448)

Institute of Hydrobiology and Aquatic Ecosystem Management (IHG)

University of Natural Resources and Life Sciences, Vienna

Supervised by

Ao.Univ.Prof. Dr.phil. Herwig Waidbacher

Dipl.-Ing. Silke-Silvia Drexler

Dipl.- Ing. Dr. techn. Roza Allabashi

Table of content

Acknowledgement

Firstly, I want to thank my father who is the main person responsible for the creation of this study to research social problems, and for constantly claiming that a member of the family Fraunbaum can do anything. Although this statement has threatened many emotional near-explosions on my part, we managed it, which makes me quite proud.

Second biggest thanks clearly belong to Silke Drexler with her enthusiastic nature always providing a motivation to continue and to just do it. Thank you for your professional and mental support.

Wiggerl is a human like a rock who gives you the last essential input and is available to help at any time, whenever it is necessary. Great freedom was given to me for my first own project which let my character grow. Thank you.

Roza Allabashi and Eva-Maria Mattausch are clear protagonists in this master thesis, since they developed the cortisol method years ago and adapted it for me to analyse blood and small sample sizes. Thank you for volunteering your valuable time.

Erwin was the magical hand, explaining the complicated statistic methods, guiding me through the analyses and creating hypothesis. Now I am not afraid to open the software SPSS anymore. Big thanks for always be here although you are in North Germany or I am in Norway.

I also want to thank Gerhard Schleining for his expertise and the use of the texture analyser for free.

During the long period of realisation of this Master thesis, family and friends supported me in the whole process, right from the start, finding a financing until the Defensio. Sometimes they would strengthen my self-confidence or remind me that they love me, even without a master's degree. This created a much more productive working environment because pressure was replaced by fun and curiosity. Big thanks to my steady life companions: Mum, my grandparents and Theresa Mayringer, and to the ones who entered into my life more recently: Laura Klüber and Sarah Unterkofler.

Last but not least I want to thank my native speakers for the proofreading of the whole thesis: Chad Meinel and Anja Hummer.

I still want to mention that I explained quite often that I think my father would switch his daughter during this project, especially on the day he wondered about finding himself sitting in his fish farm with 10 µL pipettes and collecting blood for my thesis.

Abstract

In former times the most farmed salmonids in Austria were different species of trout, a shift to char is evident due to economic and quality issues. This study investigates parameters of welfare during slaughter in the crossbreeding "Alsatian" *Salvelinus alpinus x S. fontinalis (*LINNAEUS 1758, MITCHILL 1814*)*. Stunning methods are the percussive method, and the wet electrical method, since they are the only legal procedures to use in Austria. Blue light and water temperature reduction are investigated as parameters influencing fish stress and welfare. Blood plasma cortisol and relative Elasticity (rE) are identified to be indicator parameters for this investigation and are analysed in 88 samples (fish) from one experiment. The blood plasma cortisol is determined using liquid chromatography-mass spectrometry (LC-MS/MS). rE is determined using a texture analyzer TA.XT.plus (Stable Micro Systems) using the relaxation method. The results show lower cortisol levels in electrical stunning compared to percussive stunning. Blue light decreases the cortisol level and shows a positive effect on the rE. The water temperature reduction shows a clear increasing effect to the stress level and a negative impact to the rE. A general correlation of both parameters is not found. Different correlations are found separated for stunning methods. The parameter cortisol is affected by the stunning methods. In case of the rE the tests don't yield convincing results either way, it is possible that a different parameter for flesh quality could show a clearer result.

1 Introduction

The aquaculture sector is one of the fastest expanding agricultural industries in the world. Resource availability and use have allowed a more than three times faster sector growth compared with terrestrial farm animal meat production (FAO, 2017). As clearly proven in many terrestrial meat animals, good welfare means good production. The end of animal life, i.e. the pre-slaughter procedures management and slaughter methods can heavily influence the expression of quality and the correlating changes during storage of the final product (Poli et al. 2005). In the last century there has been discussion about a fishes ability to feel pain and react on stress. Around fifty years ago research demonstrated that fish have sense organs able to detect painful stimuli, sensory pathways for processing such stimuli and brain mechanisms that process this information and generate behavioral responses (Wendelaar Bonga, 1997). Fish stunning and killing represent a highly stressful moment which can cause a strong impact on flesh quality (Errikson, 1999). Stress leads to production of cortisol and reduction of glycogen in the muscles resulting in an earlier rigor mortis onset leading to poor quality meat (Stamer, 2009). Accordingly, there is also an economic interest to treat fish in a good welfare for the purpose of a higher quality product. It is human's responsibility to look on animal welfare simply because of respect to our environment. Reality is that most fish produced worldwide are killed with little or no consideration of their welfare (Lines & Spence, 2014). Science shows attention but the realization states a challenge mainly because killing takes time and time is money. Luckily in the EU there are restrictions, which regulate this issue. Fish must get stunned and killed due to the law. Laws are an important tool but are often disregarded because of lack of enforcement. It is important to create awareness and to create the possibility to improve and develop. Nowadays there are plenty of researchers working on fish and their stress behavior. Fish feel pain and stress but there is a huge gap in handling in practice. In Austria we have strict laws and a perfect environmental situation to farm fish. So, it is quite sad that only 6% of the consumed fish comes from Austria (Statistik Austria, 2016). Instead of importing such a big amount of fish products we should focus on our national fish production for environmental, economic, health and quality reasons. Due to the excellent water quality in Austria there is the possibility to farm salmonids. The rainbow trout is the most frequently farmed fish with 2000t production per year in Austria (BMLFUW, 2017). In recent years there is a shift from trout to char. The genus char and trout show similar requirements to water quality. The reason for this shift to char is due to economic issues (Bayerische Landesanstalt für Landwirtschaft, 2016). The producer gains 30% more profit because of a higher market price for the char. The production of fingerlings is riskier. Consequently, fingerlings of char are also more expensive but it is still worth it to switch to chars. This observation was implemented three years ago in my own fish farm. Consequently, the most interesting thing for me is the reaction of chars to different stunning methods. Due to the national change and the low amount of scientific work, it is useful to collect data, because the shift is happening. We live in a time where people are willing to pay for a high-quality product and chars may show a higher flesh quality which is more tasteful

than the one of trout. The decrease of stress in fish is maybe not the main topic today, but it will become one. In Austria, stunning methods which are allowed are percussive and electrical. Both methods show a low impact on fish (Poli et al. 2005; Robb and Kestin 2002). There is no information available which stunning method is the best choice. Consequently, I wondered how I should stun the fish on my fish farm and this was the starting point for this work. The first goal was to find the better option: electrical or percussive. As I started my research, the topic got extended by further stress reduction parameters: blue light and water temperature reduction.

Keywords: fish, salvelinus, char, "Alsatian", slaughter, stunning method, flesh quality, stress reduction, temperature, blue light, water temperature reduction, cortisol, flesh texture

2 Problem statement

In Austria percussive and electrical stunning are allowed by law (§10 Tierschutz-Schlachtverordnung). Both stunning methods show a low impact on fish welfare as well as on flesh quality. The producers in Austria want to stun fish in the best possible way, to increase fish welfare and quality. Therefore, research on both stunning methods and correlated stress behavior and flesh quality parameters is necessary. Moreover, two possible stress reduction parameters will be analyzed, water temperature reduction and blue light environment. Percussive and electrical stunning combined with blue light and water temperature reduction should decrease stress in fish. The assessment of the flesh quality and stress level is done by measuring the cortisol level of blood plasma and the fillet texture.

3 Objective and Hypotheses

The central subject-theoretical question is:

Do the anesthetic methods influence the stress level and thus influence the flesh quality?

This central question can be split into three scientific-empirical questions (F_j).

F_1. Do the eight STUNNING METHODS (effect variable / treatment) influence the STRESS LEVEL K (cortisol [ng / mL])?

F_2. Do the eight STUNNING METHODS (effect variable / treatment) influence the FLESH QUALITY (Relative elasticity RE = F3 / F1) * 100 [%])?

F_3. Is there an effect of STRESS LEVEL K (Cortisol [ng / mL]) on FLESH QUALITY (Relative elasticity RE = F3 / F1) * 100 [%])?

For every scientific-empirical question (Fj) one or more empirical hypotheses are formed, which are listed in detail in chapter 6. Results.

List of variables characterizing the stunning method. Table 1 shows all settings.

P: "Stun the fish by a blow on the brain"

E: "Stun the fish by an electric current in the water"

B: "Blue light as a factor for reducing stress"

C: "Reduction of water temperature for reducing stress"

Table 1: Characteristics of the eight stunning methods

Methods - Character	*Symbolism*
Electrical	*{ E }*
Electrical & Blue	*{ E & B }*
Electrical & Cooling	*{ E & C }*
Electrical & Cooling & Blue	*{ E & C & B }*
Percussive	*{ P }*
Percussive & Blue	*{ P & B }*
Percussive & Cooling	*{ P & C }*
Percussive & Cooling & Blue	*{ P & C & B }*

4 Abbrevations

Table 2: An overview of the used abbreviations during this Masterthesis

ACN	Acetonitril
AM	Arithmetic Mean
ESI	Elektrospray-Interface
ETA^2	Degree of effect in ANOVA
K	Cortisol (stress level)
LC-MS/MS	Liquid chromatography mass spectrometry / mass spectrometry
rE	Relative elasticity
Rpm	Rounds per minute
R^2	Determination coefficient
SIG	Institute of Sanitary Engineering and Water Pollution Control
SPE	Solid Phase Extraction

5 Literature review

5.1 Char

5.1.1 General Information

Chars are a genus out of the family of the salmonids, showing the typical adipose fin. Salmonids general need low water temperatures and a high oxygen saturation. The family of whitefish *Corregonidae* and the family of grayling *Thymallidae* show the typical adipose fin but are handled as extra families. Consequently there are four autochthonous species of salmonids in Austria: The Brown trout *Salmo trutta fario* (LINNAEUS 1758), the Danube salmon *Hucho hucho* (LINNAEUS 1758), the Arctic char *Salvelinus alpinus* (LINNAEUS 1758) and the Lake trout *Salmo trutta* (LINNAEUS 1758). Each of them shows phenotypes which are typical place specific forms adapted to the local nature. Around 1880 two more species have been introduced from North America: The Rainbow trout *Oncorhynchus mykiss* (WALBAUM 1792) and the Brook char *Salvelinus fontinalis* (MITCHILL 1814). Although they become introduced anthropologically they are handled nowadays as natives in Austria due to their introduction year (Leitfaden zur Fischkunde und Angelfischerei, 2012, page 58).

Arctic char *Salvelinus alpinus* (Linnaeus, 1758)

The species arctic char is a relict from the glacial period. Consequently, it is a stenotherm, stagnophilic species which needs a high oxygen saturation in the water. The natural habitats of the arctic char are alpine lakes in an altitude of 1000m – 2500m with cold temperatures, high oxygen saturation and a low offer on nutrients. In the different lakes endemic species become adapted due to the spatial isolation. Their spawning habitats are in a depth of 2 – 80m on gravel (Leitfaden zur Fischkunde und Angelfischerei, 2012, page 31 – 32, 117). The body shows a lot of small yellow dots (Figure 1). The back is grey, green to brown. The dorsal is yellow to red. Especially during the spawning season the dorsal is red sometimes also white (Figure 1). The body shape is long and slim and shows a torpedo form. The jaw is terminal and reaches the eye. Upper and lower jaw show a lot of small teeth. The adults show a high variation in size from 10 to 70cm due to their habitat.

Figure 1: Salvelinus alpinus, male (above) and female (below) Source: https://www.lfl.bayern.de/ifi/forellenteichwirtschaft/030013/index.php; 31.10.2018

Brook char *Salvelinus fontinalis* (Mitchill, 1814)

The species Brook char is a cold-stenotherm, rheophilic species which needs a high oxygen saturation. The natural habitats of the Brook char are rivers, showing cold temperatures, high oxygen saturation and a medium offer of nutrients. The Brook char is not as dependent on "hiding structures" as the brown trout. Their spawning habitats are on gravel in rivers. The body shows a high quantity of yellow dots, and in between there are red dots with a blue border. The basal color is brown to dark olive green. The dots change on the back and at the vertical fin to marbling (Figure 2). At the dorsal site they show typical yellowish or reddish colors. Another characteristic are their dorsal fins with the black line. The body shape is like a torpedo. The chaw is terminal, at the inside dark and reaches behind the eyes which is untypical for salmonids and thereby an excellent identification property. Upper and lower chaw have a lot of small teeth. The average adult size range is 35 – 55cm. An adult Brook char can reach an age of eight to twelve years (Leitfaden zur Fischkunde und Angelfischerei, 2012, page 97).

Figure 2: *Salvelinus fontinalis,* male (above) and female (below)https://www.Bayerische Landesanstalt für Landwirtschaft.bayern.de/ifi/forellenteichwirtschaft/030013/index.php; 31.10.2018

"Alsation" *Salvelinus alpinus x S. fontinalis*

In fish farming hybrids of the Brook charr and Arctic charr are preferred to realize the benefits of both species. Experiments at the institute of fishery in Bavaria compare reciprocal hybrids and pure cultures of chars. Arctic chars show a higher hatchability but lower survival rates in sizes of 0,1 – 1,0g weight mainly caused by cannibalism. Furunculosis occurs more often in arctic char farming too (Furunculosis is a bacterial disease caused by the pathogenic agent Aeromonas salmonicida). Arctic char shows better results in slaughter characteristics in the filet quantity and -quality. The Brook char shows better performance in growth and in food-growth ratio but shows an early sexual maturity resulting in a lower quality of flesh, especially during the spawning season from September to December. In general, pure Brook chars show a better growth performance and pure arctic chars show better slaughter

characteristics. Creating hybrids was successful with the Alsatian char (Salvelinus alpinus x fontinalis). One more char hybrid is common for edible fish, a hybrid of Brook char and brown trout called “tiger trout” (Salmo trutta fario x Salvelinus fontinalis), which is sterile (Bayerische Landesanstalt für Landwirtschaft, 2016).

5.1.2 Fish farming

In the year 1765 the reproduction of fish eggs and a successful fertilisation succeeded for the first time. Almost eighty years later, in 1842 this possibility was of a commercial interest. In Austria the first fish farm for Salmonidae was built in 1854. These days one of the biggest issues was the availability of the food. The production of dry feed highly increased after the wars. Nowadays the current angling situation is not imaginable without farming and stocking (Leitfaden zur Fischkunde und Angelfischerei, 2012, page 86).

Char farming

The genus char shows an extraordinary diversity of species. Chars are a delicious edible fish. The farming is complicated due to their high needs on the ecology. The interest in char farming increased in the last decades and mainly in recent years. Trout and char show almost the same needs in a fish farm, but the mortality of the fingerlings is higher in chars (Bayerische Landesanstalt für Landwirtschaft, 2016; Leitfaden zur Fischkunde und Angelfischerei, oÖ. Landesfischereiverband, 2012). Also, the growing process takes longer, resulting in a higher market price of a finished edible char. Chars have around a 20% - 30% higher market price than trouts (Bayerische Landesanstalt für Landwirtschaft, 2016).

5.2 Stunning methods

The fish harvesting process is a very traumatic time for fish, because of the struggling and crowding that occur during capture (Poli et al., 2005). The consciousness of animal welfare is increasing. Past research showed that slaughter methods, which cause little stress for animals influence the product quality positively (Erikson et al., 1999). Different kinds of stress parameters became recognized as well as applied in past publications (Poli et al., 2005).

A few decades ago there existed controversy about pain stimuli in fish but nowadays it is clearly proven that fish recognize pain and react with stress (Wendelaar Bonga, 1997).

Worldwide there exist many different slaughter methods and many of them do not consider fish welfare at all. Table 3 shows an overview of frequently used global methods. Each slaughter method shows negative effects on fish and on flesh quality. Methods, which cause lower stress show a smaller influence. Because of this, and because of respect to animals, these methods should be used. Table 3 shows that percussive and electrical stunning are less stressful for fish compared to other methods. The lowest effect is shown during stunning with the use of an anaesthetic. Unfortunately there is no product available which is allowed for use on edible fish (Stamer, 2009; Concollato et al., 2016; Poli et al., 2005). Therefore, percussive and electrical are only allowed in Austrian law (§10 Tierschutz-Schlachtverordnung, 2015). This work focuses on these two methods. Also these methods can have a poor welfare outcome if insufficient consideration is given to the needs of the fish or if the equipment is not properly designed (J. A. Lines and Spence 2014). In Austria it is obligatory to stun a fish before killing. Therefore, a fish gets stunned and immediately afterwards killed by bleeding out. The goal of stunning is the loss of perception and feelings, and as a result there should be no reaction to external stimulus. This can be proven by the eye-moving-reflex or the breathing-reflex. Stunned fish can still show movement of muscles or fins.

Table 3: Overview of worldwide applied stunning/slaughter methods and flesh quality in fish (Robb and Kestin, 2002)

Slaughter/stunning method	Effect on fish	Effect on flesh quality
Asphyxiation	Strong negative	Strong negative
Thermal shock	Negative	Little
Bleeding out	Very strong negative	Strong negative
Ammoniac bath	Very strong negative	Strong negative
CO_2 narcosis	Strong negative	Strong negative
Use of anesthetic	Really Little	Really Little
Percussive	Really little	Little
Electrical	Really little	Little

Electrical stunning

The basis of electrical stunning is a current field in the water. A cathode and an anode are in the water which create a field where the electrodes flow. There are two different ways of stunning fish with an electrical field. During (1) electrofishing there are low amperages used which make fish motionless, but they still do feel pain. The anode is the dipnet and the cathode is a cable coming from the backpack-aggregation.

For (2) stunning before killing the fish the amperages are higher, and these cause a dysfunction of higher nerves. Consequently, the fish immediately lose their sensitivity to their environment. There can be used alternating current (AC 50 Hz) or direct current (DC). Amperages used for Rainbow trout are 0,10A/dm² (ca. 3V/cm) (Teitge, 2016).

The actual mechanisms that cause the death of fish by the action of current are not known. A ventricular fibrillation does not seem to be the real reason, as a normal heart rhythm can be observed. The actual cause of death could be a complete and irreversible depolarization of the nervous system and respiratory arrest (J. Lines and Kestin 2004). Most systems use alternating current at a frequency of 50 Hz, which is applied to water. Only if the voltage is sufficiently high, it leads to continuous unconsciousness. The higher the voltage and the longer the exposure time, the longer the period of unconsciousness with the fish and the higher the proportion of dead fish. Disagreement prevails over the required frequency of the alternating voltage in the electrical killing. Investigations and recommendations range from 50 Hz to 100 Hz and depends on the species (J. Lines and Kestin 2004). Higher alternating current (AC) voltage frequency appears to reduce the risk of tissue bleeding. At even higher frequencies (up to 2000 Hz), on the other hand, the duration of unconsciousness in fish decreases and the proportion of fish killed decreases, too (Robb et al. 2002). In this study, a self-built unit with AC was used (Figure 3), which transforms 230 Volt into 48 Volt. The fish get stunned for 120 seconds.

Figure 3: Electrical stunning unit

Percussive stunning

The function of percussive stunning is to create a concussion. It is necessary to locate the brain, as it is shown in Figure 4. There are machines available for salmon but not for trout or chars due to the smaller size. A correctly executed blow causes irreversible loose of the consciousness.

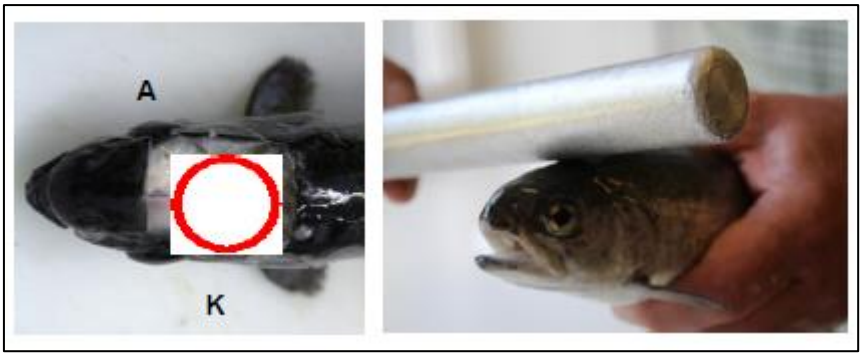

Figure 4: Location for a successful blow on the brain
Source: http://docplayer.org/59195558-Tiergerechtes-betaeuben-und-schlachten-von-regenbogenforellen.html

5.3 Existing stress-, muscle tissue-, quality- and stress-reduction parameters

A reliable assessment of animal welfare and on product quality requires a multidisciplinary approach (Poli et al., 2005).

5.3.1 Existing stress parameters

Fish react with a well-characterised neuroendocrine "stress response" to their environment (Wendelaar Bonga, 1997). These neuroendocrine "stress response" can be expressed due to changes in specific ranges of temperature, pH and solute concentration of muscle-tissue, which are necessary for the normal physiological functions. Fish react with a massive release of adrenaline and noradrenalin. Both values are hard to determine, because adrenaline and noradrenaline are fast removed from the blood and because they are elusive. This release is followed by a release of Adrenocorticotropin, which is also not measurable. The release of Adrenocorticotropin is followed by the corticosteroids, such as cortisol. Cortisol is widely used as long- and short-term stress index (Pickering & Pottinger, 1985), although it varies with feeding, reproductive cycles, seasonal cycles and husbandry conditions. The neuroendocrine stress response causes a higher heartbeat, oxygen uptake and energy mobilization as well as plasma glucose production.

The determination of plasma glucose is easy and therefore often used, although some researches could show a delay in its release (Barry et al., 1993). Higher energy mobilization initiates the anaerobic glycolysis and a related increase in plasma lactate (Erikson et al., 1999). The determination of plasma lactate is used, but most fish store lactate in muscle tissue which does not show the short-term stress and thus it is not suitable for the experiment. The higher metabolic rates cause an increase in the number of erythrocytes and of the haematocrit value, which is easy to determine as well (Poli et al., 2005).

Since experiments with cortisol already have been done at the University of Life Sciences and Natural Resources (BOKU) the decision was on cortisol. Cortisol is a steroid hormone, in the glucocorticoid class of hormones with the molecular formula $C_{21}H_{30}O_5$. Cortisol is the principal corticosteroid in bony/teleost fishes and its plasma concentrations rise during stress (Mommsen, Vijayan, and Moon 1999). It is released in response to stress. It activates reduction processes to release energy and make it available. The use of mammalian paradigms to explain the teleost situations is inappropriate (Mommsen, Vijayan, and Moon 1999). The absence of a unique mineralocorticoid and likely minor importance of glucose in fishes means that cortisol serves both glucocorticoid and mineralocorticoid roles; the unusual structure of the fish glucocorticoid receptor may be a direct consequence of this duality. Cortisol affects the metabolism of carbohydrates, protein and lipid (Mommsen, Vijayan, and Moon 1999). Cortisol is hyperglycaemic, primarily as a result of increases in hepatic gluconeogenesis

initiated as a result of peripheral proteolysis (Mommsen, Vijayan, and Moon 1999). The increased plasma fatty acid levels during hypercortisolaemia may assist to fuel the enhanced metabolic rates noted for a number of fish species (Mommsen, Vijayan, and Moon 1999). Cortisol is an essential component of the stress response in fish, but also plays a significant role in osmoregulation, growth and reproduction. Interactions between cortisol and toxicants may be the key to the physiology of this hormone. Cortisol is responsible for important housekeeping functions (Mommsen, Vijayan, and Moon 1999) and thereby necessary for the function of an organism.

5.3.2 Existing tissue parameters

Long- and short-term stress can influence fish post mortem biochemical processes. A major role takes the anaerobic glycolysis and Adenosine Tri-Phosphate (ATP) degradation rate. Figure 5 shows the complex biochemical processes in fish. These influence the onset and release of rigor mortis, which effects involution rate of fish freshness and thereby is an important parameter for the marketability of the product. Higher mobilization rates cause an early lack of ATP, which results in an early onset of rigor mortis. Higher mobilization means higher anaerobic glycolysis activity and a muscle lactic acid increase and pH decrease within the first day after death, which is clearly linked to stress (Barry et al., 1993).

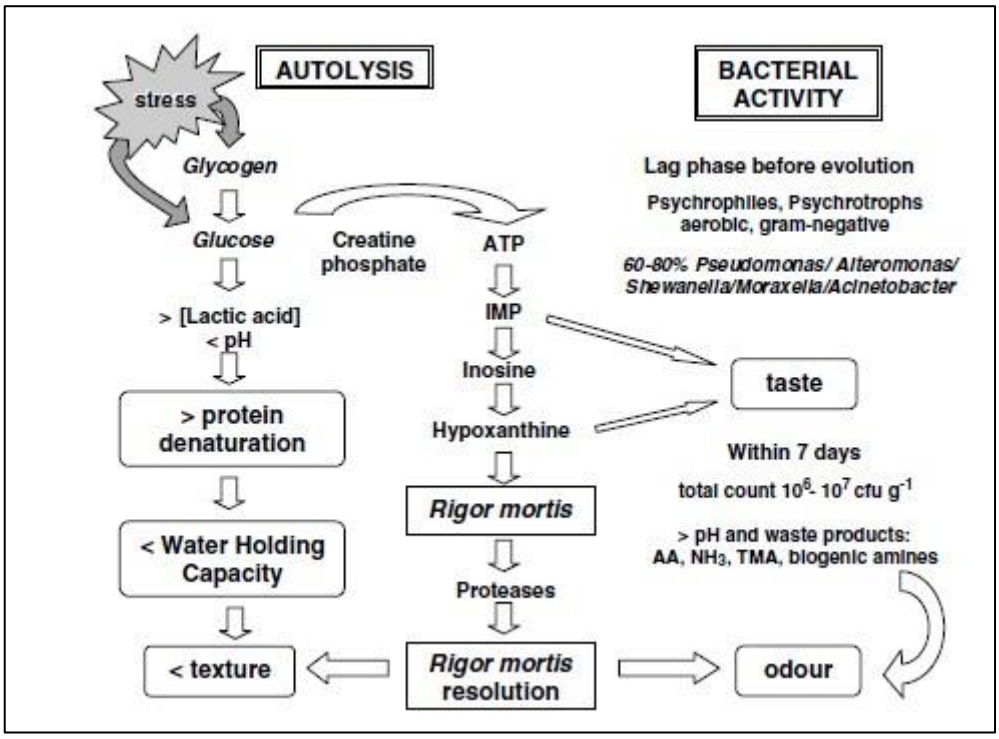

Figure 5: Tissue stress and quality indicators (Poli et al., 2005)

5.3.3 Fish quality indicators

Sensory qualities are a common indicator for the freshness of slaughtered fish, e.g. the optic of eye, gills and mucus (Bahuaud et al., 2010; Cheng et al., 2014; Concollato et al., 2016; Hanz, 2008). Texture of raw fish fillet is generally measured in the industry by the "finger method," which is an indication of the suitability for further processing and is mainly dependent on the firmness. The evaluation of firmness is usually performed through pressing on the skin or the fillet of fish by finger. And this method depends to a large extent upon subjective assessments of the expert panel. Sensory methods are the most widely used methods to evaluate fish freshness. Often used sensory parameters are appearance, odour, mucus

of skin, texture of flesh, pupil brightness and colour of gills (Barbosa and Vaz-Pires 2004). Compared with sensory evaluation, it has been proved that textural measurements by instrumental analysis methods are better due to reducing the variations during measurements arising from the human factor. The puncture, compression, shear, and tension are four main instrumental techniques and methods that are used to measure and evaluate the texture of fish from a force-deformation curve, displaying the value of force, deformation, slope, and area (Casas et al. 2006).

5.4 Existing stress reduction parameters

Studies show that reduction of stress increases the flesh quality in fish (Stamer, 2009; Poli et al., 2005; Bahuaud et al., 2010). Therefore, an economical interest for the reduction of slaughter stress exists.

5.4.1 Colour

The visual environment of fish is blue, green or near infrared and fish have cone cells which enable them to discriminate colours (Levine and MacNichol 1982). Few studies worked with the effects of background or light colours on fish, some interesting effects have been reported on egg development, schooling, and fright reaction (Volpato and Barreto 2001). Also, Loukashkin and Grant (1965) showed that fish are attracted to green and blue lights and avoid red light. Additionally, a previous study of the characid fish *Brycon cephalus* (LATREILLE 1825) has suggested that these colours may affect reaction to stressors Volpato and Barreto (2001) showed that blue light prevents the cortisol stress-response in Nile tilapia (*Oreochromis niloticus* (LINNAEUS 1758)). Figure 6 shows the difference in plasma cortisol levels between the basal and post-handling conditions.

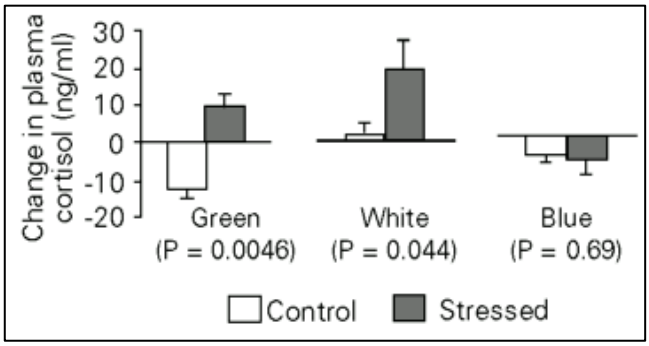

Figure 6: Effect of light colours on stress response of adult Nile tilapia (Volpato and Barreto, 2001)

5.4.2 Water temperature reduction

Fish are poikilothermic animals, meaning a lower environment temperature results in a lower metabolic rate, which could decrease their stress reaction during slaughter as well. Several studies worked already on this issue with different settings (Sandblom et al. 2013)(Lyytikäinen et al. 2002).

6 Methodology

6.1 Funding

This work is not part of an existing long-term project of the University of Life Sciences and Natural Resources. In April 2017 a suitable subsidy was found from the BOKU, called “BOKU-Förderungsstipendium”. The “BOKU-Förderungsstipendium” supports Master thesis with a maximum of 3600€ and is intended for Masterthesis with a high financial effort. Therefore, a proposal was submitted in June 2017. In July 2017 the subsidy was approved with the maximum of 3600€. The great cooperation between three different institutions have made this work possible. The ordinary cost of the study would exceed the budget by far. The fish were sponsored by my fish farm. A lot of friends helped to make this project possible with sharing their equipment or supporting us with their time. Otherwise this work would have cost around 10.000€.

6.2 Experiment design

The experiment design shows eight different stunning methods, which are realised in four treated identical tanks: one control tank, one tank treated with blue light, one tank treated with reduction of water temperature and one tank treated with blue light and reduction of water temperature in combination (Figure 7) (compare Table 1).

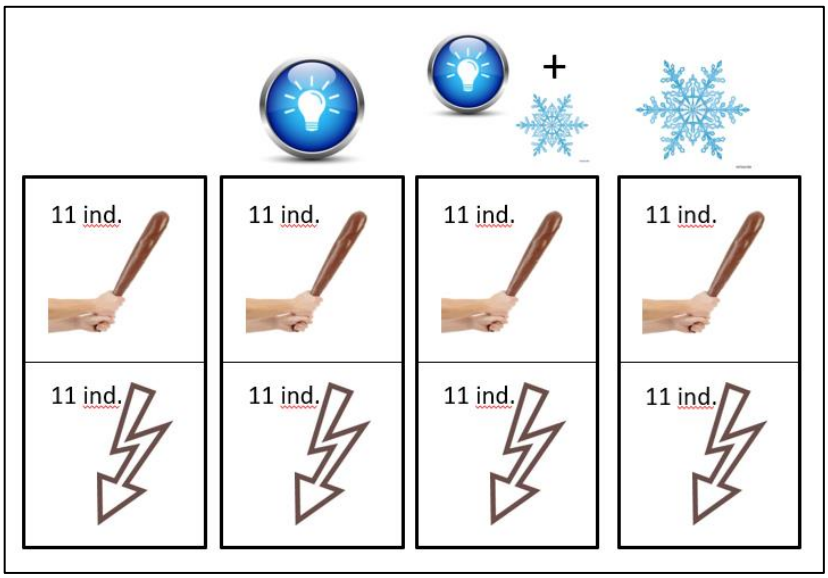

Figure 7: Overview of the eight fixed stunning settings

6.3 Experimental Setup

6.3.1 Preparation of the construction

The dimension of the four tanks are 3.0 x 0.5 x 0.5m which result in a volume of 600L per tank. Since four tanks were needed, it results in weight around 2.4 tons. Two identical tanks existed in the fish farm and two more became produced for the study. A place with a fundament for the tanks was prepared due to the high weight. Bushes and trees have been removed from the experimental area. A construction out of wood was prepared and balanced to ensure an equal water level in the lower and upper end of the tanks (Figure 8). The construction has been buried on the upper side and elevated on the lower side. Grids were used to make it possible to walk in between the tanks. Then the construction was ready to place the tanks. Each tank was mechanically separated in the middle and covered with a grid. The separations were made by colleges.

Figure 8: Construction with working water supply (left), Construction for the tent (right)

6.3.1.1 Realisation of water supply for the tanks

The water supply was created by the use of water from a pond situated higher than the tanks location, so the water entered the tank without any pumps. The water pipe was constructed with PVC-pipes of different diameters.

6.3.1.2 Realisation of the influence of the blue light

Two out of four tanks were treated with blue light. A tent was built to eliminate the influence of the daylight and to ensure the influence for the blue light. A construction was built which became covered by a canvas to realise the tent. Three lamps for each tank were installed and a clock-timer to simulate a day-night-rhythm. Figure 9 shows the final influence of blue light.

Figure 9: Influence of the blue light

6.3.1.3 Realisation of the water temperature reduction

The eight stunning methods were planned and the realisation effort was underestimated, especially for the water temperature reduction. The first idea was simply to put ice into the water. Running the calculation of the quantity of necessary ice for the temperature reduction showed a high quantity which was not possible to realise and would had implement a great disturbance for the fish.

Colleagues gave us great options for the realisation but with a high financial effort. Since money was rare a creative solution was necessary. One idea was to put the fish into a freezer with cold water. This method would had included a thermal shock which is firstly not allowed in Austria and secondly the idea was to reduce the water temperature slowly to avoid a shock event for the fish (§10 Tierschutz-Schlachtverordnung, 2015). Finally, the idea was to get an old freezer and put water in the freezer to cool the water directly in the freezer and pump it into the tanks. Pre-experiments were performed to find the correct cooling time. The cooling tanks became isolated to reduce the influence from the surrounding higher temperature (Figure 10). Another challenge was the water connection from the freezer to the tanks. A pump with a water level activated switch was installed to control the unit in combination with an on-off-unit. The on-off-unit differs to a common clock-timer by shorter intervals. Finally, a temperature reduction of 5°C between the tanks was achieved. During the experiments a temperature logger was used to observe the reduction in water temperature. Temperature loggers (iButton-Logger Maxim Integrated - DS1922L) with an accuracy of 0.0625°C packed in a waterproof aluminium capsule were installed. The loggers were programmed with the Temperature Measurement Package (Te.M.P. 2016).

Figure 10: Isolated tanks

6.3.2 Procedure of experiments

6.3.2.1 Moving the fish from the pond to the tanks

Fish were caught by pulling a net through the pond to get the desired size. Fish were then moved into the tanks (88 individuals in total, 11 individuals for each stunning setting, 22 individuals in each tank). The tanks were already prepared with blue light and the water temperature reduction unit (chapter 5.3.1). The fish were kept for two weeks to avoid the impact from moving. The tanks with water temperature reduction were not treated at this time. The water temperature reduction started two days before stunning. The blue light influence was performed during the two weeks.

6.3.2.2 Experiment day

After two weeks eight different stunning settings were performed. The fish were netted out with a dipnet and put into a container where they were stunned by using the percussive or electric method. In the case of the percussive stunning, each fish was taken and got a blow on their head and each fish was put in a separate plastic bag to identify them afterwards. In case of the electrical stunning, all fish of one stunning setting were put into one container and exposed for 120 seconds to the electrical field. To accomplish this, an anode and cathode were held under water and turned on. The blood samples were taken immediately after the procedure. The killed, chilled fish were moved in labeled plastic bags at the fish farm and moved to St.Margarethen an der Sierning to fillet them. Afterwards, the blood samples and the fillets were moved to Vienna for further analyses.

6.4 Used parameters

There are many options to analyze stress (Cheng et al., 2014; Bahuaud et al., 2010, Poli et al., 2005; Barry et al., 1993). This work focused on two parameters. One stress parameter: blood plasma cortisol level and one flesh quality parameter: fillet texture.

6.4.1 Blood plasma cortisol

Cortisol in blood or other biological tissues is widely used both as long term and as short term stress condition index (Pickering and Pottinger 1985). Fish show 2% blood of their bodyweight, so it is a challenge to gain enough blood. The analysis was done at the University of Natural Resources and Life Sciences in Vienna, at the Institute of Sanitary Engineering and Water Pollution Control (SIG). The blood samples are analyzed by Liquid chromatography-Mass Spectrometry (LC-MS/MS).

6.4.1.1 Blood sampling

Heparinized capillary tubes (Hämatokritkapillaren O.D. 1.5-1.6, HIRSCHMANN) with a volume of 100µL were used to collect blood and to avoid coagulation. The low amount of blood required pre-experiments to ensure the function of the analyses. The earlier pre-experiments were done with a higher amount of blood due to the use of mixture samples of different fish. The fish got stunned, immediately killed, and bled out by cutting their caudal vein (Figure 11 -1). Blood is collected using heparinized capillary tubes (2). For further analysis blood plasma was needed. The blood from the heparinized capillary tubes was put into Safe-Lock Eppendorf tubes with a volume of 1.5mL (3) and centrifuged (VWR MINISTAR SILVERLINE EU) at 6000 rotations per minute (rpm) for ten minutes (4) to separate the solid from the liquid fraction of the fish blood (5). The blood plasma got pipetted (6), and transported frozen to the Institute of Sanitary Engineering and Water Pollution Control, BOKU, Vienna.

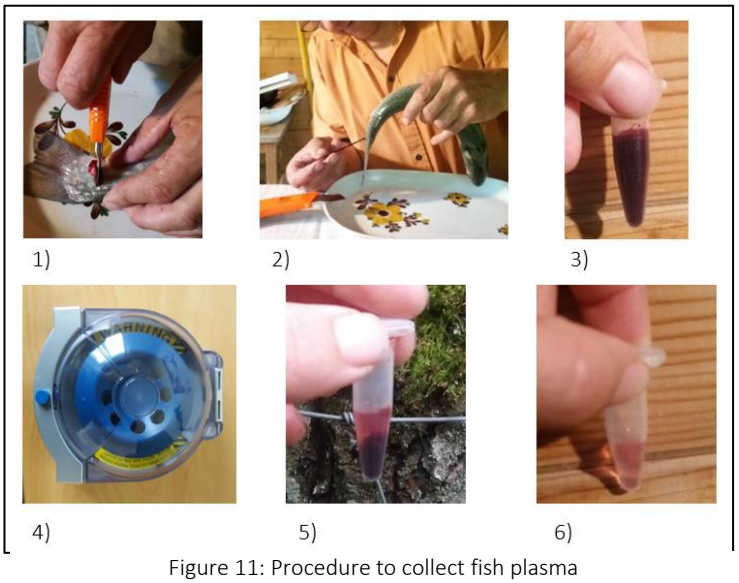

Figure 11: Procedure to collect fish plasma

6.4.1.2 Method of analysis

The procedure described follows Hunt K.E. et al. (2006). The method specifies the determination of hydrocortisone in aqueous matrix in mass concentrations from 1-2 µg / l as well as in solid samples in concentrations of 0.5ng / g. The method for biological solid samples (fish excrement) described in the literature was further developed and validated at SIG.

The frozen plasma was removed from the freezer on the day of analysis. When working with LC-MS/MS a time intensive preparation of the samples is necessary, so called Solid Phase Extraction (SPE). SPE is a sample preparation method which separates compounds that are dissolved or suspended in a liquid from other compounds in the mixture according to their physical and chemical properties (Figure 12). Analytical laboratories use SPE to concentrate the analyte and purify samples for analysis. SPE can be used to isolate analytes of interest from a wide variety of matrices including urine, blood, water, beverages, soil, and animal tissue (Solid phase extraction, Affinisep 2018).

SPE uses the affinity of solutes dissolved or suspended in a liquid (known as the mobile phase) for a solid through which the sample is passed (known as the stationary phase) to separate a mixture into desired and undesired components. The result is that either the desired analytes of interest or undesired impurities in the sample are retained on the stationary phase. The portion that passes through the stationary phase is collected or discarded, depending on whether it contains the desired analytes or undesired impurities. If the portion retained on the stationary phase includes the desired analytes, it can then be removed from the stationary phase for collection in an additional step in which the stationary phase is rinsed with an appropriate eluent (Solid phase extraction, Affinisep 2018).

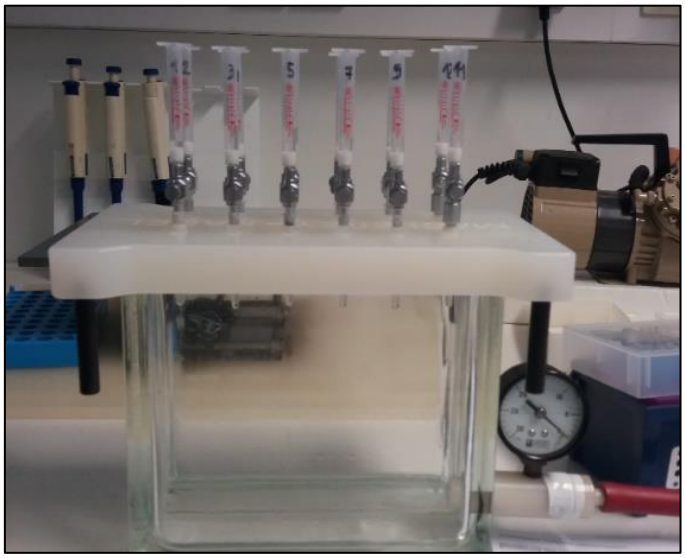

Figure 12: SPE unit

The solid phase unit involved twelve columns (Figure 12), whereas position one was always the blank and position two the control sample, which is a standard solution used to calculate the recovery rate. During the sample preparation and analysis a loss of analytes is possible. To minimise this the internal standard was used, which is a substance quite similar to the analyte. A common method to create the internal standard is deuteration. Deuteration is a technique whereby molecules get marked by replacing hydrogen atoms with an isotope of hydrogen, so called deuterium, or heavy hydrogen. A heavy hydrogen atom consists of one proton and one neutron, where common hydrogen has no neutron. Isotopes show the same number of protons but differ in neutrons, resulting in showing the same atomic number but differ in their mass. Deuteration causes no changes in chemical properties only in physical properties. Deuteration is the most used method to mark isotopes. The internal cortisol (internal standard) was put in each sample to eliminate variances. The standards were fixed due to the expected range of the analyte with 0, 0.5, 1.5, 5, 10, 20, 25, 30, 50, 70, 75 and this calibrations were done for each LC-MS/MS run (Figure 13).

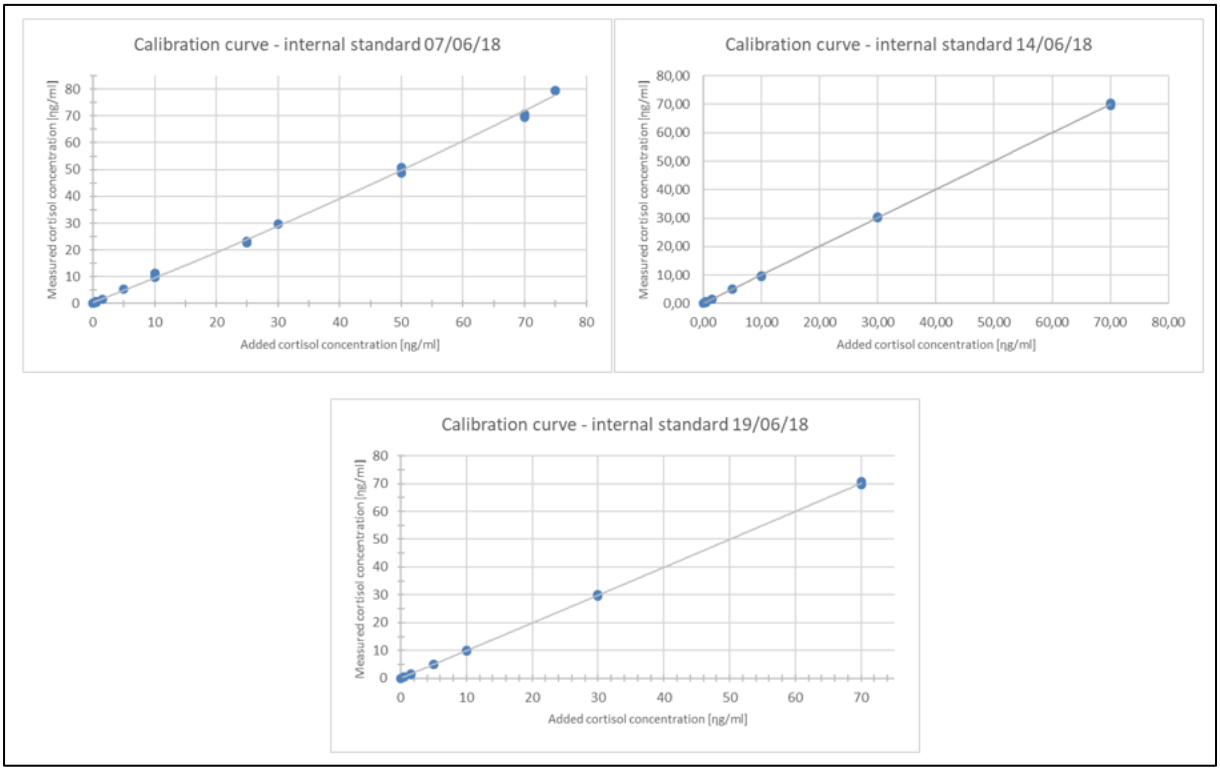

Figure 13: Calibration curves of the three runs (07/06/18, 14/06/18, 19/06/18)

Detailed SPE-steps

This method was established in earlier experiments at the SIG lab using fish faeces. Pre-experiments, performed for this study, had shown that the method is suitable for blood plasma too, with minor variations. The following steps are performed:

1. Conditioning of column (column: Strata TM – X33 µm Polymeric Reversed Phase 30mg/1mL, Tubes) with 1ml methanol (MeOH) and 1ml deionized water
   The column was equilibrated with a non-polar or slightly polar solvent, which wetted the surface and penetrated the stationary phase (MeOH). Next deionized water is washed trough the column to wet the silica surface (Figure 14).
2. Application of sample (plasma)
   The samples, the blanks and control samples were applied (Figure 14)
   Preparation of samples: available plasma (volume was noted) + 50µL internal cortisol
   Preparation of blank solution: 200µL deionized water + 50µL internal cortisol

   Preparation of control sample: 200µL deionized water + 50µL internal cortisol + 25µL cortisol. The control sample was used to calculate the recovery rate.
   As the sample passes through the stationary phase, the polar analytes interact and retain on the polar sorbent, while the solvent and other non-polar impurities pass through the column.
3. Column washing: after the sample was loaded, the column was washed with a non-polar solvent, deionized water to remove further impurities. Afterwards it was washed with 1ml ionised water and 1ml of 40% MeOH and dried by N_2 for ten minutes (Figure 14).

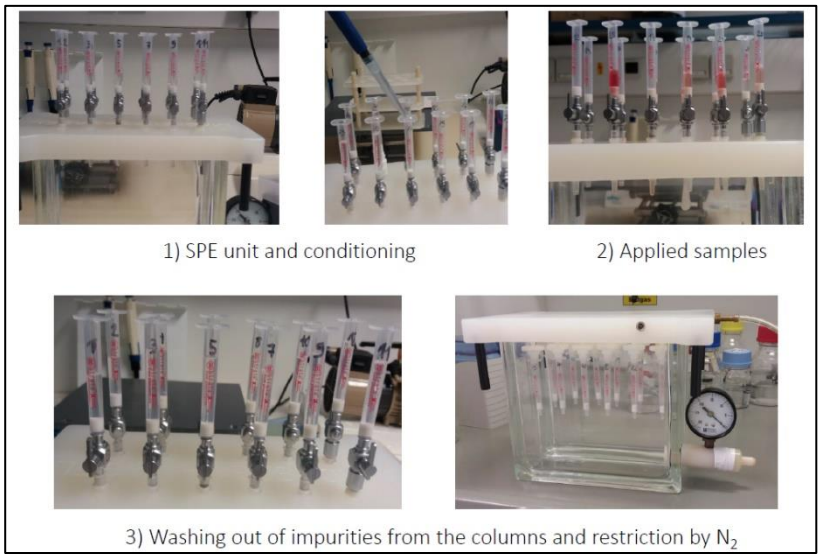

Figure 14: SPE-steps (1 – 3)

4. Elution: with 1ml MeOH to release the cortisol from the stationary phase (Figure 15).
5. Drying of the samples by using N_2 until no liquid phase was left (around 1 hour) (Figure 15).
6. Reconstitution of the sample with 500µL of 20% Acetonitrile (ACN). To get a homogenous sample it was vortexed for one minute using the AUTOVORTEX SA6 (Figure 15).
7. Pipetting the samples using a Pasteur pipette into the vials via a 0,2µL filter (GHP-Acrodisc 13mm Syringe Filter with 0.2µm GHP Membrane) to remove particles before LC-MS/MS analysis (Figure 15).
8. Cleaning of the Solid Phase Extraction unit with MeOH and ionised water (Figure 15).

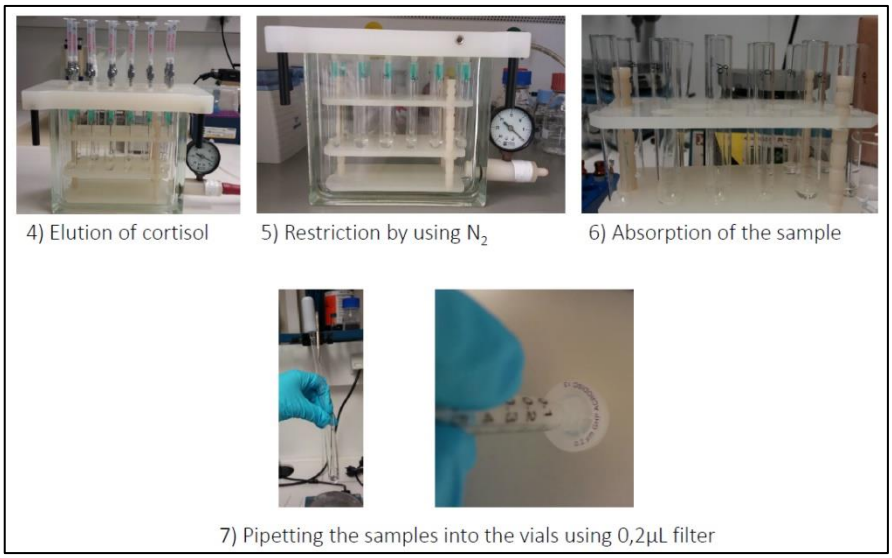

Figure 15: SPE-steps (4 – 7)

6.4.1.3 Liquid chromatography–mass spectrometry (LC-MS/MS)

LC-MS/MS describes a modern detection method and is defined by linking more mass spectrometer (MS) in series as well as a liquid chromatography (LC). This combination of detectors creates the identification and quantification of pure and mixed substances. Mass spectrometry is a detection method that relies on the mass-to-charge ratio of a compound. The MS/MS technology is only used when a single ionization is not adequate to achieve the desired sensitivity.

Function of LC-MS/MS

The tandem-MS has three Quadripoles (triple-quad), whereas in the first and in the third of these quadripoles the measurements take place. The first unit of mass spectrometry shows the whole mass range. The second one is responsible for the fragmentation. Further specificity can be obtained when multiple ionization events occur. The second ionization process fragments the molecules into pieces that each have their own mass-to-charge ratio. These pieces are called daughter ions and can be detected. Each parent molecule fragments in a predictable fashion, thus even if two parent molecules have the same mass-to-charge ratio, if they break into different daughter ions, these can be differentiated. The multiple ionization event method is called tandem mass spectrometry (or MS/MS). The last mass spectrometry unit determines the desired fragment. Commonly a LC-MS/MS is combined with an electrospray- Interface (ESI-Interface). The ESI-Interface creates the fumigation of the liquid sample in a high-voltage field (Lebensmittelchemisches Institut des Bundesverbandes der Deutschen Süßwarenindustrie e.V., 2002).

The LC-MS/MS was done with the TSQ Vantage Instrument (Triple Quadrupole LC/MS; Thermo Fisher Scientific), last modified on the 6th of November 2018 14:21:28 PM by Vantage. The following settings were used:

Method Type: EZ Method
MS Run Time (min): 16.00
Experiment Type: SRM
Chrom Filter Peak Width (s): Not used
Collision Gas Pressure (mTorr): 1.5
Use Tuned S-Lens Value: Yes
Q1 Peak Width (FWHM): 0.70
Q3 Peak Width (FWHM): 0.70
Display Time Range for SRM table: Yes
Cycle Time (s): Not used
DCV (V): Not used

Tune Method - Adjustable parameters:
Capillary Temperature: 350.0
Vaporizer Temperature: 350.0
Sheath Gas Pressure: 35.0
Ion Sweep Gas Pressure: 0.0

Aux Valve Flow: 10.0
Spray Voltage: Positive polarity - 3300.0 , Negative polarity - 3000.0
Discharge Current: Positive polarity - 4.0 , Negative polarity - 4.0

Divert Valve: in use during run
Divert Time (min) Valve State
==========
0.00 Inject \ Waste
5.48 Load \ Detector
8.84 Inject \ Waste

Used Column: Zorbax Eclipse XDB-C18, 2.1 (diameter) x 150mm (length), 5 Micron (size of particles), material of column C18, Producer: Agilent;

Measurement

Figure 16 presents the procedure of one measurement. Solvent A was ionised water and solvent C was ACN. The operation temperature of the oven was fixed at 40°C. At first step, solvent A (ionised water) was pumped into the system with 80% and solvent C (ACN) with 20%. During the measurement the concentration of solvent C is increased from 20% to 80%.

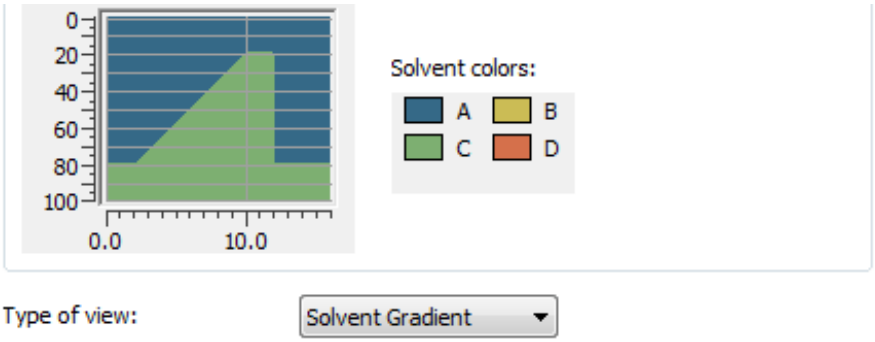

Figure 16: Visualisation of the used solvents

Figure 17 (1) shows an example for the measurement of the cortisol standard. One measurement takes sixteen minutes and two minutes are saved to gain the cortisol data around the cortisol peak at minute seven. Figure 17 (2) shows the blank value, the so-called primer noise. Figure 17 (3) shows an example of the measurement of one sample. The cortisol peak at minute seven is clearly visible. In comparison with the primer the values are low which means that only small amounts of cortisol are measured in the shown examples.

(1)

(2)

(3)

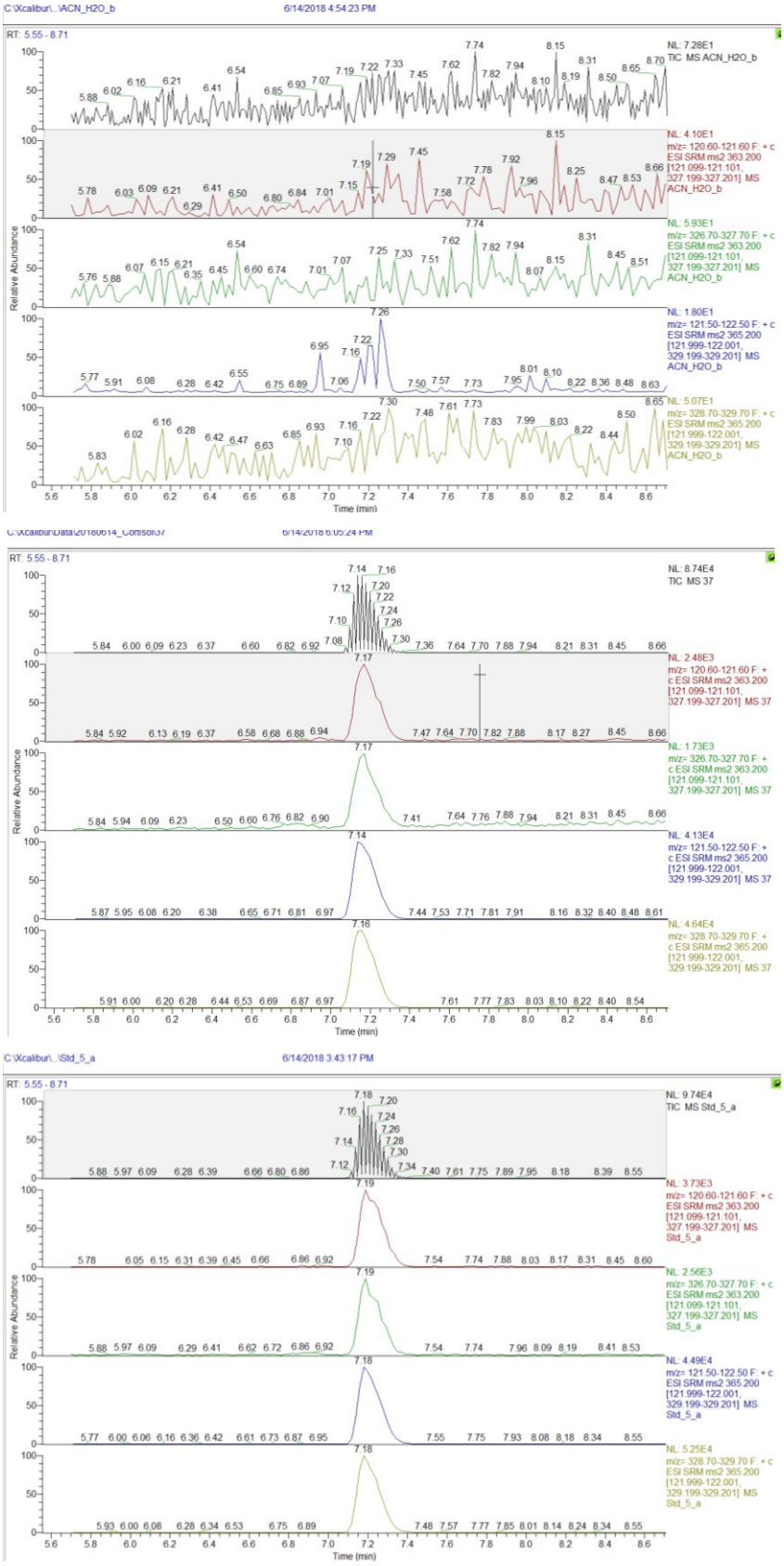

Figure 17: Measurement of the cortisol standard (1), blank value (2) and one sample (3)

6.4.1.4 Evaluation of gained data

The LC-MS/MS unit provides the cortisol values in ŋg/ml = µg/l. Since the plasma volume of the samples varies, a standardisation is necessary. Therefore, a calculation regarding the volume was done.

$$K = \frac{K_{LC-MS/MS} * V_{ACN}}{V_{sample}}$$

$K_{Lc\text{-}MS/MS}$ .....Value from the LC-MS/MS measurement [ng/mL]

V_{ACN}.....volume of ACN [mL]

V_{sample} .....applied volume of sample [mL]

K.....Cortisol [ŋg/mL]

A detection limit is necessary to create trustful data and eliminate values below the detection limit. During two runs the detection limit was calculated and fixed (Table 4).

Table 4: Calculation of the detection limit

07.06.2018	STD0,5	STD0,5	STD0,5	STD0,5	STD0,5	STD0,5	AV	SD	RSD	Detectionlimit
Substance	µg/l	µg/l	µg/l	µg/l	µg/l	µg/l	[µg/l]	[µg/l]	[%]	[ŋg/ml]
Cortisol_121	0,5	0,73	0,69	0,72	0,68	0,67	0,67	0,08	12,64	0,84

14.06.2018	STD0,5	STD0,5	STD0,5	STD0,5	STD0,5	STD0,5	AV	SD	RSD	Detectionlimit
Substance	µg/l	µg/l	µg/l	µg/l	µg/l	µg/l	[µg/l]	[µg/l]	[%]	[ŋg/ml]
Cortisol_121	0,561	0,529	0,457	0,535	0,469	0,662	0,5355	0,07	13,79	0,74

STD…Standard

AV…average of the measured values of the standards

SD…standard deviation = $s = \sqrt{\frac{1}{n-1}\sum_{i=1}^{n}(X_i - \bar{X})^2}$

RSD…Relative Standard deviation = SD*100/AV

Detection Limit = 10 x SD

The statistical analyses of the plasma samples and the fillets texture are explained in the Chapter 5.5 Statistical Data Analysis.

6.4.2 Fillet texture – Relative elasticity

Fillet texture is one of the most important quality parameters in fish, and in contrast to mammalian meat, consumers generally prefer firm and elastic fish fillet (Rasmussen 2001). An excessive softening of fish flesh usually makes it too fragile for processing (smoking, freezing, slicing, etc.). The fillet texture was measured mechanically by a Texture Analyzer. The texture analyzing is based on the principle that external force results in changes in intermolecular forces. Flesh absorbs this energy and this energy causes tensions inside the flesh. The absorbed energy can be completely or partially diffused by changes in texture or converted to frictional heat (Hanz, 2008). The smoother the force development, the more elastic the fish fillet becomes, resulting in a higher quality. The analysis is done at the University of Natural Resources and Life Sciences, Vienna at the Institute of Food Science. Pre-rigor fillets are required for the analysis. The fillets were analyzed two days post-mortem to eliminate an effect of the rigor mortis (Bahuaud et al., 2010). Pre-experiments were carried out to find a method to differentiate flesh qualities. Different fixtures of the Texture-Analyzer TA.XT.plus (Stable Micro Systems) were tried, i.e. SMSP/0-5 and the ball with a diameter of 10mm (Figure 18). The pre-experiments show small fixtures produce more reliable data. The reason to use the ball was to imitate the fingertip freshness in an objective way. The experiment analyses will be done with the ball. The pre-experiments showed different values at the posterior and at the anterior end, compared to the mid-region. Therefore, the measurements were carried out in the mid-region in between the pelvic and the anal fin, three cm from the anterior end to 3 cm to the posterior end. Three measurements for each fish were completed at a distance of 1cm. The fillets must be tempered and were in plastic bags to protect them against drying-up and to ensure comparable data (Hanz, 2008).

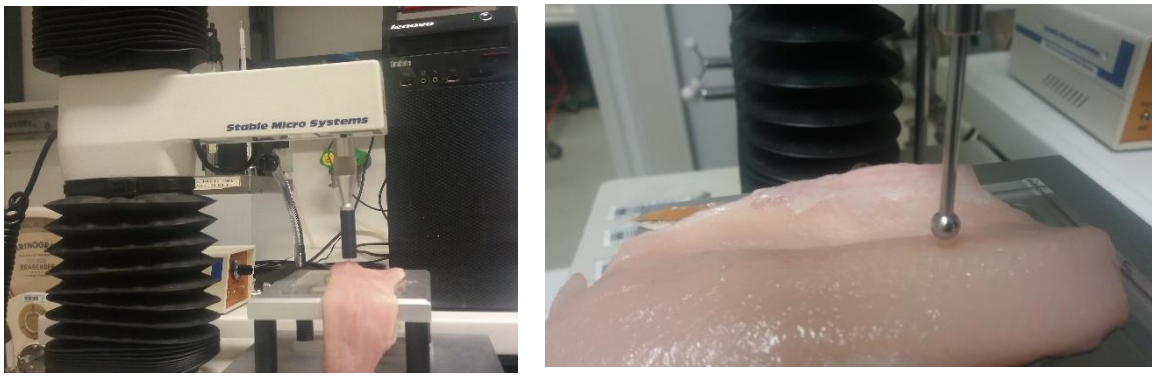

Figure 18: Testing the fillet texture with the SMSP/0-5 (left) and the ball with a diameter of 10mm (right)

Texture analyser settings

The system is determined by three components: (1) Testbed, (2) control panel to regulate the testbed, (3) computer, an optional part of the control panel. Before the measurements can begin, a calibration of the force and probe head is necessary. For the probe head calibration, the probe head moves downwards with a speed of 0,001 m/s until it reaches the test bed and sets this as zero point (Hanz, 2008). For the calibration of the force, a load cell of 5kg was used. According to the results of the Master thesis of Hanz, the Relaxation method is the best choice to analyse fish fillets. For this method, the penetration depth was fixed at 2mm, as soon as this depth is reached, the position of the ball was held for 20 seconds. The pre-Test Speed was 0,5mm/sec. When the analyser recognized the fillet the speed automatically changed to 0,80mm/sec until the penetration depth of 2mm was reached. This position was then held for twenty seconds before the ball was removed with a post-Test Speed of 10mm/sec. The applied trigger force was 0,049N (Figure 19). For processing the data, a software application was required. The software is provided by the same company, Micro Stable Components and is called Exponent for TA.XT.plus.

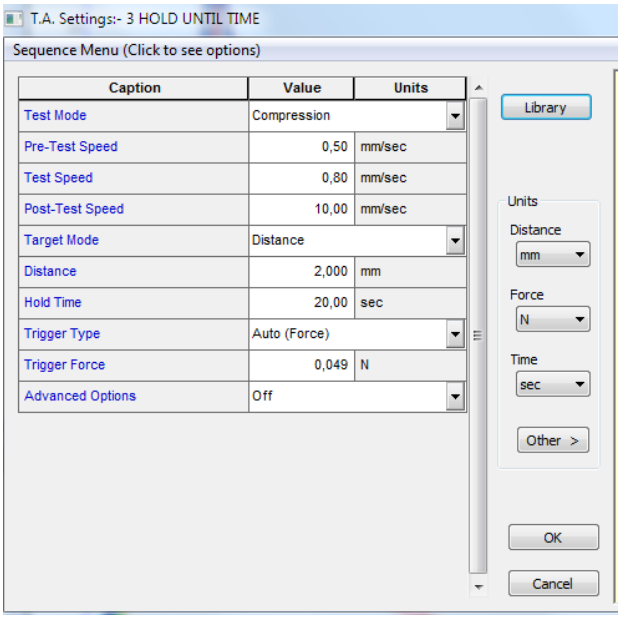

Figure 19: Settings for the measurements

Different parameters were fixed and saved as a Makro. These parameters were then calculated automatically by the software. The chosen parameters for this experiment were F1, F2, F3, T50. Figure 20 shows an example of a measured curve. Table 5 shows the output of a measurement for one fish.

F1…force at fixed penetration depth [N]

F2…F1/2 [N]

F3…Force after holding time [N]

T50…time at F2 [sec.]

The parameters F1 and F3 were used to calculate the Relative elasticity (rE). rE is used for further analyses. Other flesh parameters were not used in this Master thesis.

rE [%] = F3 / F1 * 100

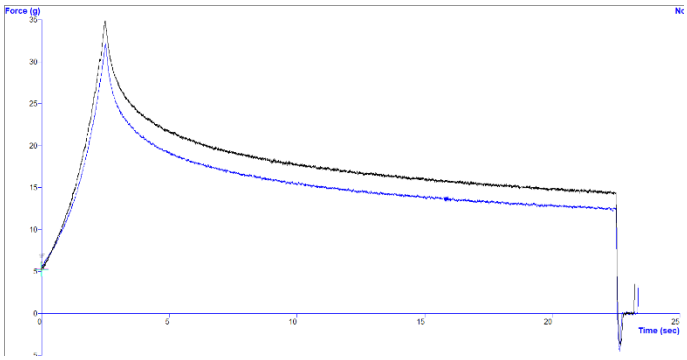

Figure 20: Example of a texture-analyse (relaxation method)

Table 5: Measurement of one fish fillet

Test ID	Batch	Force 1	Time 1	Area F-D 1:2	Force 2	Time 2	Force 3
		N	sec	N.mm	N	sec	N
		Force 1	Time 1	Area F-D 1:2	Force 2	Time 2	Force 3
Start Batch E	E						
E01	E	0,454	2,510	0,449	0,228	11,780	0,201
E02	E	0,513	2,505	0,468	0,256	12,110	0,232
E03	E	0,573	2,505	0,494	0,286	12,330	0,255
End Batch E	E						
Average:	E (F)	0,513	2,507	0,471	0,257	12,073	0,229
S.D.	E (F)	0,060	0,003	0,022	0,029	0,277	0,027
Coef. of Variation	E (F)	11,609	0,115	4,760	11,416	2,293	11,818

6.5 Statistical Data Analysis

The observed data were implemented in a prepared data mask in Microsoft Excel (Table 6). This Excel data matrix was exported to a SPSS file for the statistical Analysis. The hypotheses guided analyses were carried out with the IBM SPSS Statistics 21 software.

Table 6: Data File (Excel data matrix)

Identifer	Fish ID	Stunning method	K [ng/ml]	F1 [N]	F2 [N]	T2 [sec]	F3 [N]	rE [%]
1	1	Percussive	23,8	0,286	0,143	12,125	0,131	46
2	1	Percussive	23,8	0,286	0,143	11,47	0,129	45,1
3	1	Percussive	23,8	0,235	0,117	11,3	0,106	45,2
4	2	Percussive	41,6	0,328	0,164	10,43	0,14	42,6
5	2	Percussive	41,6	0,334	0,166	10,55	0,147	43,9
…	…	…	…	…	…	…		…
239	88	Electrical & Cooling & Blue	5,9	0,191	0,095	11,395	0,084	43,9
240	88	Electrical & Cooling & Blue	5,9	0,203	0,102	11,425	0,088	43,4

The data evaluation focuses primarily on two Hypotheses, which focus on the different levels of two variables (Cortisol and relative Elasticity) in dependence on the stunning methods. For the global analysis, the simple Variance Analysis was used. The post hoc analysis was done with the DUNCAN test for the parametric tests (local/pairwise) and the median test (global and post hoc local/pairwise) for the non-parametric test .

The third Hypothesis relates to the intercorrelation between K and rE and to the one-sided regression (K → rE), both global and divided according to the stunning methods. Linear, quadratic and cubic regression models were used for this purpose. The empirical results were interpreted based on the strength of the degree of determination (R^2) and the empirical error probability (p).

7 Results

The effects of the stunning method on the plasma cortisol level was analysed.

7.1 Cortisol

Verification of empirical hypotheses H1.1 to H1.3

The verification of the empirical hypotheses H1.1 to H1.3 refers to the mean level (location) of the variables "stress level" and "relative elasticity". Parameters of the middle level (location) are the **arithmetic mean AM ($\overline{x}$)** and the **median ($\tilde{x}$).** In each case certain conditions must be considered.

The statistical hypotheses (NULL HYPOTHESIS H0 and ALTERNATIVE HYPOTHESIS H1) in orientation for the parameter **MEAN ($\overline{x}$)** are:

global:

H_0: $\overline{x}_1 = \bar{x}_2 = \bar{x}_3 = \bar{x}_4 = \bar{x}_5 = \bar{x}_6 = \bar{x}_7 = \bar{x}_8 = \overline{x}$

H_1: $\bar{x}_1 \neq \bar{x}_2 \neq \bar{x}_3 \neq \bar{x}_4 \neq \bar{x}_5 \neq \bar{x}_6 \neq \bar{x}_7 \neq \bar{x}_8 \neq \overline{x}$;

post-hoc local:

H_0: $\overline{x}_i = \bar{x}_j$

H_1: $\bar{x}_i \neq \bar{x}_j$

and for the **MEDIAN ($\tilde{x}$):**

global:

H_0: $\tilde{x}_1 = \tilde{x}_2 = \tilde{x}_3 = \tilde{x}_4 = \tilde{x}_5 = \tilde{x}_6 = \tilde{x}_6 = \tilde{x}_7 = \tilde{x}_8 = \tilde{x}$

H_1: $\tilde{x}_1 \neq \tilde{x}_2 \neq \tilde{x}_3 \neq \tilde{x}_4 \neq \tilde{x}_5 \neq \tilde{x}_6 \neq \tilde{x}_6 \neq \tilde{x}_7 \neq \tilde{x}_8 \neq \tilde{x}$

post-hoc local:

H_0: $\tilde{x}_i = \tilde{x}_j$

H_1: $\tilde{x}_i \neq \tilde{x}_j$.

The one-factorial univariate analysis of variance (ANOVA) wass used for the global hypotheses on the mean. For the post-hoc local hypothesis the multiple test strategy of DUNCAN was used. The median test was performed globally followed by the post-hoc local/multiple.

The requirements for the ANOVA are:

[1] Normal distribution of the two dependent variables – cortisol K [ηg / mL]) and relative elasticity rE [%] - in all treatments of the anaesthetic methods and

[2] Variance homogeneity of both variables between all treatments of the stunning methods.

DEPENDENT VARIABLE: stress level (cortisol K [ηg / mL]

INDEPENDENT VARIABLE: stunning method (Treatment)

The validity for a normal distribution of the dependent variable stress level (cortisol K [ηg / mL]) was not given. The required relationship $\{ \hat{x} = \tilde{x} = \bar{x} \}$ cannot be observed (Table 7). The hypothesis of the variance homogeneity of cortisol K [ηg / mL] must be rejected as well (LEVENE TESTS: F = 16.77, df1 = 7, df2 = 220, p = 0.000).

Table 7: Location parameters (Mean, Median, Modus) of the variable cortisol K [ηg / mL] differentiated by stunning method; several modes available, the lowest value is displayed.

Cortisol K [ηg/mL]					
E	Mean	6.31	P	Mean	18.92
	Median	4.47		Median	17.98
	Mode	1.83		Mode	9.11
E & B	Mean	5.30	P & B	Mean	6.00
	Median	5.04		Median	6.13
	Mode	0.51		Mode	2.93
E & C	Mean	41.03	P & C	Mean	33.26
	Median	34.67		Median	29.47
	Mode	26.90		Mode	20.01
E & C & B	Mean	21.67	P & C & B	Mean	20.11
	Median	15.50		Median	16.34
	Mode	2.12		Mode	9.30

If both conditions / prerequisites - normal distribution and variance homogeneity - were met (!), The following picture would emerge for the ANOVA (Table 8):

Table 8: Sample size (n) mean and standard deviation of the parameter cortisol [ŋg / mL] of the different stunning methods

Cortisol K [ŋg/mL]			
Treatments	n	Mean	Std. Deviation
E	27	6.31	4.80
E & B	30	5.30	2.88
E & C	30	41.03	17.55
E & C & B	30	21.67	17.59
P	30	18.92	9.83
P & B	27	6.00	2.79
P & C	27	33.26	10.84
P & C & B	27	20.11	11.13
Total	228	19.22	16.45

ANOVA: F = 42.05; p= 0.000; ETA^2 = 0.572.

note: interpretation (see Cohen 1988)

ETA^2: 0.00 - 0.08 - no influence or heterogeneity

ETA^2: 0.09 - 0.14 - weak influence or weak heterogeneity

ETA^2 => 0.15 - medium to strong influence or medium to strong heterogeneity.

The global null hypothesis H0 was rejected in favour of the two-sided alternative hypothesis H1. The heterogeneity hypothesis was globally valid. ETA^2 is a parameter for the assessment of the effect strength or the degree of heterogeneity.

In this case: ETA^2 is 0.572, which means that the amounts of cortisol differed significantly. The statistical significance p = 0.000 indicated high security. There was a general/global connection between the independent variable stunning method (treatment) and the dependent variable cortisol. Thus, the stunning methods were an influencing parameter on the cortisol level.

The decision for the global statistical alternative hypothesis of heterogeneity required an exact localization of the heterogeneities. For this purpose, post-hoc pairwise local tests according to DUNCAN were carried out. The statistical hypotheses for this test are:

$$H_0: \bar{x}_i = \bar{x}_j \quad \text{versus} \quad H_1: \bar{x}_i \neq \bar{x}_j.$$

Table 9: Results of the post-hoc Duncan test of the variable / character cortisol [ng / mL], tested on basis of the mean according to anaesthetic methods

Results: Post-Hoc-Test: Duncan-Test				
Treatments	N	Subset		
		1	2	3
		Mean ($\bar{X}$)		
E & B	10	5.30		
P & B	9	6.00		
E	9	6.31		
P	10		18.92	
P & C & B	9		20.11	
E & C & B	10		21.67	
P & C	9			33.26
E & C	10			41.03
Sig. (p)		.857	.622	.139

The mean levels ($\bar{X}_j$) within a group/subset column were statistically significantly homogeneous. The mean levels between the subset columns were statistically significantly heterogeneous. Table 9 shows three homogeneity groups in total. The cells labelled dark blue indicate the treatments in which $\bar{X}_j$-cortisol levels are equally low: E & B, P & B and E (5.30 to 6.31), the light blue cells the treatments with equivalent high $\bar{X}_j$-cortisol values: P & C and E & C with 33.26 and 41.03. Cortisol values of the treatments P, P & C & B, and E & C & B (18.92 to 21.67) were not homogeneously extreme (neither low nor high). The AM of the cortisol of the treatment P was statistically significantly higher (18.92), compared to the cortisol value in the treatment E (6.31).

A further specific variant of the multiple post-hoc comparison strategy with the AM ($\bar{X}_j$) is the decision-tree analysis. The result was an optimal grouping of the stunning methods regarding the mean cortisol level (X) in the context of multiple ANOVA's. The decision tree anlaysis facilitated the interpretation. The groups of the tree diagram (Figure 21) were identical with those of the multiple DUNCAN test. ETA² = 0.558 highlighted again the high degree of heterogeneity of the mean level of cortisol between the three homogeneous stunning method groups.

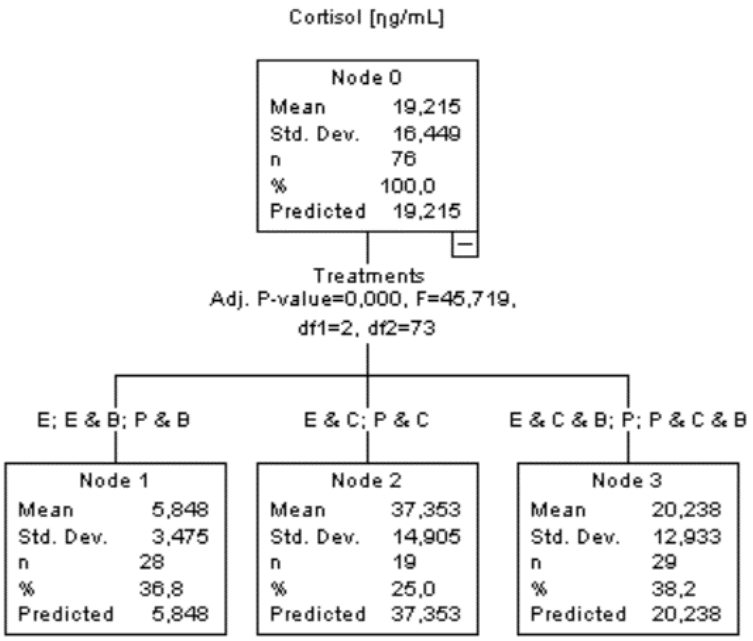

Figure 21: Tree analysis of the variable cortisol depending on the stunning method

$ETA^2 = 0.558$

Since - as had already been stated - the two requirements for a comparison by means of the AM (parametric test) were not met, the median test (non-parametric test) must be applied globally and locally as an alternative to analysis of variance (ANOVA / Duncan test). The statistical hypotheses for the global median test are:

$$H_0\colon\ \tilde{x}_1 = \tilde{x}_2 = \cdots = \tilde{x}_8 = \tilde{x} \quad \text{versus} \quad H_1\colon\ \tilde{x}_1 \neq \tilde{x}_2 \neq \cdots \neq \tilde{x}_8 \neq \tilde{x}.$$

These hypotheses are analysed by the Independent-Samples-Median-Test (Figure 22):

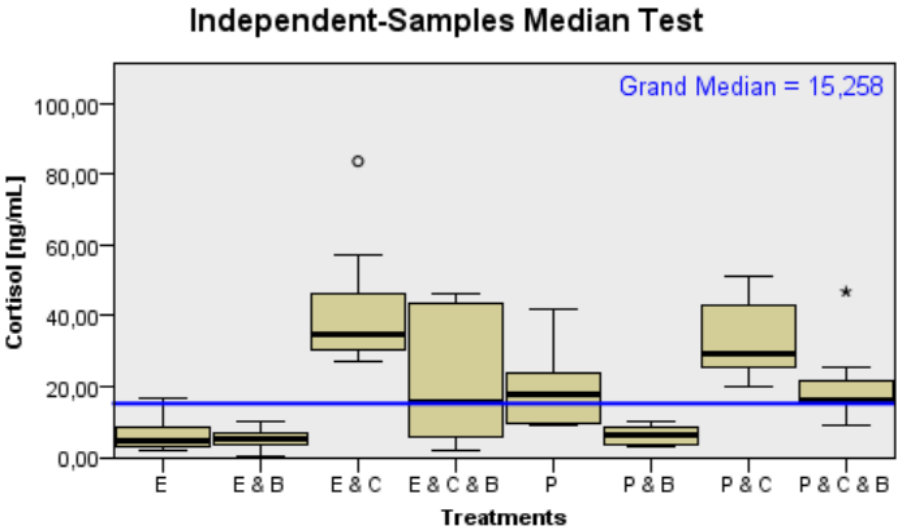

Figure 22: Boxplot diagram of the medians of the variable cortisol [ŋg / mL] according to the different treatments

Mediantest: Chi² = 15,26; df = 7; p = 0.000

The global null hypothesis H0 of equal medians was not valid because statistically significant differences (heterogeneity) were reported. The differences become located with the pairwise post-hoc median test. For this, the following local statistical hypotheses are used:

$$H_0\colon \tilde{x}_i = \tilde{x}_j \quad \text{versus} \quad H_1\colon \tilde{x}_i \neq \tilde{x}_j \,.$$

		Subset		
		1	2	3
Sample[1]	E	4,472		
	E & B	5,041		
	P & B	6,132		
	E & C & B	15,495	15,495	
	P & C & B		16,340	
	P		17,978	
	P & C		29,471	29,471
	E & C			34,665
Test Statistic		3,422	7,244	1,351
Sig. (2-sided test)		,331	,065	,245
Adjusted Sig. (2-sided test)		,552	,125	,675

Figure 23: Results of the post-hoc median test of the variable cortisol [ηg / mL] according to the different treatments

The Duncan test (AM) shows three homogenous groups which are also shown in the median test (Figure 23). Equal low medians were found in group 1: E, E & B, P & B (4.47 - 6.13). The stunning method E & C & B was divided into two groups: group 1 and group 2. As the difference to group 2 was significantly smaller, E & C & B was further treated in group 2. Thus, group 2 is represented by E & C & B, P & C & B and P (15.50 - 17.98). P & C is in group 2 and group 3. Since the deviation to group 3 is smaller, P & C was treated as belonging to group 3. P & C and E & C are the group with the highest cortisol levels (29.47 - 34.67).

Both the variance analysis (= parametric test) and the median test (= non-parametric test) consequently led to identical results. Because the requirements for parametric tests (ANOVA and DUNCAN test) were not met, the test decisions of the nonparametric median test, globally and locally, are valid. The median test confirms the test decision for the local H1: There is special cortisol heterogeneity.

The first hypothesis:

H1.1: The category of the percussive effect variable *(P)* has a higher CORTISOL level *(K)* than the electric stunning *(E).*

H1.1 is confirmed based on the results (Figure 23), category P is in Subset 2, and Category E is in Subset 1 with a lower stress level.

The second hypothesis refers to the influence of blue light and was formed as follows:

H1.2: The categories of the blue light effect variable *(B)* show a lower CORTISOL level *(K)* than the non-blue light categories: [E & B, P & B, E & C & B, P & C & B} vs. [E, P, E & C, P & C}.

H1.2 is analysed using the already performed median test (Figure 23). E & C & B and P & C & B show a lower cortisol level (15.50, 16.34) than the non-blue light stunning methods: E & C and P & C (29.47 and 34.67). P has a higher cortisol value (17.98) than P & B (6.13). Only E already has a very low value (4.47) and cannot be further differentiated from E & B (5.04). Because of this result, H1.2 is discarded. However, a stress-reducing influence of the blue light can be considered given. Only in the lower cortisol area this is not visible.

The third hypothesis deals with the influence of water temperature reduction.

H1.3: The categories of effect variables with reduction of water temperature *(C)* show a lower CORTISOL *(K)* level than the categories without reduction of water temperature {E & C, P & C, E & C & B, P & C & B} vs. {E, P, E & B, P &B}.

H1.3 is tested using performed median test (figure xx). P & C and E & C are in Group 3 and have the highest cortisol levels of 29.47 and 34.67. P is in Group 2 and E in Group 1, so the categories without water temperature reduction have a lower cortisol value. The categories P & C & B and E & C & B are in group 2 (15.50 - 16.34) and show a higher cortisol level than E & B and P & B in group 1. None of the water temperature reduction categories show a lower cortisol level compared to the categories without reduction of water temperature. Thus H1.3 is clearly refuted.

The empirical question ***F1***: "Do the eight STUNNING METHODS (effect variable / treatment) influence the STRESS LEVEL (cortisol K [ŋg / mL])?" can be answered with a YES.

7.2 Relative elasticity

To determine a possible effect from the stunning methods on the second parameter, the flesh quality was tested. The empirical question F_2 refers to the parameter **r**elative **E**lasticity **rE** = (F3 / F1) * 100 [%] and is tested based on three hypotheses (H2.1, H2.2 and H2.3).

DEPENDENT VARIABLE: Relative elasticity

INDEPENDENT VARIABLE: Stunning method (treatment)

The validity of the statistical hypothesis H2.1 to H2.3 was tested based on the arithmetic mean ($\bar{x}$) and the median ($\tilde{x}$). Regarding the AM, one-factorial univariate analysis of variance (ANOVA) was used. Based on the median, the median test was used.

At first the global test with the orientation on the AM was created (x = Relative elasticity [%]):

$$H_0: \bar{x}_1 = \ \ldots\ = \bar{x}_8 = \ \overline{X} \qquad \text{versus} \qquad H_1: \bar{x}_1 \neq \ \ldots\ \neq \bar{x}_8 \neq$$

It should be mentioned again that two conditions are required to use the ANOVA: **[1]** Normal distribution of the dependent variable rE in all eight treatments and **[2]** Variance homogeneity of the dependent variable RE between all eight treatments.

Table 10: Location parameters (Mean, Median, Modus) of the variable RELATIVE ELASTICITY rE [%] differentiated by stunning method

Relative elasticity RE = (F3/F1) * 100 [%]					
E	Mean	43.21	P	Mean	43.08
	Median	43.28		Median	43.64
	Modus	38.77		Modus	35.30
E & B	Mean	42.92	P & B	Mean	41.49
	Median	43.38		Median	41.12
	Modus	36.83		Modus	36.79
E & C	Mean	41.54	P & C	Mean	42.58
	Median	41.42		Median	41.77
	Modus	41.13		Modus	38.01
E & C & B	Mean	43.06	P & C & B	Mean	43.79
	Median	43.12		Median	43.32
	Modus	39.80		Modus	39.06

The condition for the normal distribution ($\{\hat{x} = \tilde{x} = \bar{x}\} \rightarrow NV$) was not given (Table 10). The hypothesis of variance homogeneity wass proven in this case (Levene-test: F = 1.60, df1 = 7, df2 = 232, p = 0.135 n.s.).

If both (!) conditions were met, the ANOVA would show the following picture (Table 11):

Table 11: Mean and standard deviation of the variable relative elasticity [%] of the different stunning methods

Dependent Variable: RE = (F3/F1) * 100 [%]			
Treatments	n	Mean	Std. Deviation
E	30	43.2	2.4
E & B	30	42.9	2.6
E & C	30	41.5	2.3
E & C & B	30	43.1	1.5
P	30	43.1	2.8
P & B	30	41.5	2.4
P & C	30	42.6	2.9
P & C & B	30	43.8	2.0
Total	240	42.7	2.5

ANOVA: F = 3.045; p = 0.002; ETA² = 0.094

Table 11 shows very small differences between the stunning methods. This statement is intensified by a small ETA². The measure of the effect size ETA² indicates a very weak influence of the stunning types on the rE but an influence nonetheless, with a value of 0.094. This result is statistically verified with p = 0.002.

The alternative statistical hypothesis H_1 applies: $\bar{x}_1 \neq \ \ldots \ \neq \bar{x}_8 \neq \ \bar{X}$.

The choice of the global alternative statistical hypothesis H1 of heterogeneity requires the exact location of heterogeneity. For this purpose, post-hoc pairwise local tests after DUNCAN (Table 12) and the decision tree analysis (Figure 24) were carried out. Both methods of analysis also try to identify homogeneous groups (subsets) based on AM.

The statistical hypotheses are:

$$H_0\colon \bar{x}_i = \bar{x}_j \qquad \text{versus} \qquad H_1\colon \bar{x}_i \neq \bar{x}_j.$$

The Post-Hoc-Duncan test forms two homogeneous groups of the same AM $(\bar{x})$. P & B and E & C are in subset 1 (41.5). Group 1 has the lowest relative elasticity and thus the lowest flesh quality. P & C has been assigned to both subset 1 and subset 2. The deviation to group 2 is smaller, putting P & C into group 2 with a higher elasticity and flesh quality. The decision-tree analysis shows identical groups to

the post-hoc Duncan test and P & C is clearly assigned to subset 1, which represents the higher flesh quality.

Table 12: Results of the post hoc Duncan test of the variable relative elasticity [%], based on the AM differentiated on treatments; red = group 1; blue = group 2;

Dependent Variable: rE = (F3/F1) * 100 [%] – DUNCAN-test			
Stunning methods	N	Mean	
		1	2
P & B	30	41.5	
E & C	30	41.5	
P & C	30	42.6	42.6
E & B	30		42.9
E & C & B	30		43.1
P	30		43.1
E	30		43.2
P & C & B	30		43.8
Sig. (p)		.097	.088

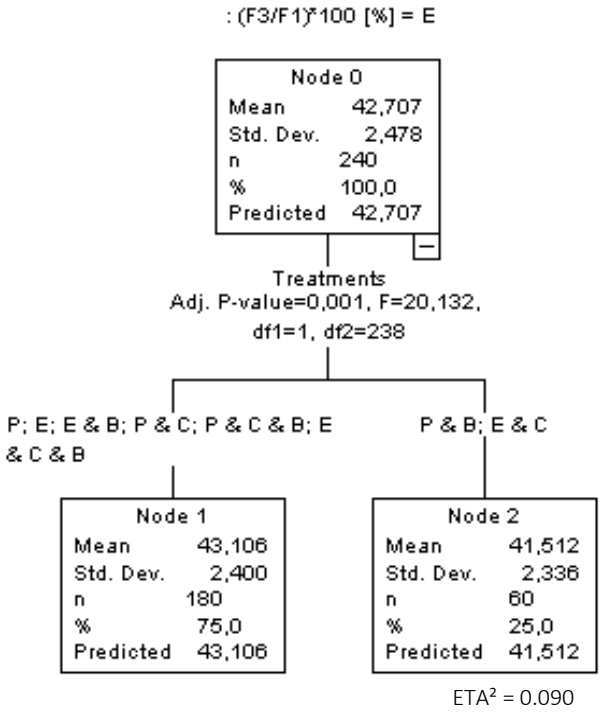

Figure 24: Decision-tree Diagram of variable rE

However, the conditions for the test based on the AM ($\bar{x}$) were not given. Thus, tests are performed using the median (statistical hypotheses: H_0: $\tilde{x}_1 = \cdots = \tilde{x}_8 = \tilde{x}$ versus H_1: $\tilde{x}_1 \neq \cdots \neq \tilde{x}_8 \neq \tilde{x}$) which then showed the following picture (Figure 25):

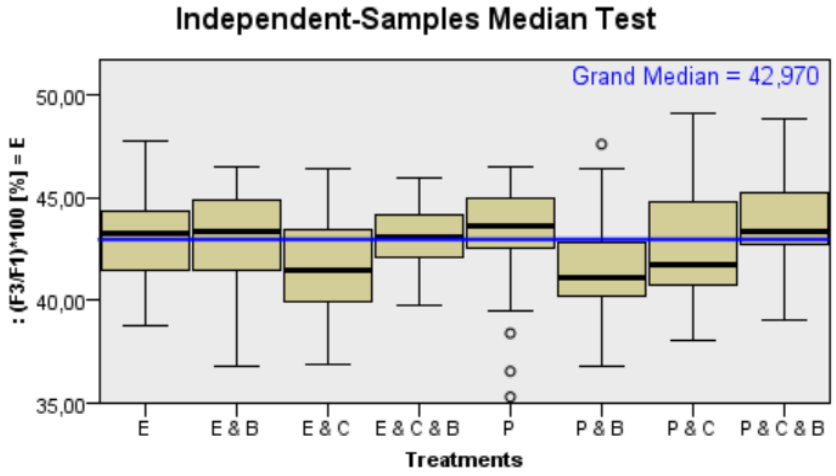

Figure 25: Boxplot diagram of the medians of the variable relative elasticity [%] according to the treatments

The differences in rE in median are lower compared to the parameter cortisol. According to the test result, the decision applies to the global alternative hypothesis H1: the median heterogeneity. To answer the empirical hypotheses H2.1 - H2.3, the localization of heterogeneity was necessary. For this purpose, the following hypotheses are tested:

$$H_0: \tilde{x}_i = \tilde{x}_j \quad \text{versus} \quad H_1: \tilde{x}_i \neq \tilde{x}_j$$

The output is shown in Figure 26, group 1 includes the treatments P & B, E & C and P & C showing the lower medians (41.12 to 41.77). According to the median test, treatment P & C could belong to both subsets. The difference to subset 1 is less than to subset 2. Treatment P & C was assigned to subset 1. Subset 1 is then formed by P & B, E & C and P & C, which shows a variation to the Duncan-test. Group 2 is characterized by a higher rE (higher flesh quality) with the treatments E & C & B, E, P & C & B, E & B and P (43.12 to 43.64). The requirements for the Duncan-test were not met. Thus, the groups based on the median were used in the following.

		Subset	
		1	2
Sample[1]	P & B	41,124	
	E & C	41,419	
	P & C	41,771	41,771
	E & C & B		43,122
	E		43,284
	P & C & B		43,323
	E & B		43,380
	P		43,635
Test Statistic		1,067	6,667
Sig. (2-sided test)		,587	,247
Adjusted Sig. (2-sided test)		,905	,314

Figure 26: Results of the multiple post-hoc median test for the variable rE; red = group 1; blue = group 2;

The first hypothesis of the empirical questions F_2 is:

H2.1: The category of the percussive effect variable (P) has a low RELATIVE ELASTICITY rE (= low flesh quality) compared to electrical stunning (E).

The categories of the effect variable Percussive (P) and electrical (E) show an identical rE since they are in the same homogenous subset so H2.1 is rejected.

The second hypothesis H2.2 on the empirical question F_2 refers to the influence of the blue light:

H2.2: The categories of blue light effect variables *(B)* have a higher RELATIVE ELASTICITY *rE* than the non-blue light categories: *{E & B, P & B, E & C & B, P & C & B}. {E, P, E & C, P & C}.*

E & B is in group 2 with E, so there is no influence of the blue light. P & B is in group 1 and P in group 2 with the higher flesh quality. P & C & B and E & C & B are in Group 2 with the higher flesh quality. In contrast, P & C and E & C without blue light are in Group 1 and have lower qualities. The blue light has

an influence on the flesh quality, this is especially obvious in P & C and E & C vs. P & C & B and E & C & B. H2.2 must be discarded, as this effect cannot be proven in all categories, but a tendency is observed.

The third hypothesis on the empirical question F_2 contains the influence of water temperature reduction:

H2.3: The categories of effect variables with water temperature reduction (*C*) have a higher RELATIVE ELASTICITY r*E* than the categories without water temperature reduction: *{E & C, P & C, E & C & B, P & C & B}* vs. *{E, P, E & B, P & B}*.

E & C and P & C are in group 1 with the lower flesh quality. The categories E and P are in group 2 and have a higher quality. A negative influence of the temperature reduction on the flesh quality becomes apparent. E & C & B, P & C & B and E & B are in Group 2. P & B is in Group 1. In this comparison, there is no difference between E & B and E & C & B. P & B has the lowest quality, P & C & B shows a higher quality. The empirical hypothesis H2.3 is clearly rejected. The results are diffuse, but it is interesting that P & C and E & C are in the group 1 showing the lowest flesh quality and the highest cortisol levels.

At this point, a problem in the method of this thesis needs to be discussed: For each fish, there is one cortisol value and three relative elasticity values. The above analyses were carried out with the complete elasticity values with three rE values or each fish. To create no wrong results, tests are carried out with the reduced data. Reduced data sets consist of one calculated AM for each fish. The results are in Figure 27.

		Subset	
		1	2
Sample[1]	P & B	40,736	
	E & C	41,180	41,180
	P & C	41,755	41,755
	P & C & B	43,149	43,149
	E & C & B	43,434	43,434
	E	43,481	43,481
	P		43,483
	E & B		43,767
Test Statistic		8,000	9,600
Sig. (2-sided test)		,156	,143
Adjusted Sig. (2-sided test)		,203	,143

Figure 27: Results of the post hoc Duncan test of the variable relative elasticity [%] based on the median according to the stunning methods (REDUCED DATA); red = group 1; blue = group 2;

The analyses with the reduced data set also form two subsets. The differentiation is overlapping here resulting in more diffuse data. Most of the treatments are assigned to both subsets.

The differentiation between P & B on the one hand and P and E & B on the other is clear. Considering a certain "willingness to compromise", one could also decide as follows:

Due to the small differences, P & B, E & C and P & C could also be assigned to subset 1 and the remaining stunning methods to subset 2. This assumption would then meet the results with the complete data set. The conclusion of this analysis is that there is no difference using the reduced or the complete data set.

The empirical question F_2: Do the eight STUNNING METHODS (effect variable / treatment) influence the FLESH QUALITY (relative elasticity rE = F3 / F1) * 100 [%])? can be answered with a weak and cautious yes.

6.3 Correlation of cortisol and relative elasticity

According to other literature the stress level shows influence on the flesh quality. Since the single parameters, cortisol and relative elasticity are tested, it is of interest whether the stress level influences the flesh quality. The empirical question F_3 deals with the general relationship of cortisol and relative elasticity:

H3.1: There is a general relationship between the stress variable CORTISOL (*K)* and the flesh quality Variable RELATIVE ELASTICITY (*RE)*.

The statistical hypotheses are:

$$H_0: \rho = 0$$
$$H_1: \rho \neq 0$$

Three different models were analysed (linear, quadratic, cubic) to identify a possible relation of cortisol and relative elasticity.

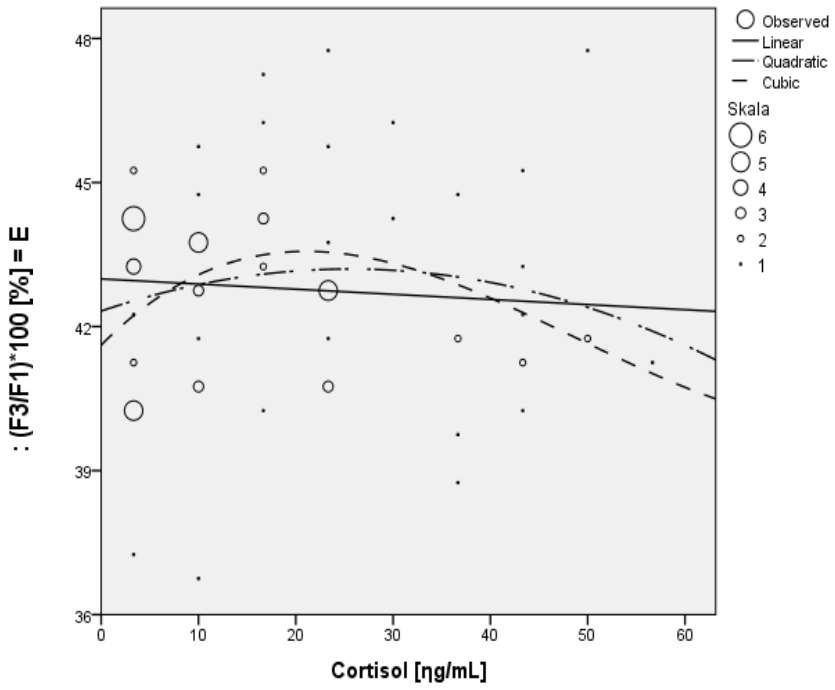

Figure 28: Regression analysis of cortisol and relative Elasticity

Table 13: General model summary of a relation between cortisol and relative elasticity

Model Summary and Parameter Estimates						
Dependent Variable: (F3/F1) * 100 [%] = RE						
Equation	Model Summary		Parameter Estimates			
	R^2	Sig.	Constant	b1	b2	b3
Linear	.007	.481	42.992	-.011		
Quadratic	.067	.079	42.323	.068	-.001	
Cubic	.100	.053	41.615	.204	-.006	0.00004
The independent variable is Cortisol K [ŋg/mL].						

Table 13 shows the highest determination coefficient (R^2) in the cubic model with 0.100, which means that 10% of the data are explained by this model. The R^2 of the linear and the quadratic model are even lower. Based on this result, H3.1 is discarded and an in-depth regression analysis is not useful with these low determination coefficients.

H3.2: A higher stress level *(K)* leads to a lower flesh quality *(RE).*

The model (Table 14) above also indicates the gradients (b1, b2, b3). The gradients give information about the correlation of both parameters. A direct correlation means increasing cortisol leads to an increase in relative elasticity. In the technical view a positive prefix means higher cortisol leads to a better flesh quality. An indirect correlation / a negative prefix means increasing cortisol leads to a decrease in relative elasticity (worse flesh quality).

B1, b2 and b3 (Table xx) show the influence of the independent parameter cortisol on the dependent parameter, relative elasticity, and are low. B1 is negative in the linear model, meaning increasing cortisol decreases the flesh quality. In the quadratic model, b1 is positive and b2 is negative. According to the quadratic model, cortisol and relative elasticity are directly related until a certain point followed by an indirect correlation. The cubic model shows a positive b1 value, a negative b2 value and again a positive b3 value. The indirect correlation is just given in the linear model, H3.2. is rejected.

Since surprisingly no general / global relationship was demonstrated, the examination of the relationship between CORTISOL and the RELATIVE ELASTICITY was now differentiated according to the stunning methods (Table 14). The results show a differentiated picture. The interpretation should consider the small number of cases caused by data splitting by treatment method.

Table 14: Model summary and parameter estimates

Model Summary and Parameter Estimates							
Dependent Variable: RE = (F3/F1) * 100 [%]							
Treatment		Model Summary		Parameter Estimates			
		R^2	p	Constant	b1	b2	b3
Electrical	Linear	**.582**	**.017**	40.969	.339		
	Quadratic	**.589**	.070	41.345	.207	.008	
	Cubic	**.788**	.039	34.197	4.340	-.564	.021
Electrical & Blue	Linear	.158	.255	41.279	.309		
	Quadratic	**.420**	.149	38.372	1.707	-.127	
	Cubic	**.860**	**.006**	34.506	6.490	-1.314	.077
Electrical & Cooling	Linear	.151	.266	43.353	-.044		
	Quadratic	.159	.545	44.913	-.112	.001	
	Cubic	.193	.710	58.088	-.977	.018	.000
Electrical & Cooling & Blue	Linear	.033	.615	43.354	-.013		
	Quadratic	.046	.847	43.064	.028	-.001	
	Cubic	.177	.739	41.815	.375	-.021	.000
Percussive	Linear	.021	.690	42.318	.040		
	Quadratic	.064	.795	39.977	.280	-.005	
	Cubic	.136	.814	27.807	2.355	-.105	.001
Percussive & Blue	Linear	.124	.354	40.484	.235		
	Quadratic	**.640**	**.047**	31.404	3.679	-.270	
	Cubic	**.664**	.116	24.267	7.623	-.920	.033
Percussive & Cooling	Linear	.185	.249	46.115	-.098		
	Quadratic	.201	.511	41.773	.169	-.004	
	Cubic	.201	.511	41.773	.169	-.004	0.000
Percussive & Cooling & Blue	Linear	.309	.120	41.920	.098		
	Quadratic	**.439**	.177	45.454	-.219	.006	
	Cubic	**.717**	.077	29.494	2.285	-.108	.001
The independent variable: K = Cortisol [ŋg/mL].							

The differentiation gives surprising information, due to the low R^2 results from the general model. The p-values indicate the general validity of this analysis ($p > 0.05$ no general validity) which means these data cannot be generalized and the results can only be applied to these data.

In four treatments (E, E & B, P & B, P & C & B), R^2 is found to be above 0.40, meaning more than 40% of the results are explained by the model differentiated by anaesthetic methods. A significant linear

relationship is only seen in the treatment E: R^2 is 0.582. The significance is also given (p = 0.017). This is a very strong significance compared to the rest of the data. In case of the stunning method *E,* the quadratic and cubic model shows even higher values of R^2 and the significance is lower. In the treatments E & B, P & B and P & C & B, there are correlations shown above 0.40 in the quadratic and cubic model.

The different orientations of b1 in the linear model are worth mentioning. Category E, E & B, P, P & B and P & C & B have a positive b1 value, meaning a direct correlation between cortisol and relative elasticity. The categories E & C, E & C & B and P & C have a negative b1 value, therefore, as stress increases the flesh quality decreases, however, the gradients are low.

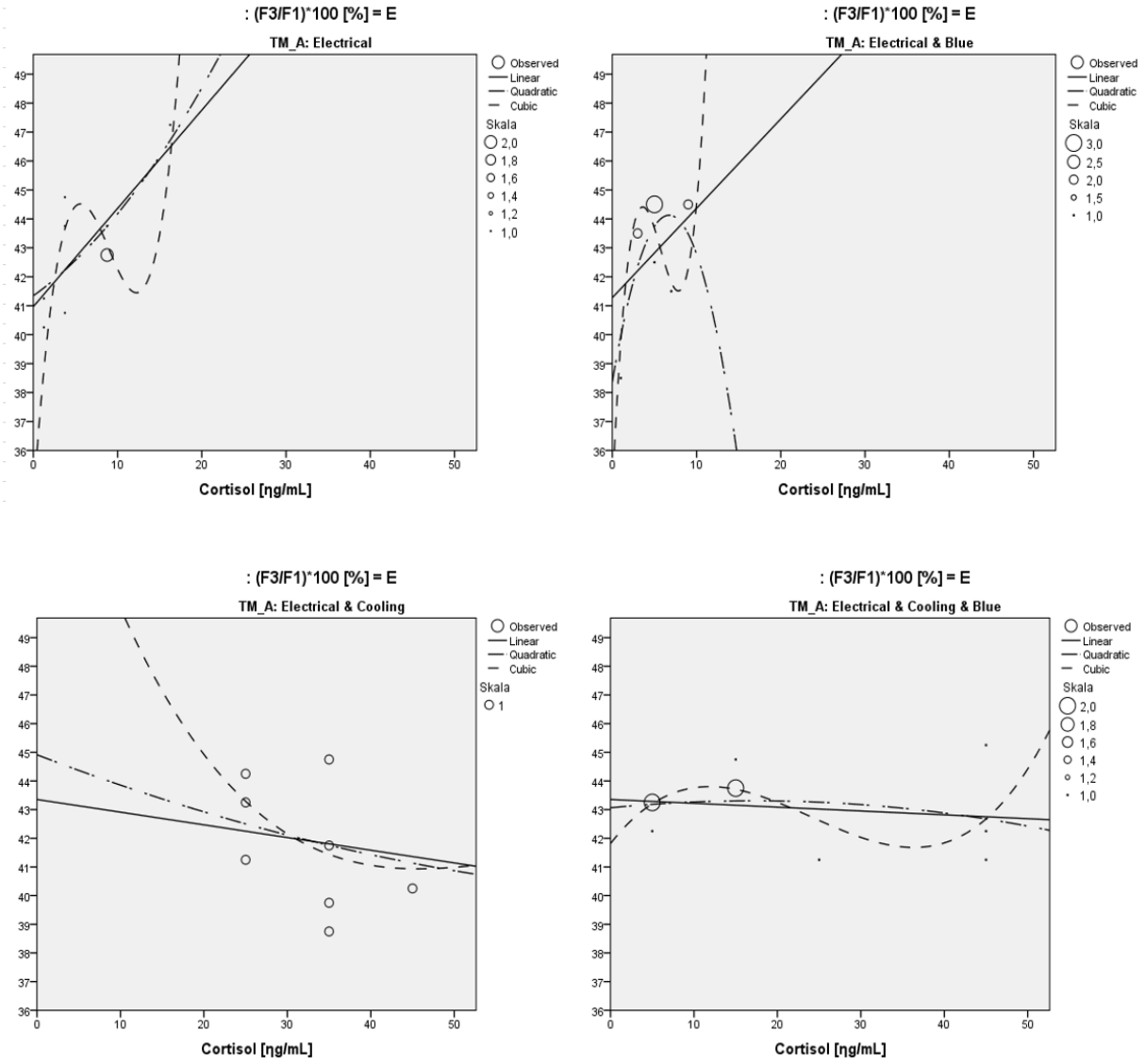

Figure 29: Linear, quadratic and cubic model of all electrical stunning methods; conditional (controlled) regression; conditional variable = stunning method (independent variable = cortisol; dependent variable = relative elasticity)

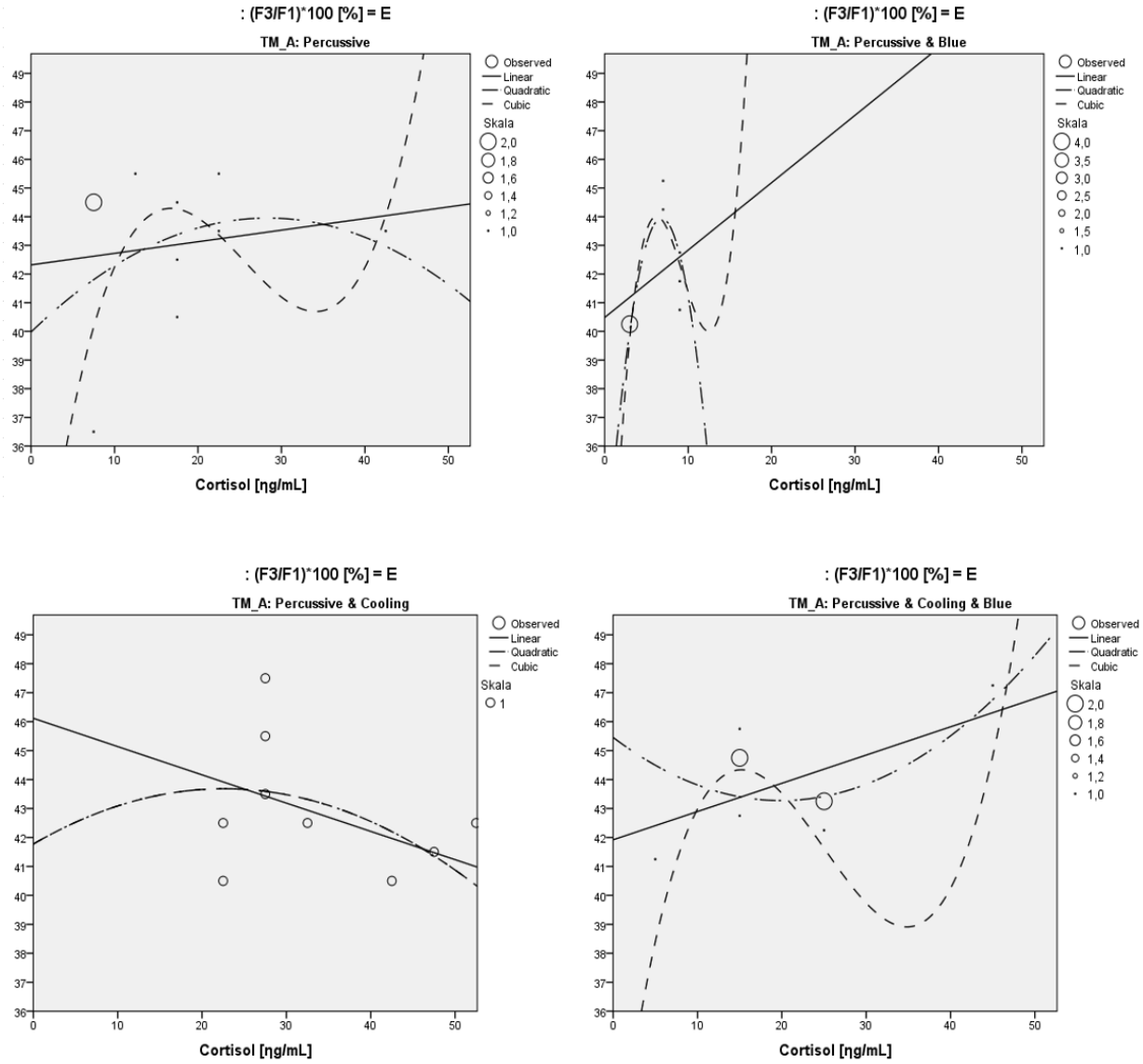

Figure 30: Linear, quadratic and cubic model of all percussive stunning methods; conditional (controlled) regression; conditional variable = stunning method (independent variable = cortisol; dependent variable = relative elasticity)

Having a look at this data with the homogenous groups of the cortisol parameter shows an interesting trend: The categories that are found in variable cortisol in subsets 1 and 2 (lower stress levels) all have a positive b1 value, except for E & C & B. The categories in subset 3, with the higher stress level, have a negative b1 value. This suggests that lower cortisol levels have no negative impact on flesh quality. High cortisol levels could have a negative impact.

The graphic output clearly shows that there are big differences in the correlation of K and rE when using different stunning methods (Figure and Figure). The stunning methods E and E & B show stronger

correlations compared to E & C and E & C & B. In case of the percussive stunning the correlation is generally weaker and shows different patterns due to stunning combinations.

8 Discussion

Since the requirements for the ANOVA were not fulfilled, neither in cortisol nor in relative Elasticity, the tests were done by the non-parametric analyses, the median test. Due to personal interests the analyses were created with the parametric and non-parametric tests and we could show that the results do not differ significantly.

8.1 Cortisol

Cortisol concentrations in fish varies with age, season, gender, feeding, daytime, stocking density and are individual (Poli et al. 2005). E.g. variations in cortisol concentrations are higher for chars that spent a lower proportion of time on moving in the field than for chars that spent a higher proportion of time on moving (Farwell et al. 2014). Since the char used for this study come from the same generation, the cortisol variations caused by stunning methods take luckily precedence over the individual cortisol variations.

The percussive stunning clearly shows a higher median of cortisol concentration , with a value of 17.98 ŋg/mL, compared to electrical stunning, at 4.47 ŋg/mL. In case of percussive stunning, a lot of handling and touching stress is involved. A human grab the fish, kept in the same tank as the other fish, so the last fish could be more stressed than the first ones. Analyses to find this pattern are not possible with the low amount of data in this study, but no trend to prove this pattern can be observed. Moreover, the fish are touched and removed from water, where the fish cannot breathe, which is an added stressor. It was harder to gain enough blood from the percussive group. It is assumed that the blow on the head implements a hematoma in the brain where the blood gets clumped.

In contrast, electrical stunned fish are moved from the pond/tank into the tank where they get stunned (in case of wet electrical stunning). All fish are stunned at the same time without being touched by human hands. The cathode and anode are put into the water and the electricity is switched on. Immediately, the fish get immobilized which seems to reduce the stress. A plasma cortisol level during wet electrical stunning of 4.47 ŋg/mL is evident. Literature shows that plasma cortisol can rise rapidly a few minutes after exposure to a stressor and may remain elevated for several hours (Wendelaar Bonga 1997) (Barton 2002). The cortisol level can increase for up to 90 minutes after an acute stress event (Lyytikäinen et al. 2002) (Pottinger 2010) (Seth et al. 2013). In case of this study, the samples were taken immediately after stunning. It took almost one hour to collect the blood samples of each setting before the eleventh fish could be sampled, so it is likely that the last fish handled shows a higher cortisol level due to the possible increase after stressor (Wendelaar Bonga 1997). However the data do not show any increase in blood cortisol. During electrical stunning, fish are immobilized, which does not mean that the fish also lose consciousness (Gräns et al. 2016). Electrical stunning can be performed for a too short time period. Due to this immobilization, there is a considerable risk that fish recover from consciousness

before dying from exsanguination in companies (Gräns et al. 2016). This immobilization also eliminates the possibility for behavioural responses like struggling or escape responses. It is documented that fish stunned by dry electrical stunning show double the plasma concentration than fish stunned with CO_2 narcosis, although the individuals stunned by electrical stunning do not show struggling or escape behaviour (Gräns et al. 2016). Dry electrical stunning involves more handling stress for the fish than wet electrical stunning. The fish are removed from the water and put on an electrical conveyor to lose their consciousness (Gräns et al. 2016). Gräns et al. (2016) show in Arctic char a plasma cortisol level of around 5 ŋg/mLin the control group. Cannulated arctic chars clearly show higher plasma cortisol levels with 20 ŋg/mL(Seth et al. 2013) (Sandblom et al. 2012). During dry electrical stunning, the Arctic char shows a clear increase in cortisol levels, at around 35 ng ml^{-1}. Future studies should include measurements of consciousness, this knowledge gap currently prevents accurate welfare assessment of fish during stunning. Evidence of unconsciousness and insensibility of the 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 salmons after percussive stunning using an air pressure of 8.1 to 10 bars, while 8 fish were considered conscious at pressures below 8.1 bars, although some exhibited seemingly unconscious on behaviour (Lambooij et al. 2010). For percussive stunning, we conclude that if sufficient force is used, the fish will be rendered unconscious and insensible which results in damage of the carcass. For electrical stunning a combined AC and DC can be recommended (Lambooij et al. 2010). There are difficulties in understanding why a combined AC/DC current supply turns out to be more efficient than a single AC or DC source. It is well known that AC currents in the range of 50 – 100 Hz will cause substantial injuries in salmon (Roth, Moeller, and Slinde 2004).

Influence of colour on stress response

Volpato and Barreto (2001) show the influence of blue, green or white light on the stress response of the Nile tilapia, *Oreochromis niloticus (L.).* Blue light decreases the plasma cortisol in Nile tilapia (Volpato and Barreto 2001). This study also shows a stress reduction effect of blue light in Salvelinus alpinus x fontinalis. The stress reduction effect of blue light is also known in humans in colour therapy. Colour effects on a fish's life seem to be a species-dependent phenomenon which may be related to specific habitat characteristics (Volpato and Barreto 2001). In the chapter 6.1 the cortisol results show that blue light decreases the plasma cortisol level. Although the blue light is probably not practical in farms, experiments would be interesting with coloured inner walls of tanks for transports or stunning to reduce stress. Different effects of light are discussed in literature, not only on cortisol level but also on weight gain, feed efficiency, resistance to disease and survival (Mclean et al. 2008). Volpato and Barreto (2001) clearly show a cortisol reduction effect due to blue light.

Influence of water temperature reduction on stress response

The study in Lake Inari with Arctic Char (*Salvelinus Alpinus)* showed the fastest growing rate at 10.3 - 14.1°C and was clearly lower at 18.1°C (Lyytikäinen et al. 2002). During our experiments the stunning temperature was set to 10.50°C in tank 3 and 7.80°C in tank 4. The hybrids used in this study prefer slightly warmer temperatures than the Arctic Char used in the Lyytikäinen study. Seth et al. (2013) showed that CO_2 stunning elicits physiological and behavioural stress response that are not (!) reduced by hypothermia, a rapid temperature drop from 10°C to 0.25°C show even a slight elevation. In this work the water temperature reduction happened slowly but still showed an increase in cortisol level. The reduction was done completely different,– with the idea to avoid stress and a thermal shock - and a temperature difference of 5°C over a period of two days was created. The results were surprising which is reflected in the rejection of the Hypothesis H1.3. and H2.3. A stress reduction caused by water temperature reduction was expected but a different picture was observed. The results show a strong picture: P & C and E & C are alone in group 3 with the highest, clearly elevated cortisol levels. Thus, a shock behaviour does not make sense. Still the increase in cortisol is obvious, it seems that the chars are stressed by the changed environment and the reduction of metabolism is not counteracting the effect of the environmental changes. Consequently the water temperature reduction shows an increase in cortisol level as it is shown in chapter 6.1. Behavioural differences could not be visually observed. The group with water temperature reduction was harder to stun, especially percussive stunning requires a higher number of blows compared to the control group. It seems that the reduction of water temperature leading to higher cortisol levels creates a more stable metabolism due to the reduced metabolism. The idea behind this hypothesis was to cool the water down to 1°C, keep it there for a distinct time (around 1 day), allowing the fish a chance to adapt to their low surrounding temperature and then stun the fish to reduce the stress in the fillet. Due to a lack of available equipment for realisation, this experiment was not possible and would be of high interest for future studies. In this experiment, the fish would be almost frozen and therefore their metabolism should be low which could also increase their resilience against stunning. Although this is an interesting idea it should be mentioned that these extremely low temperatures are not comfortable temperatures for char. It may cause stress for the fish although they can handle these temperatures in nature.

8.2 Texture

The texture and structure of fish muscle are important freshness quality attributes that depend on several parameters such as hardness, cohesiveness, springiness, chewiness, resilience, and adhesiveness, as well as the internal cross-linking of connective tissue and the detachment of fibers (Cheng et al. 2014). Group 1, with P & B and E & C, present a lower flesh quality compared to the other settings. Recognizing only minor differences, it is assumed that different parameters, i.e. the rigor mortis onset, or the use of other characteristic parameters of mechanical texture analyses, e.g. absolute Elasticity or Term50 would show more significant differences. The texture measurement by itself is highly complex and a lot of measurement options are possible. Since there is no standard method in literature, a method was created. The complexity is high, Hanz (2008) perfomed her Masterthesis about texture analyses in fish as there are a lot of variations possible, e.g. variation in fixtures, in penetration depth (percentage or absolute), in test speed, in the method (Relaxation method), in holding time, in applied force and many more.

Analysis with the AM of the three values per fish compared to the use of all individuals' values give the same information but still the use of all individual values gives a clearer picture. The calculation of the AM creates a loss of data. Since the heterogeneity of fish fillet is high and shows variations due to the position, there should always be more than one measurement and if the work is not focused on the texture measurement there should be a focus neither on the dorsal or the pelvic area. Creating an AM of both areas is not recommended due to the high heterogeneity which was visible in the pre-experiments for determining a reliable method. Also, measurement on the lateral line are a bad choice since the upper and lower loin are highly heterogenic. The anterior and posterior areas should not be involved in analyses apart from tests exclusively focusing on texture analyses. Hanz (2008) showed in her master thesis that processing reduces the variations in the fillet. It could be wise to avoid processing of a high-quality flesh. Literature shows that, for fresh fillets the filleting method was the predominant factor for the end quality overshadowing the influence of pre-slaughter conditions and stunning method. Salting and smoking eliminated differences caused by slaughter or filleting methods. It was concluded that the quality of Atlantic salmon is influenced in the following order: stunning method < pre slaughter conditions < filleting method < processing by salting and smoking (Roth, Birkeland, and Oyarzun 2009). Cheng et al. (2014) concluded that all texture parameters changed with post-mortem storage, and most of them decrease significantly within five days of storage. In particular, the values of hardness, gumminess, and chewiness all decreased significantly, by about one-half, in comparison with the values observed at pre-rigor (Cheng et al. 2014).

In general it states a question if sensory parameters would make more sense due to the high heterogeneity of fish fillet and the simplicity of the sensory parameters, in special the parameter rigor

mortis onset. The Rigor mortis onset gives information about slaughter stress and storage life. A correlation analyses of the fillet texture and rigor mortis would be interesting. The problem hereby is that for the fillet analyses pre-rigor fillet are necessary which make it impossible to analyse the rigor-mortis onset and fillet texture of one fish, which was the reason choosing the more objective parameter in this thesis.

8.3 Correlation

The general model shows no correlation between cortisol and relative Elasticity , but correlations are shown when separating by stunning methods, still yielding high variations. A general fitting model cannot be observed because depending on the stunning methods different models show a better fitting. An interesting observation was made in regards to the best fit models for the stunning methods: For all stunning methods, percussive and electric, the linear model gives the highest variations whereas the cubic model always shows the best fit. A linear model is straight and there is little place to adapt to data whereas the quadratic and the cubic models are more flexible to adapt to the data resulting in higher R^2. A correct interpretation of a cubic model is challenging and should be done with attention.

Having a look at this data with the homogenous groups of the cortisol parameter shows an interesting trend: The categories that are found in variable cortisol in subsets 1 and 2 (lower stress levels) have a positive b1 value, except for E & C & B. The categories in subset 3, with the higher stress level, have a negative b1 value. This suggests that lower cortisol levels have no negative impact on flesh quality. High cortisol levels could have a negative impact.

9 Outlook

Further experiments would be of high interest. There are interesting topics, especially irreversible tests are of interest, e.g. cortisol analyses with cortisol abstraction from water. There are also open literature issues, e.g. whether cortisol can also be found in the fillet/tissue and whether is absorbed by humans after fish consumption. The cortisol concentration in the tissue may show the long-term stress. The combination of the cortisol concentration in the tissue and the one in blood plasma show a long- and short-term stress evaluation. Also, a cooperation with the food science department makes sense to determine the best option for a differentiation method. The implementation of the pH of the fillet or the rigor mortis onset are also cheaply observable quality parameter and states a wise parameter for ongoing research. In the scientific community heart rate tags are also often used in combination for stress determination. This would be quite interesting. Therefore, an implantation or an external fixing is possible.

10 References

Bahuaud, D., M. Gaarder, E. Veiseth-Kent, and M. Thomassen. 2010. "Fillet Texture and Protease Activities in Different Families of Farmed Atlantic Salmon (Salmo Salar L.)." *Aquaculture* 310(1–2): 213–20.

Barbosa, Alexandra, and Paulo Vaz-Pires. 2004. "Quality Index Method (QIM): Development of a Sensorial Scheme for Common Octopus (Octopus Vulgaris)." *Food Control*.

Barry, Terence P., Anita F. Lapp, Terrence B. Kayes, and Jeffrey A. Malison. 1993. "Validation of a Microtitre Plate ELISA for Measuring Cortisol in Fish and Comparison of Stress Responses of Rainbow Trout (Oncorhynchus Mykiss) and Lake Trout (Salvelinus Namaycush)." *Aquaculture* 117(3–4): 351–63.

Barton, Bruce A. 2002. "Stress in Fishes : A Diversity of Responses with Particular Reference to Changes In." *Integ. and Comp. Biol.*

Casas, C. et al. 2006. "Textural Properties of Raw Atlantic Salmon (Salmo Salar) at Three Points along the Fillet, Determined by Different Methods." *Food Control*.

Cheng, Jun Hu, Da Wen Sun, Zhong Han, and Xin An Zeng. 2014. "Texture and Structure Measurements and Analyses for Evaluation of Fish and Fillet Freshness Quality: A Review." *Comprehensive Reviews in Food Science and Food Safety* 13(1): 52–61.

Cohen, Jacob. 1988. "Statistical Power Analysis for the Behavioral Sciences." *Technometrics*.

Concollato, A et al. 2016. "Effects of Stunning/Slaughtering Methods in Rainbow Trout (Oncorhynchus Mykiss) from Death until Rigor Mortis Resolution." *Aquaculture* 464: 74–79. https://www.scopus.com/inward/record.uri?eid=2-s2.0-84974559681&doi=10.1016%2Fj.aquaculture.2016.06.009&partnerID=40&md5=4d0297f80e5d0529475fbce96a8c893f.

Erikson, U. et al. 1999. "Contribution of Bleeding to Total Handling Stress during Slaughter of Atlantic Salmon." *Aquaculture International* 7(2): 101–15.

Farwell, Michelle, Megan L.M. Fuzzen, Nicholas J. Bernier, and Robert L. McLaughlin. 2014. "Individual Differences in Foraging Behavior and Cortisol Levels in Recently Emerged Brook Charr (Salvelinus Fontinalis)." *Behavioral Ecology and Sociobiology*.

Gräns, A. et al. 2016. "Stunning Fish with CO2 or Electricity: Contradictory Results on Behavioural and Physiological Stress Responses." *animal*.

Hunt K.E. et al., 2006, Analysis of fecal glucocorticoids in North Atlantic right whale (Eubalaena glacialis), *General and Comparative Endocrinology* 148 (2006) 260-272

Lambooij, E. et al. 2010. "Percussion and Electrical Stunning of Atlantic Salmon (Salmo Salar) after Dewatering and Subsequent Effect on Brain and Heart Activities." *Aquaculture*.

Levine, J. S., and E. F. MacNichol. 1982. "Color Vision in Fishes." *Scientific American*.

Lines, J A, and J Spence. 2014. "Humane Harvesting and Slaughter of Farmed Fish." *Rev. sci. tech. Off. int. Epiz* 33(1): 255–64.

Lines, Jeff, and Steve Kestin. 2004. "Electrical Stunning of Fish: The Relationship between the Electric Field Strength and Water Conductivity." *Aquaculture*.

Lyytikäinen, Tapani, Päivi Pylkkö, Ossi Ritola, and Pirjo Lindström-Seppä. 2002. "The Effect of Acute Stress and Temperature on Plasma Cortisol and Ion Concentrations and Growth of Lake Inari Arctic Charr, Salvelinus Alpinus." In *Environmental Biology of Fishes*,.

Mclean, E, P Cotter, Claire Thain, and N King. 2008. “TANK COLOR IMPACTS PERFORMANCE OF CULTURED FISH.” *Ribarstvo* 66(2): 43–54.

Mommsen, Thomas P., Mathilakath M. Vijayan, and Thomas W. Moon. 1999. “Cortisol in Teleosts:Dynamics, Mechanisms of Action, and Metabolic Regulation.” *Reviews in Fish Biology and Fisheries* 9(3): 211–68.

Pickering, A.D., and T.G. Pottinger. 1985. “Cortisol Can Increase the Susceptibility of Brown Trout, Salmo Trutta L., to Disease without Reducing the White Blood Cell Count.” *Journal of Fish Biology* 27(5): 611–19.

Poli, B.M., G. Parisi, F. Scappini, and G. Zampacavallo. 2005. “Fish Welfare and Quality as Affected by Pre-Slaughter and Slaughter Management.” *Aquaculture International* 13(1–2).

Pottinger, T. G. 2010. “A Multivariate Comparison of the Stress Response in Three Salmonid and Three Cyprinid Species: Evidence for Inter-Family Differences.” *Journal of Fish Biology*.

Rasmussen, Richard Skoøtt. 2001. “Quality of Farmed Salmonids with Emphasis on Proximate Composition, Yield and Sensory Characteristics.” *Aquaculture Research* 32(10): 767–86.

Robb, D. H.F., and S. C. Kestin. 2002. “Methods Used to Kill Fish: Field Observations and Literature Reviewed.” *Animal Welfare* 11(3): 269–82.

Robb, D. H.F., M. O’Callaghan, J. A. Lines, and S. C. Kestin. 2002. “Electrical Stunning of Rainbow Trout (Oncorhynchus Mykiss): Factors That Affect Stun Duration.” *Aquaculture*.

Roth, Bjorn, Sveinung Birkeland, and Fernando Oyarzun. 2009. “Stunning, Pre Slaughter and Filleting Conditions of Atlantic Salmon and Subsequent Effect on Flesh Quality on Fresh and Smoked Fillets.” *Aquaculture*.

Roth, Bjorn, Dag Moeller, and Erik Slinde. 2004. “Ability of Electric Field Strength, Frequency, and Current Duration to Stun Farmed Atlantic Salmon and Pollock and Relations to Observed Injuries Using Sinusoidal and Square Wave Alternating Current.” *North American Journal of Aquaculture*.

Sandblom, Erik et al. 2012. “Effects of Electric Field Exposure on Blood Pressure, Cardioventilatory Activity and the Physiological Stress Response in Arctic Char, Salvelinus Alpinus L.” *Aquaculture*.

———. 2013. “Stress Responses in Arctic Char (Salvelinus Alpinus L.) during Hyperoxic Carbon Dioxide Immobilization Relevant to Aquaculture.” *Aquaculture*.

Seth, H. et al. 2013. “Physiological Responses and Welfare Implications of Rapid Hypothermia and Immobilisation with High Levels of CO2at Two Temperatures in Arctic Char (Salvelinus Alpinus).” *Aquaculture*.

Stamer. 2009. “Betäubungs- Und Schlachtungsmethoden Für Speisefische.”

Volpato, G. L., and R. E. Barreto. 2001. “Environmental Blue Light Prevents Stress in the Fish Nile Tilapia.” *Brazilian Journal of Medical and Biological Research* 34(8): 1041–45.

Wendelaar Bonga, S E. 1997. “The Stress Response in Fish.” *Physiol. Rev.* 77(3): 591–625. http://physrev.physiology.org/cgi/content/abstract/77/3/591.

10.1 Internetreferences

BMLFUW, Ministerium für ein lebenswertes Österreich of 30th of May 1017, URL: https://www.bmlfuw.gv.at/land/produktion-maerkte/tierische-produktion/fischzucht-

oe/aquakultur.html. Acces on 18th of December 2017.

FAO, Aquaculture resources, 2017, URL: http://www.fao.org/fishery/resources/aquaculture/en. Access on 18th of December 2017.

o.V. "Saiblinge und Saiblingskreuzungen in der Forellenteichwirtschaft" at Bayerische Landesanstalt für Landwirtschaft , 2016.

URL: https://www.lfl.bayern.de/ifi/forellenteichwirtschaft/030013/index.php. Access on 29th of October 2018.

o.V. "Solid Phase Extraction" at Affinisep, 8th of July 2018, URL: https://www.affinisep.com/technology/solid-phase-extraction/ . Access on 19th of February 2019.

o.V. "Fisch" at Statistik Austria 2016, 14th of June 2019, URL: https://www.ages.at/themen/lebensmittelsicherheit/tierische-lebensmittel/fisch/

Süsswaren. „LC-MS/MS – Was ist das eigentlich?" at Lebensmittelchemisches Institut des Bundesverbandes der Deutschen Süßwarenindustrie e.V., 2002 Heft 9, URL: http://www.lci-koeln.de/deutsch/veroeffentlichungen/lci-focus/lc-ms-ms-was-ist-das-eigentlich-. Access on 15th of February 2019.

Teitge Felix, Tiergerechtes Betäuben und Schlachten von Regenbogenforellen at Stiftung Tierärztliche Hochschule Hannover, 23rd of November 2016, URL: http://docplayer.org/59195558-Tiergerechtes-betaeuben-und-schlachten-von-regenbogenforellen.html. Access on 26th of November 2018.

10.2 Bookreferences

Hanz, K. (2008), Texturale und farbliche Veränderungen im Herstellungsprozess von Räucherlachs (Salmo salar). Masterthesis, University of Life Sciences and Natural Resources

Leitfaden zur Fischkunde und Angelfischerei, herausgeben vom Oö. Landesfischereiverband, 4., erweiterte Auflage 2012, S.58

Verordnung der Bundesministerin für Gesundheit über den Schutz von Tieren bei der Schlachtung oder Tötung (Tierschutz-Schlachtverordnung) (BGBl. II Nr. 312/2015)

Eidesstaatliche Erklärung

Ich, Katharina Fraunbaum, erkläre eidesstattlich, dass ich die Arbeit selbständig angefertigt habe. Es wurden keine anderen als die angegebenen Hilfsmittel benutzt. Die aus fremden Quellen direkt oder indirekt übernommenen Formulierungen und Gedanken sind als solche kenntlich gemacht. Diese schriftliche Arbeit wurde noch an keiner Stelle vorgelegt.

_____

Katharina Fraunbaum