Anaesthesia of farmed fish with special emphasis on Atlantic cod (*Gadus morhua*) and Atlantic halibut (*Hippoglossus hippoglossus*)

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Scientific environment

The work presented in the current thesis was conducted at the Institute of Marine Research, Bergen, Norway, between 2004 and 2008. The experiments upon which the thesis is based were carried out at the Institute of Marine Research in Bergen, at Matre Research Station, and at Austevoll Research Station. The work of the first paper included in the thesis was conducted in collaboration with the Swedish University of Agricultural Sciences, and the Norwegian School of Veterinary Science.

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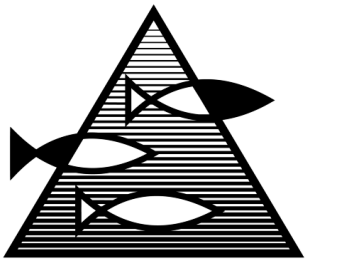

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Summary

During the life cycle as farmed animals there are numerous situations where fish are subjected to handling and confinement. Netting, weighing, sorting, vaccination, transport and, at the end, slaughter are common events under farming conditions. As research animals fish may also undergo surgical procedures, ranging from tagging, sampling, and small incisions, to larger operations. Under these varying situations treatment with anaesthetic agents might be necessary in order to ensure the welfare of the fish. Anaesthetic protocols for new species that are introduced to research or cultivation are generally based on protocols developed for the more established species. In Norway the anaesthetic protocols for Atlantic cod (*Gadus morhua*) and Atlantic halibut (*Hippoglossus hippoglossus*), which were introduced to fish farming in the 1980's, have thus been based on protocols used for salmonid species. Atlantic salmon (*Salmo salar*) has been farmed since the 1960's and is the most important species in Norwegian fish farming.

The main objective of the current investigation was to gain more knowledge regarding the effect of anaesthetic agents in farmed fish, with special emphasis on Atlantic cod and Atlantic halibut. Large variations in response to anaesthetic agents exist both between and within fish species. Factors such as body weight, water temperature and acute stress may be important for the response and were studied in the current investigation. The anaesthetic agents benzocaine, metacaine (MS-222), metomidate hydrochloride, isoeugenol, 2-phenoxyethanol, and quinaldine were used. In Atlantic cod and Atlantic halibut the agents were studied with regards to efficacy, assessed as induction and recovery times as well as reaction to handling under anaesthesia. In Atlantic salmon pharmacokinetic properties of the agents were examined.

Both in Atlantic cod and Atlantic halibut large differences in efficacy between the different anaesthetic treatments were found. Induction and recovery times varied both in relation to body weight and in relation to water temperature. The main trend observed in Atlantic cod at high water temperature was shorter induction and recovery times for all weight groups and treatments. Whereas in Atlantic halibut higher water temperature resulted in shorter induction times, longer recovery times, and increased responsiveness to handling. Atlantic halibut of large body size displayed longer induction times, shorter recovery times,

and reduced responsiveness to handling in comparison with fish of smaller body size. However, in Atlantic cod no uniform trend was found in the relationship between the size of the fish and anaesthetic efficacy. In Atlantic cod induction and recovery times were found to increase with increasing body weight for benzocaine and MS-222. For metomidate the recovery time increased with increasing weight whereas there were no weight related differences in induction time. No differences in either induction or recovery times associated to body weight were found for 2-phenoxyethanol. The pharmacokinetic study in Atlantic salmon showed that the anaesthetics were rapidly eliminated and that elimination was related to the water soluble characteristics of the agents. The recovery times were shorter in fish that were given artificial gill ventilation. In the assessment of the importance of acute stress prior to anaesthesia of Atlantic cod it was found that the stress resulted in significantly shorter induction time and prolonged recovery time, as well as deeper anaesthetised fish. The anaesthetic dosage had to be reduced in order to avoid mortality in fish anaesthetised subsequent to acute stress.

Anaesthetic protocols for fish have generally comprised one single agent, whereas protocols of human and veterinary medicine comprise combinations of several drugs, each one contributing with effects needed in the anaesthesia. Stress prior to anaesthesia may result in abnormal reactions, as seen in Atlantic cod in the current study, and may require dosage adjustments of drugs both for induction and maintenance. Pre-anaesthetic sedation is therefore commonly used in order to avoid stress and is an integrated part of the veterinary protocols. In the current study, protocols comprising combinations of two anaesthetic agents, one agent to induce sedation followed by one agent to induce anaesthesia, were tested in Atlantic cod and Atlantic halibut. In both species combination anaesthesia allowed a reduction of the dosages used for inducing anaesthesia. In Atlantic cod combination anaesthesia resulted in markedly reduced recovery times compared to agents administered individually. In Atlantic halibut combination anaesthesia had no effect on the induction times in small fish in comparison with individual agents, but resulted in significantly shorter recovery times and reduced responsiveness to handling. In Atlantic halibut of large body size combination anaesthesia gave rise to shorter induction times than individual administered agents whereas no uniform trend was observed in recovery times and no differences in responsiveness to handling were noticed.

Anaesthetic agents are commonly used in fish farming to avoid stress during various farming practices. While several studies report that anaesthetic agents are effective in reducing the stress associated with confinement and handling, there are also reports indicating that the exposure to anaesthetics may in itself induce a stress response, measured by increased levels of cortisol. In order to examine stress induced by exposure to anaesthetic agents the release of cortisol to water following anaesthetic exposure was examined in Atlantic cod, Atlantic halibut and Atlantic salmon. In this examination the fish were not subjected to any concomitant handling in connection to anaesthesia or sampling. The plasma cortisol concentration during anaesthesia was examined in Atlantic salmon, however this examination included some degree of handling. All of the anaesthetics tested induced a release of cortisol to water in all three species, with maximum release rates measured 0.5-1 hour post exposure. This also complied with the plasma cortisol levels measured in Atlantic salmon. MS-222 elicited the highest cortisol release rates while benzocaine caused a bimodal response where the initial peak in cortisol release rate was followed by a second and smaller peak. Metomidate induced the lowest release of cortisol of the agents tested in both Atlantic halibut and Atlantic cod, but resulted in a bimodal response in Atlantic salmon where the initial increase in cortisol release was followed by an even larger increase. The stress induced in Atlantic salmon by isoeugenol resembled that of MS-222, but did not reach the same elevated level. Over all the cortisol release was most profound in Atlantic salmon followed by Atlantic halibut and Atlantic cod.

Based on the findings in the current study it is recommended that anaesthetic protocols should always be tested on a few fish under prevailing conditions in order to ensure an adequate level of depth while avoiding overdosing. This recommendation applies whether one single agent or a combination of agents are used although it was found here that protocols comprising combinations of agents provide wider margins of safety. While exposure to anaesthetic agents was found to elicit a stress response, displayed as increased levels of cortisol, the amount of cortisol released in response to anaesthesia was low compared to what is reported following strong stressors such as handling and confinement. Stress caused by anaesthetic agents may however represent an extra load during otherwise stressful circumstances.

List of publications

This thesis is based on the following papers, referred to in the text by their Roman numerals:

Paper I

Kiessling, A., Johansson, D., Zahl, I.H., Samuelsen, O.B., 2009. Pharmacokinetics, plasma cortisol and effectiveness of benzocaine, MS-222 and isoeugenol measured in individual dorsal aorta-cannulated Atlantic salmon (*Salmo salar*) following bath administration. Aquaculture 286, 301-308.

Paper II

Zahl, I.H., Kiessling, A., Samuelsen, O.B., Hansen M.K., 2009. Anaesthesia of Atlantic cod (*Gadus morhua*) – Effect of pre-anaesthetic sedation, and importance of body weight, temperature and stress. Aquaculture 295, 52-59.

Paper III

Zahl, I.H., Kiessling, A., Samuelsen, O.B., Hansen M.K., Anaesthesia of Atlantic halibut (*Hippoglossus hippoglossus*) – Effect of pre-anaesthetic sedation, and importance of body weight and water temperature. Aquac. Res., Available online 30.Nov.2010, DOI: 10.1111/j.1365-2109.2010.02711.x.

Paper IV

Zahl, I.H., Kiessling, A., Samuelsen, O.B., Olsen, R.E., 2010. Anesthesia induces stress in Atlantic salmon (*Salmo salar*), Atlantic cod (*Gadus morhua*) and Atlantic halibut (*Hippoglossus hippoglossus*). Fish Physiol. Biochem. 36, 719-730.

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1. Introduction

1.1 Background

Commercial fish farming has since the breakthrough in the 1970's become one of the largest industries in Norway. Salmonid fish species dominate, with Atlantic salmon (*Salmo salar*) constituting nearly 90% of the total Norwegian production reaching an estimated harvest quantity of more than 735 000 metric tonnes in 2008 (Statistics Norway[1]). Commercial farming of Altantic cod (*Gadus morhua*) and Atlantic Halibut (*Hippoglossus hippoglossus*) was introduced in the early 1980's. In 2008 the estimated harvest quantities of these species reached 18 000 and 1 500 metric tonnes respectively (Statistics Norway[1]). By the end of 2008 the live stock counted over 320 million salmonid fish, nearly 25 million Atlantic cod and 2 million Atlantic halibut (Statistics Norway[1]).

According to the National Animal Research Authority of Norway, 97% of the 1.92 million animals used for research in Norway in 2008 were fish (Volckmar et al., 2009). The majority were among salmonid species, which for the most part were included in aquaculture field trials regarding development of fish vaccines. Atlantic cod and Atlantic halibut were primarily used in pure research. Additionally, approximately 3 million fish kept in research laboratories are not included in the statistics. This group of fish comprise fish that were not subjected to procedures that would affect their normal way of life or produce non-physiological states, in addition to surplus fish not used in research, and these fish are thus not defined as research animals.

1.2 Fish welfare

It is our responsibility to ensure good welfare of the fish that are in our care, whether they are held as pets, ornamental, under farming conditions or as research animals. Although fish show a wide range of signs in response to impaired wellbeing and recent studies suggest that fish are sentient creatures with the capacity to suffer (Braithwaite and Huntingford, 2004; Chandroo et al., 2004; Huntingford et al., 2006; Sneddon, 2003b; Sneddon et al.,

[1] www.ssb.no (Statistics Norway)

2003) the concept of fish welfare is still under discussion (Arlinghaus et al., 2007; Braithwaite and Huntingford, 2004; Braithwaite and Boulcott, 2007; Huntingford et al., 2007; Rose, 2002; 2007; Sneddon, 2003a). Several definitions based upon different perspectives of animal welfare are being applied (Huntingford and Kadri, 2009; Huntingford et al., 2006; Volpato, 2009). Animal welfare issues are implemented in international and national legislation, regulations and standards for both cultivation and research. According to the Norwegian Animal Protection Act from 1974 (Lov om dyrevern, 1974) animals shall be treated well and consideration shall be given to the instinctive behaviour and natural needs so that there is no risk of causing unnecessary pain or suffering. The revised Animal Protection Act, the Animal Welfare Act (Lov om dyrevelferd, 2010), which entered into force in 2010 takes in that animals have an intrinsic value in addition to a useable value, and includes the respect for animals in the intention of the act. The World Medical Association Declaration of Helsinki, developed in 1964 to govern ethical principles regarding human experimentation in medical research, has also included the welfare of research animals as a basic principle in all medical research. Medical and surgical interventions should be carried out with respect to the welfare of the animal and protect the animal's performance and its quality of life.

During cultivation the fish encounter several situations where they are subjected to handling. Netting, weighing, sorting, vaccination, transport and, at the end, slaughter are regular events during fish farming. As research animals fish may also undergo surgical procedures, ranging from tagging, sampling, and small incisions to larger operations. Under these varying situations treatment with anaesthetic agents might be necessary in order to ensure the welfare of the fish.

1.3 Anaesthesia of fish

Anaesthetic agents have been used for fish since the beginning of the last century (McFarland, 1959; McFarland and Klontz, 1969; Schoettger and Julin, 1967). They were initially applied in order to facilitate handling, but an increased knowledge of fish physiology and a better understanding of the importance of anaesthetic treatment have led to an increased use. Anaesthetics are now applied widespread, ranging from light sedation in

order to reduce stress during handling and non-invasive procedures to full anaesthesia in order to avoid inflicting pain during surgery and larger interventions (Ackerman et al., 2005; Neiffer and Stamper, 2009; Ross and Ross, 2008; Summerfelt and Smith, 1990). The most common route of administration is via inhalation. Agents are absorbed over the gills when the fish are exposed to anaesthetic agents dispersed in water. The effect is usually assessed by induction and recovery times, reflex reactions to external stimuli and responsiveness to handling.

The progress of induction, and the depth of anaesthesia, is generally divided into different stages and planes (Table 1). Common parameters that are evaluated in order to determine these stages and planes during anaesthesia of animals include behaviour, activity, eye signs, muscle tone, reflexes, respiratory rate, heart rate, and blood pressure. As several of these parameters are difficult to assess in fish it may be difficult to distinguish one stage or plane from the other, especially in situations where induction is fast. In fish stages and planes are therefore usually described by changes in swimming activity, balance, respiratory frequency as well as reactions to external stimuli (Table 1).

Table 1 Stages of anaesthesia in fish

Stage	Plane	Description	Behaviour	Activity	Equilibrium	Responsiveness	Muscle tone	Respiration	Heart rate
0		Normal	Normal	Swimming	Normal	Reactive	Normal	Normal	Normal
I		Light sedation	Disoriented	Reduced	Normal	Slightly reduced	Normal	Normal	Normal
II		Excitatory stage	Excited	Increased	Struggles to maintain balance	Normal or exaggerated	Normal	Irregular or increased	Irregular, may increase
III	1	Light anaesthesia	Anaesthetised	Stopped	Lost	Reacts to strong tactile stimuli	Decreased	Normal or decreased	Regular
	2	Surgical anaesthesia	Anaesthetised	Stopped	Lost	None	Relaxed	Shallow	Depressed
	3	Deep narcosis	Anaesthetised	Stopped	Lost	None	None	Nearly absent	Depressed
IV		Impending death	Moribund	Stopped	Lost	None	None	Stopped	Cardiac arrest

Adapted from Bell, 1987, Burka et al., 1997, McFarland, 1959, McFarland and Klontz, 1969, and Summerfelt and Smith, 1990.

1.4 Anaesthetic agents

Anaesthesia (from Greek: an- 'without', and aisthesis 'sensation') consists of several components, including relaxation, immobilisation, unconsciousness, amnesia, and analgesia. These components can be obtained by various anaesthetic agents, each giving rise to one or several components. A large selection of agents is being used for fish. Among the most common are MS-222, benzocaine, isoeugenol, metomidate, 2-phenoxyethanol and quinaldine (Ackerman et al., 2005; Neiffer and Stamper, 2009; Ross and Ross, 2008; Summerfelt and Smith, 1990).

Much of the knowledge regarding the effect of many of the agents used for anaesthesia of fish is based on data from other vertebrate species. Extrapolating data on the effects between different species should be exercised with great caution. This also applies to fish. There are over 30.000 different species of fish (Froese and Pauly, 2010) inhabiting environments of high diversity, varying in salinity, temperature and depth. The route of administration of anaesthetic agents may differ between different species. General anaesthesia of terrestrial animals is often obtained by i.v administration of anaesthetics, whereas in fish the anaesthetics are most commonly administered via inhalation by exposing the fish to the agents through bath immersions.

1.4.1 Metacaine and benzocaine

Metacaine (ethyl 3-aminobenzoate, tricaine methanesulphonate, MS-222) (Figure 1) and benzocaine (ethyl 4-aminobenzoate) (Figure 2) are the two most common anaesthetics used for fish in Norway (Anon., 2007). They are approved for use in food fish production with a withdrawal period of 21 days.

MS-222 and benzocaine are both local anaesthetics that inhibit the initiation and propagation of action potentials by blocking voltage-sensitive sodium channels (Frazier and Narahashi, 1975; Neumcke et al., 1981). This class of drugs are used in human and veterinary medicine as topical analgesics. When administrated to fish via bath immersion they enter the circulation and produce anaesthesia by inhibiting neural signal transmission ranging from the periphery to higher parts of the nervous system. The exact mechanism in the central part of the nervous system is not fully understood (Hara and Sata, 2007; Hedrick

and Winmill, 2003; Ueta et al., 2007) but many of the unwanted side effects caused by this class of drugs in man are related to CNS effects.

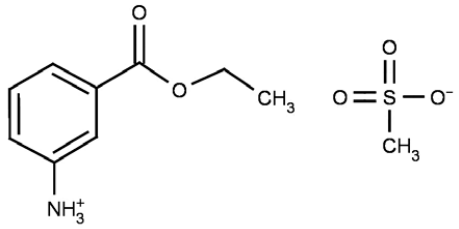

Figure 1 MS-222 ($C_{10}H_{15}NO_5S$)

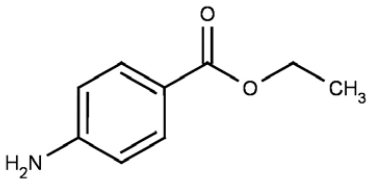

Figure 2 Benzocaine ($C_9H_{11}NO_2$)

A range of adverse effects of MS-222 and benzocaine have been reported in fish. The initial response to these agents is characterised by a period of increased heart rate and respiration accompanied by increased levels of blood glucose, followed by a depression of cardiovascular and respiratory function that eventually may come to a complete stop (Hill et al., 2002; Houston et al., 1971a; Lochowitz et al., 1974; Randall, 1962; Ryan, 1992). This will subsequently lead to hypoxaemia, observed as changes in partial pressure of arterial O_2 and CO_2 accompanied by decreased blood glucose levels and increased levels of lactate, increased hematocrit and haemoglobin, and erythrocyte swelling (Bourne, 1984; Hill and

Forster, 2004; Holloway et al., 2004; Houston et al., 1971b; Iwama et al., 1989; Ortuno et al., 2002; Ryan, 1992; Soivio et al., 1977; Thomas and Robertson, 1991; Velisek et al., 2009). Increased plasma levels of catecholamines have also been observed (Gingerich and Drottar, 1989; Iwama et al., 1989; Wedemeyer, 1970). Benzocaine has also been found to have immunodepressant effects (Cuesta et al., 2004; Ortuno et al., 2002).

1.4.2 Metomidate

Metomidate hydrochloride (methyl 3-(1-phenylethyl) imidazole-4-carboxylate hydrochloride) (Figure 3) is a methyl analogue of the imidazole derivate etomidate, a non-barbiturate hypnotic that activates and modulates inhibitory gamma-aminobutyric acid type A ($GABA_A$) receptors, thus affecting higher regions of the nervous system (Ashton and Wauquier, 1985; Yang and Uchida, 1996). Etomidate has a strong selectivity for $GABA_A$ receptors producing sedation and hypnosis, but has limited analgesic and immobilising effect (Grasshoff et al., 2006).

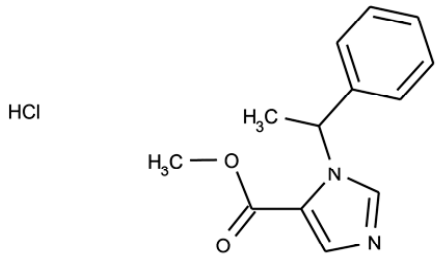

Figure 3 Metomidate hydrochloride ($C_{13}H_{14}N_2O_2 \cdot HCl$)

Etomidate is commonly used in human and veterinary medicine as a sedative and for inducing anaesthesia (Ching and Baum, 2009; Darrouj et al., 2009; Falk and Zed, 2004; Sams et al., 2008). One of the side effects of the drug is suppression of adrenal

steroidogenesis, leading to an inhibition of the synthesis of cortisol (Vanden Bossche et al., 1984; Wagner et al., 1984) (man, cow, rat). Metomidate has also been found to have an inhibitory effect on cortisol in fish (Davis and Griffin, 2004; Olsen et al., 1995; Small, 2004; Thomas and Robertson, 1991) but efficiency and dose dependence of this inhibition seems to be unclear (Eliason et al., 2007; Olsen et al., 1995; Sandodden et al., 2001). Other adverse effects of metomidate in fish are related to depressant effects on respiration and circulation subsequently leading to hypoxemia observed as decreased partial pressure of O_2, increased partial pressure of CO_2 and reduced pH of the blood (Hill and Forster, 2004; Hill et al., 2002; Iwama et al., 1989).

1.4.3 2-phenoxyethanol

2-phenoxyethanol (Figure 4) is a compound with antibacterial properties used as a preservative in vaccines, in dermatological products and as fixative for perfumes. The exact mechanism for the anaesthetic effect in fish has to my knowledge not been reported but it has been suggested that it involves an expansion of neuronal cell membranes (Burka et al., 1997). 2-phenoxyethanol has been found to inhibit the activity of excitatory *N*-methyl-D-aspartate (NMDA) receptors in *Xenopus* oocytes (Musshoff et al., 1999) and the anaesthetic effect may thus be related to suppression of neural activity in higher regions of the nervous system. NMDA receptor inhibition is linked to analgesic effect (Grasshoff et al., 2006).

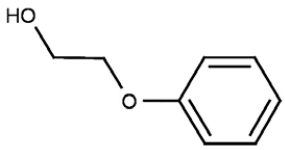

Figure 4 2-phenoxyethanol ($C_8H_{10}O_2$)

Adverse effects of 2-phenoxyethanol in fish include reduced ventilation, decreased heart rate and blood pressure, reduced blood partial pressure of O_2 accompanied by increased CO_2 and decreased blood pH, as well as increased plasma levels of adrenaline and glucose

(Fredricks et al., 1993; Iwama et al., 1989; Lambooij et al., 2009; Ortuno et al., 2002). 2-phenoxyethanol has also been found to have immunodepressant effects (Cuesta et al., 2004; Ortuno et al., 2002).

1.4.4 Isoeugenol

Isoeugenol (2-methoxy-4-prop-1-enyl-phenol) (Figure 5) is an oily liquid that is found in ylang-ylang and other essential oils. It is structurally similar to eugenol, a widely used analgesic in dentistry which inhibits sodium, potassium, and calcium channels, inhibits NMDA receptors and potentiates $GABA_A$ receptors (Aoshima and Hamamoto, 1999; Lee et al., 2005; Li et al., 2007; Park et al., 2006; Wie et al., 1997). Isoeugenol is, together with eugenol, among the constituents of clove oil, another agent commonly used for anaesthesia of fish. Isoeugenol is the active ingredient in Aqui-S®, which has approval for use in food fish production in New Zealand, Australia and Chile with no withdrawal period. In Norway Aqui-S® is used for brood stock and in research.

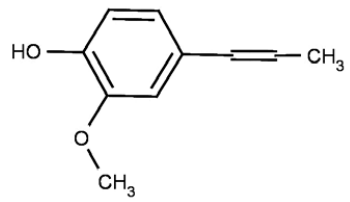

Figure 5 Isoeugenol ($C_{10}H_{12}O_2$)

Adverse effects of isoeugenol (Aqui-S®) in fish include reduced ventilation and depression of the cardiovascular system, which result in slower heart rate, decreased cardiac output, in addition to reduced blood pressure and vascular tone (Hill and Forster, 2004; Hill et al., 2002; Rothwell and Forster, 2005). Elevated plasma levels of catecholamines have also been seen observed as well as increased hematocrit (Hill and Forster, 2004).

1.4.5 Quinaldine

Quinaldine (2-methylquinoline) (Figure 6) is a colourless oily liquid that has been used to anaesthetise fish since the beginning of the last century. The exact mechanism of action has, to my knowledge, not been reported.

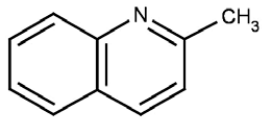

Figure 6 Quinaldine ($C_{10}H_9N$)

Quinoline family compounds have antiseptic and antipyretic properties. They are widely used as parent compounds to make drugs, especially anti-malarial medicines, and are also used in fungicides, biocides, alkaloids, dyes, rubber chemicals and flavouring agents.

Fish anaesthetised with quinaldine respond by an increased heart rate followed by bradycardia and impaired respiratory function (Lochowitz et al., 1974). Elevated concentrations of plasma cortisol and glucose have also been observed as well as increased levels of serum IgM (Cuesta et al., 2004; Davis and Griffin, 2004; Ortuno et al., 2002).

1.5 Combination anaesthesia

Anaesthetic protocols of human and veterinary medicine comprise combinations of several drugs, each one contributing with effects needed in the anaesthesia. Different drugs are used for induction and maintenance, and both intra- and postoperative analgesic treatments are provided. The drugs are selected on the basis of their properties, tailored both to the surgical intervention and to the physiological state of the patient or the animal. Pre-anaesthetic sedation used in order to avoid stress prior to anaesthesia is an integrated part of the veterinary protocols. Combining drugs of different properties provides a more complete

anaesthesia than what is possible with one single substance alone. Further, synergistic effects between the different drugs may also permit a reduction of the dosage of each drug compared to drugs administered individually. This may result in smoother induction and recovery and reduce the incidence of adverse effects (Rang et al., 2003).

1.5.1 Combination anaesthesia in fish

Current protocols for fish do not typically include administration of combinations of anaesthetics. Schoettger and Steucke (1970) examined possible synergistic effects of combination anaesthesia consisting of a mixture of MS-222 and quinaldine in rainbow trout (*Oncorhynchus mykiss*) and northern pike (*Esox lucius*) and found that the mixture resulted in a safer and more effective anaesthesia. The same mixture tested in 14 freshwater species showed that the dosages needed when administered in combination were considerably lower than the dosage of each agent used alone (Gilderhus et al., 1973). It was discovered however that not only effectiveness but also toxicity increased when theses agents were combined (Dawson and Marking, 1973). Synergistic effects have also been found in combination anaesthesia consisting of a mixture of quinaldine sulphate and the benzodiazepine diazepam (Kumlu and Yanar, 1999; Yanar and Kumlu, 2001). Administrated in combination with diazepam, quinaldine sulphate could be used in lower dosages in anaesthesia of both gilthead sea bream (*Sparus aurata*) and European sea bass (*Dicentrarchus labrax*). Hyperactivity and excitement, unwanted side effects of quinaldine sulphate, were reduced and there were no mortalities (Kumlu and Yanar, 1999; Yanar and Kumlu, 2001). Combinations consisting of MS-222 anaesthesia and intraoperative injections of local analgesics have been examined during surgery of koi carp (*Cyprinus carpio*) (Harms et al., 2005). Intraoperative injections of the opiate butorphanol reduced the behavioural changes post surgery while the non steroidal anti-inflammatory drug (NSAID) ketoprofen reduced the muscle tissue damage. Protocols that include both pre-anaesthetic sedation and intraoperative local analgesic treatment in addition to general anaesthesia have been successfully applied for anaesthesia of Atlantic salmon undergoing surgery for insertion of dorsal aorta and portal vein cannulae (Eliason et al., 2007; Karlsson et al., 2006; Kiessling et al., 2003; Kiessling et al., 1995). Pre-anaesthetic sedation with metomidate followed by full anaesthesia with MS-222 combined with intraoperative injections of lidocaine at the site of incision resulted in an improved recovery in

comparison with MS-222 used individually and fish that quickly resumed normal behaviour (Eliason et al., 2007; Karlsson et al., 2006; Kiessling et al., 2003; Kiessling et al., 1995).

1.6 Factors giving rise to variations in response

There are substantial variation between the fish species in response to anaesthetic agents, and also large individual differences within each species. Variations may result from differences in pharmacokinetics, commonly explained as what the body does to the drug, and by differences in pharmacodynamics, commonly explained as what the drug does to the body. Both pharmacokinetic and pharmacodynamic variations in fish may be influenced by biological factors such as age, sex, life stage, body weight, growth rate, body composition, physiological condition and health status as well as environmental factors such as water temperature, salinity, pH and oxygen level.

1.6.1 Pharmacodynamics

The pharmacodynamic characteristics of a drug describe the outcome of the interaction between the drug and the receptor or other primary site of action and include all effects and adverse effects. As described in section 1.4.1-1.4.5 the anaesthetic agents may act by binding to receptors or by interacting with ion channels. As several tissues may contain the target molecules of a drug the primary responses may induce secondary responses or side effects that subsequently affect the primary response. Thus, pharmacodynamic effects may influence the pharmacokinetic processes, e.g. an effect on respiration and circulation will as a consequence also be of importance both for absorption, distribution and elimination of drugs administered via inhalation.

1.6.2 Pharmacokinetics

In order to induce a biological response the drug must be present at the receptor site at a sufficient concentration over a certain length of time. The concentration achieved at the receptor site is dependent on the pharmacokinetic properties of the drug i.e. how the drug is absorbed, distributed, metabolised, and eliminated. In fish the administration of drugs via bath immersion is equivalent to inhalation anaesthesia in human and veterinary medicine. The processes of absorption and elimination are determined by the diffusion rate through the

gill epithelium which mainly depends on the lipid solubility, ionisation and polarity of the drug. Following absorption a fraction of the drug will bind to plasma protein, mainly albumin, while the rest will remain unbound and be pharmacologically active. The unbound drug will be distributed to the various parts of the body, commonly separated into compartments, where it exerts its effect. The main body compartments are plasma, interstitial fluid, intracellular fluid, and adipose tissue. Lipid solubility and ionisation are important factors for the distribution of the drug. High lipid solubility results in rapid diffusion through the cell membranes and a possible accumulation of the drug within hydrophobic compartments, such as the adipose tissue and the lipid bilayers of cell membranes. Ionised molecules and molecules with high polarity on the other hand are less lipid soluble and do not easily penetrate biological membranes.

Ionisation of weak acids and bases is determined by the dissociation constant p*K*a defined as the pH where 50% of the acid or base is in ionic form (p*K*a, Table 2). When a compound is allowed to distribute itself between equal volumes of two immiscible liquids, the ratio of the concentration of the solute in the two phases at equilibrium is called the partition coefficient (logP, Table 2). For drugs, the two phases used to describe the partition coefficient are octanol and an aquatic buffer with pH of 7.4. As the the logP value describes the partition of neutral uncharged molecules it is not an exact measure for the distribution of ionisable compounds such as many anaesthetic agents. Anaesthetic agents are generally associated with high lipid solubility which may lead to an accumulation in adipose tissue both during long exposures and following repeated exposures (Rang et al., 2003).

Table 2 logP (octanol:water), p*K*a, and water solubility of anaesthetic agents

Anaesthetic	logP	p*K*a	Water solubility (mgl^{-1})
MS-222	1.8	3.8[B]	1.0×10^5
Benzocaine	1.9	2.5	1.3×10^3
Metomidate hydrochloride [A]	3.1	4.5[C]	6.3 x 10
2-phenoxyethanol	1.2	15.1	2.7×10^4
Isoeugenol	3.0	9.9	3.6×10^2
Quinaldine	2.6	5.7	5.0×10^2

Source: ChemIDplus, U.S. National Library of Medicine.
A: The values indicated for logP, p*K*a, and water solubility are applicable for etomidate.
B: source: Stenger and Maren, 1974.
C: source: Levron and Assoune, 1990.

In fish the main route of elimination of anaesthetic agents is via the gills, and the agents are mainly eliminated in unchanged form (Hayton et al., 1996; Hunn and Allen, 1974; Hunn et al., 1968; Meinertz et al., 1991; Stenger and Maren, 1974). Some may also undergo metabolism prior to elimination (Hayton et al., 1996; Meinertz et al., 1991; Ryan, 1992; Stenger and Maren, 1974).

Pharmacokinetic models are being used to describe the pharmacokinetic processes. Plasma concentration-time curves are applied to estimate the pharmacokinetic parameters and perform compartmental analyses to describe drug distribution. In the simplest compartmental pharmacokinetic model the body is regarded as one compartment where the drug is distributed evenly following absorption. This model, termed one-compartment open model with first order elimination (Figure 7A) is a simplification used to illustrate basic pharmacokinetic principles. A two-compartment open model (Figure 7B) includes a peripheral compartment representing the tissue in addition to the central compartment representing the plasma, thereby introducing a distribution process to the model. From the central compartment the drug can be removed both by distribution to the peripheral compartment and by elimination. The process of distribution is described by the initial rapid decrease in plasma concentration of the drug, entitled the α–phase. When the distribution is complete the process of elimination describes the slower decrease in plasma concentration, entitled the β-phase.

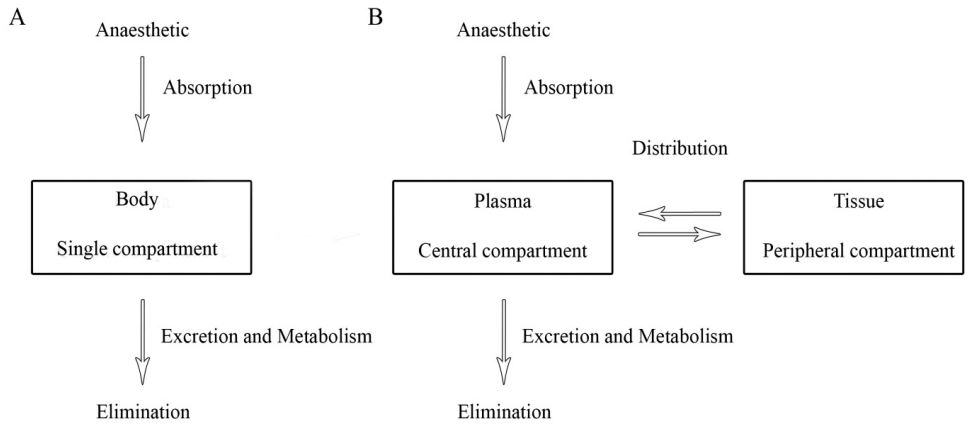

Figure 7 Pharmacokinetic models, A: One-compartment, B: Two-compartments

The distribution of a drug between the plasma and the rest of the body is described by the pharmacokinetic parameter volume of distribution (Vd). The Vd, which indicates how a drug will relocate into body compartments relative to the blood is a non-physiological volume. This volume corresponds to the volume that would be necessary in order to dilute the administered drug to produce the concentration that is present in the blood. Lipophilic drugs that have a tendency to accumulate in body fat thus have high Vd values while drugs with a high degree of plasma protein binding have low Vd values. The rate at which the drug is removed from the blood is described by the parameter clearance (C) which is defined as the volume of blood or plasma that is cleared per time unit. Together with clearance the volume of distribution determines the plasma half-life ($t_{1/2}$) of a drug. The plasma half-life is defined as the time that it takes for the plasma concentration of a drug to halve.

1.6.3 Water temperature

Temperature is an important factor for the velocity of physiological processes in ectothermic animals such as fish, and thus an important factor in the processes related to uptake and elimination of drugs. Increasing the temperature by 10°C (Q_{10}) typically doubles the basal metabolic rate of teleost fish (Clarke and Johnston, 1999). This leads to a higher oxygen demand which is met by enhanced respiration, increased cardiac output, and an increased blood flow through the gills (Graham and Farrell, 1989; Nilsson and Sundin, 1998; Webber et al., 1998). Decreased oxygen solubility of the water in relation to rising water temperature causes an additional need for enhancing respiration and blood flow.

Temperature increases have been reported to shorten induction and recovery times for a number of anaesthetic agents in several teleost species including benzocaine in striped bass (*Morone saxatilis*), 2-phenoxyethanol and clove oil in European sea bass and gilthead sea bream, isoeugenol in rainbow trout, and clove oil in rainbow trout, brown trout (*Salmo trutta*), Atlantic salmon, whitefish (*Coregonus lavaretus*), perch (*Perca fluviatilis*) and roach (*Rutilus rutilus*) (Gilderhus et al., 1991; Mylonas et al., 2005; Hoskonen and Pirhonen, 2004; Stehly and Gingerich, 1999; Woolsey et al., 2004). Reduced induction time with increasing water temperature has also been shown for common carp (*Cyprinus carpio*), rainbow trout and fathead minnows (*Pimephales promelas*) anaesthetised with MS-222 (Hikasa et al., 1986; Houston and Woods, 1976; Sylvester and Holland, 1982).

1.6.4 Body weight

The relationship between body weight and the sensitivity to anaesthetics may be influenced by several characteristics related to body weight, such as age, body composition, growth rate, and sexual maturity, as all of these characteristics influence the physiology of the fish. As the major route both for uptake and elimination is over the gills, the rate of oxygen consumption, the body volume to gill surface area, and the level of gill perfusion are of high importance. These factors vary both within and between species, with life stage and life style as well as with body size. The gill surface area decreases with increasing body size (Oikawa and Itazawa, 1985; Oikawa et al., 1999). Basal metabolism decreases in relation to increasing body size, and larger animals have thus lower oxygen consumption in relation to body size than small animals (Clarke and Johnston, 1999; Schmidt-Nielsen, 1984). The scaling relationship between metabolic rate and size is commonly described by the equation $Vo_2 = a\ M_b^{0.75}$ where the metabolic rate (Vo_2) scales as 0.75 power of body mass (M_b) and the constant *a* indicates the level of the resting metabolic rate (Schmidt-Nielsen, 1984). The constant *a* may differ between species and varies in relation to lifestyle, and in ectotherm animals such as fish it is strongly dependent on temperature. For teleost fish a scaling exponent of 0.8 has been found (Clarke and Johnston, 1999). Sedate and less active fish species have lower resting metabolic rates than more active species (Morris and North, 1984). Clarke and Johnston (1999) found that resting metabolic rate varied between taxonomic groups, gadoids having higher resting metabolic rates than pleuronectiformes and salmoniformes, although there was a high degree of overlap.

The reports regarding the effect of anaesthetic agents and the importance of body weight in fish are contradictory. Some studies show no relationship between body size and induction and recovery times (Houston and Woods, 1972; Stehly and Gingerich, 1999) whereas others indicate that a relationship exists (Gilderhus and Marking, 1987; Houston et al., 1976; Olsen et al., 1995). Hoskonen and Pirhonen (2004) observed a decrease in induction time with increasing body size in whitefish, whereas they found the opposite relationship in rainbow trout, and found no size related variations in Atlantic salmon or brown trout. Weber et al. (2009) found that induction times increased with increasing body weight in Senegalese sole (*Solea senegalensis*) anaesthetised with 2-phenoxyethanol, metomidate and clove oil, while this was not found for MS-222. The dynamics of the

recovery times were more complex and were only weight related for MS-222 (Weber et al., 2009).

1.6.5 Stress

When fish are subjected to handling, such as crowding or dip netting, they will respond by an attempt to escape. This escape reaction will lead to increased oxygen consumption due to increased physical activity. Paralleling the alterations in behaviour a cascade of physiological reactions is activated, constituting the physiological stress response, first termed the general adaptation syndrome (Selye, 1985). The physiological stress response in fish is similar to that described in other vertebrates and is characterised by three phases (Wendelaar Bonga, 1997). During the first phase the activation of two neuroendocrine systems, the hypothalamus-sympathetic-chromaffin cell axis and the hypothalamus-pituitary-interrenal axis, results in a massive release of hormones. The chromaffin cells of the head kidney of the fish release catecholamines, adrenaline and noradrenaline while interrenal steroidogenic cells produce and release corticosteroids (Barton, 2002; Mommsen et al., 1999; Randall and Perry, 1992; Reid et al., 1998; Sumpter et al., 1986). The increased levels of circulating catecholamines and corticosteroids, which are characteristic of the second phase of the stress response, induce a mobilisation of energy stores and stimulate the respiratory and cardiovascular systems (Farrell, 1984; Nilsson and Sundin, 1998). The plasma levels of glucose and fatty acids rise rapidly, promoted through a stimulation of hepatic glucose release, gluconeogenesis and lipolysis (Mommsen et al., 1999; Randall and Perry, 1992). The third phase of the stress response is characterised by the effects of the cost of the activated cells and systems. The activation is energy consuming, and the consequence of situations of severe stress, chronic stress, or recurrent incidents of stress where the fish fail to recover is an impairment of the whole organism. The detrimental effects are related to elevated levels of corticosteroids, primarily cortisol, which are involved in increased susceptibility to disease, suppression of growth rate, impairment of reproductive processes, and alterations of social interactions (Campbell et al., 1992; Pickering, 1990; Pickering and Pottinger, 1989; Schreck, 1990; Schreck et al., 2001).

The high peak of plasma concentrations of catecholamines, adrenaline and noradrenaline, observed immediately following incidents of acute stress drops rapidly

(Randall and Perry, 1992; Reid et al., 1998) whereas the plasma concentration of corticosteroids, primarily cortisol, increases more slowly and returns more slowly to basal level (Olsen et al., 2002; Olsen et al., 2008; Pickering and Pottinger, 1989; Sumpter et al., 1986). The dynamics of the cortisol response have therefore made cortisol a widely used parameter in studies of stress in fish. Large variations in the magnitude of the cortisol response to stress have been found between species (Barton, 2002; Barton and Iwama, 1991) and high and low responders within species have also been identified (Fevolden et al., 1999; Pottinger and Moran, 1993; Pottinger and Carrick, 1999; Pottinger et al., 1994; Sumpter et al., 1986).

1.6.6 Stress and anaesthesia

Stressed animals display abnormal reactions to anaesthesia and may require increased dosages both for induction and maintenance (Hall et al., 2001). Increased physical activity while attempting to escape handling, followed by the cascade of physiological alterations of the stress response, will contribute in facilitating uptake of the anaesthetic. Elevated levels of catecholamines and corticosteroids stimulate ventilation and enhance cardiac output resulting in an increased gill blood flow (Farrell, 1984; Graham and Farrell, 1989; Nilsson and Sundin, 1998). Perfusion of gill lamellae increases and lamellae that in a normal resting state are barely or not perfused at all are recruited, thus expanding the respiratory surface area and facilitating diffusion.

There are several reports showing that stress associated with handling of fish can be effectively reduced by anaesthetic agents. Metomidate was found to reduce handling stress in Atlantic salmon, Chinook salmon (*Oncorhynchus tshawytscha*), hybrid striped bass (*Morone chrysops* x *Morone saxatilis*), channel catfish (*Ictalurus punctatus*) and red drum (*Sciaenops ocellatus*), while isoeugenol has been found effective in reducing stress in Atlantic salmon and channel catfish, and MS-222 has been found to inhibit the stress response in connection to handling of rainbow trout and channel catfish (Davis and Griffin, 2004; Iversen et al., 2003; King et al., 2005; Kreiberg and Powell, 1991; Olsen et al., 1995; Small, 2003; Small, 2004; Small and Chatakondi, 2005; Thomas and Robertson, 1991). It has also been found however that exposure to anaesthetics may in itself induce a stress response. Of the agents in the present study MS-222 was found to induce stress in rainbow

trout, channel catfish, striped bass, hybrid striped bass, gilthead sea bream and red drum, while isoeugenol was found to induce stress in rainbow trout and hybrid striped bass, eugenol was found to induce stress in hybrid striped bass, and metomidate of low dosage was found to induce stress in Atlantic salmon (Barton and Peter, 1982; Davidson et al., 2000; Davis and Griffin, 2004; Davis et al., 1982; Molinero and Gonzalez, 1995; Olsen et al., 1995; Small, 2003; Thomas and Robertson, 1991). Confinement and relocation in connection to anaesthesia will however influence the responses to anaesthesia and in addition possibly mask the stress inducing effects of the anaesthetic agents themselves (Hill and Forster, 2004; Molinero and Gonzalez, 1995; Thomas and Robertson, 1991).

2. The objective of the present study

The overall objective of the current investigation was to gain more knowledge regarding the effect of anaesthetic agents in farmed fish, with special emphasis on Atlantic cod and Atlantic halibut. More specifically, to elucidate differences in effect between different agents and to examine the importance of factors such as body weight, water temperature, and acute stress.

A further objective was to determine pharmacokinetic properties of different anaesthetic agents and to relate these parameters to the observed pharmacodynamic effect and to the stress reducing potential of the agents. As anaesthetics are used in order to reduce stress in connection to handling and confinement of fish the influence of the agents themselves on the physiological stress response was investigated.

The final objective was to search for combinations of anaesthetic agents that would be superior to agents administrated individually and thereby offer an improvement to the anaesthetic protocol.

3. Summary of results

3.1 Paper I

This study reports data on plasma clearance kinetics of anaesthetics concomitant with physiological stress responses in dorsal aorta (DA) cannulated Atlantic salmon (*Salmo salar*) both with and without influence of artificial gill ventilation during recovery from anaesthesia. For MS-222 the plasma data were best described by a one-compartment open model and first-order elimination, whereas a two-compartment open model and first-order elimination best described the plasma data for benzocaine and isoeugenol. The compartment analysis was not affected by ventilation. Distribution volumes from 1.99 lkg^{-1} (isoeugenol) to 3.98 (MS-222) and 5.12 lkg^{-1} (benzocaine) showed a moderate to large distribution of the drugs from plasma to tissues. MS-222 was eliminated most rapidly, with an elimination half-life of 1.7 min, while elimination half-lives of 18.7 and 25 min were calculated for benzocaine and isoeugenol, respectively. Ventilation following exposure increased the elimination rate. Anaesthetics were administered in two steps with an initial 10 minutes of sedation by a low dosage (1×10^{-1} of full dosage) before moving the fish to a full strength anaesthesia bath. All anaesthetics used caused a marked increase in plasma cortisol, apparent already at the end of the exposure. Marked differences were noted between anaesthetics, with time to regain consciousness and responsiveness to external stimuli paralleling the plasma clearance of the anaesthetic. Recovery from anaesthesia was accompanied by higher respiration frequency. Recovery was always faster when the fish was given artificial gill ventilation.

3.2 Paper II

The efficacy of the anaesthetic agents benzocaine, metacaine (MS-222), metomidate hydrochloride and 2-phenoxyethanol was studied in Atlantic cod (*Gadus morhua*) with average body weights of 10±4 g, 99±33 g and 1022±274 g at water temperatures of 8°C and 16°C. The agents were tested individually and as combination anaesthesia comprising pre-sedation with a low dosage of metomidate or 2-phenoxyethanol followed by anaesthesia with benzocaine or MS-222. All agents were administered through bath immersion with an

exposure time of 5 minutes. The different treatments resulted in average induction and recovery times ranging from 52±6 s to 182±16 s and 77±26 s to 659±46 s respectively. Induction and recovery times varied in relation to water temperature and were generally shorter at 16°C for all weight groups and treatments compared to 8°C. For benzocaine and MS-222 induction and recovery times were found to increase with increasing body weight. For metomidate the recovery time increased with increasing weight whereas there were no weight related differences in induction time. No differences in either induction or recovery times associated to body weight were found for 2-phenoxyethanol. Acute stress prior to anaesthesia with MS-222 resulted in significantly shorter induction time and prolonged recovery time, as well as deeper anaesthetised fish. The dosage of MS-222 had to be reduced in order to avoid mortality in fish subjected to acute stress. Combination anaesthesia allowed a reduction of the dosages used for inducing anaesthesia and produced markedly reduced recovery times compared to agents administered individually.

3.3 Paper III

The efficacy of the anaesthetic agents benzocaine, metacaine (MS-222), metomidate, 2-phenoxyethanol, quinaldine and isoeugenol was studied in Atlantic halibut (*Hippoglossus hippoglossus*). Fish with average body weight of 33 g were anaesthetised at 8°C and fish with average body weight of 1243 g were anaesthetised at 8°C and 15°C. Agents were tested individually and as combination anaesthesia comprising pre-anaesthetic sedation followed by anaesthesia. Induction and recovery times varied in relation to body weight and water temperature. Large fish had longer induction times and shorter recovery times, and displayed reduced responsiveness to handling compared to small fish. Higher temperature resulted in shorter induction times, longer recovery times and increased responsiveness to handling. Lower dosages were used for all agents in combination anaesthesia. In small fish this had no effect on induction times but resulted in shorter recovery times and reduced responsiveness to handling. In large fish combination anaesthesia resulted in shorter induction times whereas no uniform trend in recovery times and no differences in responsiveness to handling were observed. Neither individual agents nor combinations blocked all reflex reactions to external stimulation in all fish of any treatment group. MS-222 and benzocaine used

separately or in combination anaesthesia were the most effective agents in reducing reflex reactions.

3.4 Paper IV

Stress in response to anaesthesia with benzocaine, MS-222, metomidate and isoeugenol was studied in Atlantic salmon (*Salmo salar*), Atlantic halibut (*Hippoglossus hippoglossus*) and Atlantic cod (*Gadus morhua*) with no concomitant stress from handling or confinement in association with anaesthesia or sampling. All of the anaesthetics tested induced a stress response in all species, displayed by a release of cortisol to the water. MS-222 anaesthesia elicited the highest cortisol release rates, reaching maximum levels 0.5 h post exposure and returning to basal levels after 3 to 4 h. Benzocaine anaesthesia caused a bimodal response where the initial peak in cortisol release rate was followed by a second increase lasting towards the end of the trial (6 h). This bimodality was more profound in Atlantic salmon than in Atlantic halibut and Atlantic cod. Metomidate anaesthesia induced the lowest release of cortisol of the agents tested in both Atlantic halibut and Atlantic cod, but resulted in a bimodal response in Atlantic salmon where the initial increase in cortisol release was followed by a larger increase peaking at 2 to 2.5 h post exposure before returning to basal levels after 5 h. The stress induced in Atlantic salmon by isoeugenol anaesthesia resembled that of MS-222, but did not reach the same elevated level. Over all the cortisol release was most profound in Atlantic salmon followed by Atlantic halibut and Atlantic cod.

4. General discussion

The aim of the current investigation was to anaesthetise the fish until stage III, plane 2 (Table 1) while obtaining induction and recovery times within the range defined for suitable fish anaesthetics, i.e. 3 and 5 minutes respectively (Bell, 1987; Marking and Meyer, 1985). The parameters assessed to determine anaesthetic depth were balance, reflex reactions to a caudal peduncle pinch, and responsiveness to handling. Induction and recovery times were set at loss and regain of equilibrium (Paper II and III). In the majority of the previous reports regarding anaesthesia of fish the fish have been anaesthetised until a pre determined stage, often corresponding to loss of equilibrium, no response to external stimuli, and reduced or even stopped respiration (Table 1, stage III plane 3 to stage IV), and directly thereafter been moved to clean water for recovery (Hoskonen and Pirhonen, 2004; Mattson and Riple, 1989; Mylonas et al., 2005; Stehly and Gingerich, 1999; Sylvester and Holland, 1982). As the induction time may vary between individual fish the exposure times have varied accordingly. In the current investigation an identical exposure time was chosen for all fish and treatments. The duration of the exposure, 5 minutes, was selected in order to ensure a sufficient margin of safety. As common field situations often involves anaesthesia of a large number of fish in one tank, the exposure times thus generally exceed the induction time for individual fish.

4.1 Pharmacokinetics

The pharmacokinetic analysis revealed large variations between the anaesthetics (Paper I). MS-222, the most hydrophilic of the agents tested (Table 2) was distributed rapidly, followed by a rapid clearance and elimination. The plasma data were best described by a one-compartment open model with first order elimination (Figure 7A), which indicates a homogenous distribution and an elimination proportional to the plasma concentration. The plasma data of benzocaine and isoeugenol were best described by a two-compartment open model (Figure 7B) indicating a rapid transfer of drug from plasma to tissues, followed by a slower process of elimination from the plasma. Both benzocaine and isoeugenol, which were found to be rapidly distributed, had rates of clearance and elimination much slower than MS-222. An initial phase of rapid distribution followed by a period of slow elimination has also been found in rainbow trout anaesthetised with benzocaine (Meinertz et al., 1996; Stehly et

al., 1998). The pharmacokinetic properties of benzocaine in rainbow trout were best described by a two-compartment model when benzocaine was administered through bath immersion while a three-compartment model was best when describing the properties following intra-arterial injection (Meinertz et al., 1996; Stehly et al., 1998). To the best of my knowledge, no compartment analysis has been reported for MS-222 or isoeugenol in fish. Guenette at al. (2007) found that eugenol, which is similar to isolegenol, was well absorbed and eliminated by rainbow trout. The plasma concentration versus time curve was biphasic, which indicates two compartments. A biphasic plasma elimination curve was also found in the elasmobranch dogfish (*Squalus acanthias*) following arterial injections of MS-222 (Stenger and Maren, 1974). MS-222 was excreted rapidly across the gills, mainly in unchanged form. In the current investigation artificial ventilation had no influence on the compartment analysis (Paper I). However, the elimination rates were faster in the groups of fish that received artificially ventilation which show the importance of the gills as pathway for drug elimination. This is in agreement with previous reports in other species (Hayton et al., 1996; Hunn and Allen, 1974; Meinertz et al., 1991) and emphasises the significance of respiratory function and flow of clean water over the gills during recovery.

4.2 Pharmacodynamics

The pharmacodynamic properties studied in Atlantic cod (Paper II) and Atlantic halibut (Paper III) showed that there were large differences within and between the two species as well as between the agents. Induction time, set at loss of equilibrium, occurred within 3 minutes for all agents tested thereby fulfilling the criteria set for suitable fish anaesthetics (Bell, 1987; Marking and Meyer, 1985). The recovery times, set at regain of equilibrium, varied extensively between the agents. Metomidate resulted in the longest recovery times in Atlantic cod, and was together with isoeugenol and 2-phenoxyethanol among the agents giving rise to the longest recovery times in Atlantic halibut (Paper II and Paper III). This complies with pharmacokinetic data (Guenette et al., 2007; Hansen et al., 2003; Kildea et al., 2004; Paper I) indicating that a slower elimination and a prolonged recovery might be the expected outcome in agents of higher lipid solubility. The recovery times in Atlantic cod were within the range defined for suitable anaesthetics, i.e. 5 minutes, (Bell, 1987; Marking and Meyer, 1985) for all agents except for metomidate but exceeded

this in Atlantic halibut. However as the parameter “recovery time” is recorded differently in Atlantic halibut compared to free swimming fish such as the Atlantic cod, values for recovery times should be compared with caution. In Atlantic cod the recovery time was set upon regain of equilibrium (Paper II) whereas in Atlantic halibut the recovery time was set when the fish turned upright after having been placed upside down in the recovery tank (Paper III).

The agents differed extensively in their capacity to depress responses to external stimulation, assessed in the current investigation as reactions to handling and to caudal peduncle pinching. Reflex reactions to the caudal peduncle pinch and responsiveness to handling were most effectively reduced by the two local anaesthetics MS-222 and benzocaine. This is probably related to the mode of action, as these substances suppress nervous signalling both in central and peripheral parts of the nervous system. However, the effect may be influenced by the route of administration i.e. whether the agents are injected directly into the target tissue, which is common in higher vertebrates, or administered via inhalation, which is the common route of administration in fish (further discussed in section 4.8). Suppression of nerve signals may lead to adverse effects. Agents administrated to fish via inhalation enter the circulation and may be distributed to the whole body. Upon passing the blood-brain barrier the agents will affect the brain and the centres that control respiration and circulation. A depression of respiration and circulation would cause hypoxemia, metabolic acidosis, and changes in blood electrolytes, and would lead to prolonged recovery times enhancing the effects of the anaesthetic.

4.3 Water temperature

Fish confined in sea cages must submit to the conditions within the cages and are therefore subjected to seasonal and daily fluctuations in physical and chemical environment. The thermally stratified water off the Norwegian coast may during the summer season reach temperatures close to 20°C (Aure, 2009). This is higher than preferred both by Atlantic cod and Atlantic halibut (Bjornsson and Tryggvadottir, 1996; Bjornsson et al., 2007; Hallaraker et al., 1995; Jobling, 1988). For both species the temperature optimum for growth drops with

body size (Bjornsson and Tryggvadottir, 1996; Bjornsson et al., 2007; Hallaraker et al., 1995; Jobling, 1988).

In the current investigation, high water temperature resulted in shorter induction times in both Atlantic cod and Atlantic halibut (Paper II and III). This is in agreement with numerous reports comprising several agents and species (Gilderhus et al., 1991; Hikasa et al., 1986; Hoskonen and Pirhonen, 2004; Houston and Woods, 1976; Mylonas et al., 2005; Stehly and Gingerich, 1999; Sylvester and Holland, 1982; Woolsey et al., 2004). The rapid induction may be related to the higher basal metabolism at higher temperatures (Clarke and Johnston, 1999; Imsland et al., 2000; Saunders, 1963; Schurmann and Steffensen, 1997) and the corresponding increase in oxygen demand leading to an increased respiration and circulation. This will facilitate a rapid absorption and distribution of anaesthetic and may also lead to a larger amount being absorbed. Significantly higher concentrations of isoeugenol have been found in the fillet of rainbow trout following exposure at 17°C compared to 7°C (Meinertz et al., 2006). In the current study Atlantic cod recovered more quickly at the higher water temperature and the therapeutic window was wider, indicating a more rapid clearance and elimination. Faster clearance rates at higher temperature have also been found in rainbow trout and silver perch (*Bidyanus bidyanus*) anaesthetised with benzocaine and isoeugenol respectively (Kildea et al., 2004; Stehly et al., 1998). Atlantic halibut on the other hand recovered more slowly at higher temperatures. But in spite of longer recovery times the fish displayed stronger reactions to handling indicating lack of anaesthetic effect. Thus, it appears that the central and peripheral effects of the anaesthetics were differently influenced by the temperature increase, central effects becoming more profound whereas peripheral effects becoming less profound. This was most prominent in benzocaine which may reflect higher lipid solubility compared to MS-222, the only two agents tested in Atlantic halibut at the higher water temperature.

4.4 Body weight

No uniform relationship was found between the effect of anaesthetic agents and the body weight of the fish (Paper II and III). In Atlantic halibut there was a trend though, with increasing induction times and decreasing recovery times with increasing body weight.

Increased induction times with increasing body weight were also seen in Atlantic cod anaesthetised with MS-222 and benzocaine. However, as opposed to Atlantic halibut also the recovery times increased in larger fish. No weight relationship was found in Atlantic cod anaesthetised with 2-phenoxyethanol whereas fish of high body weight anaesthetised with metomidate recovered more quickly than small fish. For Atlantic cod the results suggest that the body weight of the fish, or factors related to body weight, are less important for the variations in effect than the properties of the anaesthetic agents themselves.

Increased induction time in relation to increased body weight indicates that there is an inverse relationship between the uptake rate of anaesthetic and body weight. As fish of large body size have a lower basal metabolism and thus lower oxygen consumption relative to size than smaller fish and in addition have a smaller gill surface area in relation to size (Clarke and Johnston, 1999; Muir, 1969; Oikawa and Itazawa, 1985; Oikawa et al., 1999) a slower rate of absorption and elimination of anaesthetic in relation to weight could be expected. Slow absorption and thus longer time required for obtaining a blood concentration that would give sufficient anaesthetic effect may be accompanied by a slow elimination of the drug associated with prolonged recovery. A slower absorption rate may on the other hand result in a lower blood concentration following exposure which may result in shorter recovery times, the main trend observed for larger fish in the current study. The findings in the current study are in line with previous reports regarding the importance of body weight for anaesthetic effect, namely not consistent. Additional characteristics related to body weight such as age, growth, body composition, and sexual maturity may be important for the response to anaesthesia as these factors influence the physiology of the fish. Physiological differences may result in pharmacokinetic and pharmacodynamic differences and may explain some of the variations in response found in the various investigations (Gilderhus and Marking, 1987; Gilderhus et al., 1991; Hoskonen and Pirhonen, 2004; Houston and Woods, 1976; Olsen et al., 1995; Tsantilas et al., 2006; Weber et al., 2009).

4.5 Species differences

4.5.1 Dosages

The dosages that were used in the study of Atlantic halibut (Paper III) were within the range commonly used for other fish species (Ackerman et al., 2005; Neiffer and Stamper, 2009; Ross and Ross, 1999; 2008) and are similar to manufacturer's recommendations for salmonid fish (Pharmaq, ACD Pharmaceuticals, Aqui-S® New Zealand, and Syndel Laboratories ltd.). Atlantic cod was more sensitive and received lower dosages of all agents except metomidate (Paper II). Further, in Atlantic cod dosage adjustments were necessary both in relation to body weight and water temperature in order to obtain an adequate level of anaesthesia while avoiding overdosing. This indicates that the therapeutic window is narrower in Atlantic cod than in Atlantic halibut where identical dosages were used at both water temperatures and body weights. However, in the protocols comprising combination anaesthesia there were only minor differences between the dosages administrated to the two species. In comparison with individual administrated agents all dosages used in combination anaesthesia were reduced. Combination anaesthesia is further discussed in section 4.6.

4.5.2 Atlantic cod sensistivity

Data regarding anaesthesia of Atlantic cod are scarce and protocols are therefore generally based on drugs and dosages recommended for other fish species. This has resulted in recurring incidents of mortalities, reported both by experienced technical staff and scientists of the Institute of Marine Research (Hari Rudra, pers.comm.). The current investigation revealed that there were large variations between the anaesthetic agents. These variations appeared to be unrelated to mode of action as the Atlantic cod was far more sensitive to benzocaine than to MS-222 although benzocaine was used in much lower dosages than MS-222. The difference in sensitivity must therefore result from other characteristics of the substances or by differences in pharmacokinetic properties. Although most of the MS-222 and benzocaine are eliminated in unchanged form via the gills (Hayton et al., 1996; Hunn and Allen, 1974; Hunn et al., 1968; Meinertz et al., 1991; Stenger and Maren, 1974), the agents are also subjected to metabolism and have been found to undergo both acetylation and hydrolisation (Hayton et al., 1996; Hunn et al., 1968; Meinertz et al.,

1991; Ryan, 1992; Stenger and Maren, 1974). In a study of the metabolism of sulphadimethoxine, an antibacterial drug which in fish is metabolised via acetylation, it was found that the plasma concentration of the acetylated form of the substance was much lower in Atlantic cod than what had previously been observed in Atlantic salmon, channel catfish and rainbow trout (Samuelsen, 2006; Samuelsen et al., 1995; Squibb et al., 1988; Uno et al., 1993). This indicates that acetylation may be a less important metabolic pathway in Atlantic cod than in the other fish species and may result in a reduced metabolism of substances that are metabolised through acetylation. However, a complete pharmacokinetic analysis is necessary in order to determine whether this is a factor in the higher sensitivity observed in the current study.

Isoeugenol and quinaldine were also tested in Atlantic cod, but under the prevailing experimental conditions no suitable dosages were found. The fish died in the recovery bath although being almost impossible to handle due to lack of anaesthetic effect. These substances are the most lipophilic of the agents tested in this investigation (Table 2) which may indicate that there is a possible relationship between the sensitivity of Atlantic cod and the lipid solubility of the anaesthetics. Anaesthetic agents are associated with high lipid solubility and a tendency to accumulate in tissues with a high content of fat (Rang et al., 2003). This might be a problem following repeated exposures or in exposures of long duration, for instance during larger operations. Whether 5 minutes of bath exposure is sufficient for accumulation in fat rich tissues of Atlantic cod will however only remain speculations without a thorough analysis of the tissues for drug residues.

In Atlantic cod the therapeutic window was found to decrease with increasing body weight and with decreasing water temperature (Paper II). For fish of high body weight (1000 g) anaesthetised at low water temperature (8°C) the dosage of both metomidate and MS-222 had to be reduced, whereas for benzocaine no suitable dosage was found. Following 5 minutes of exposure to benzocaine the fish were reactive both to handling and to the caudal peduncle pinch, but got respiratory arrest and died in the recovery bath. The findings in Atlantic cod comply with findings in brown trout and goldfish where higher sensitivity was seen at lower water temperature during anaesthesia with benzocaine and 2-phenoxyethanol respectively (Dawson and Gilderhus, 1979; Weyl et al., 1996). The findings contradict however with observations in striped bass where higher dosages of benzocaine were used at

lower water temperature for fish of higher body weight (Gilderhus et al., 1991). In an evaluation of Aqui-S® in rainbow trout the toxicity was found to decrease with increasing temperature (Stehly and Gingerich, 1999). The higher sensitivity displayed by Atlantic cod of higher body weight may be related to a lower basal metabolism in large fish resulting in a slower turnover and prolonged effect of the anaesthetic. An alternative, or accompanying, explanation is differences in body composition between small and large fish. There is an increasing deposition of lipids in the liver with increasing body size. This may result in a higher deposition of lipophilic substances, such as anaesthetic agents, in the liver and thereby an increased risk of secondary effects due to a delayed elimination.

The liver is the main fat depot in Atlantic cod, whereas Atlantic halibut deposits fat along the dorsal and ventral fins, the fat notch, and in the tissue around the head region, and Atlantic salmon deposits fat within the muscle, dorsally within the belly flap and in the abdominal cavity around the internal organs (Aursand et al., 1994; Holdway and Beamish, 1984; Jobling et al., 1991; Martins et al., 2007; Nortvedt and Tuene, 1998). The liver lipid content which is much higher in farmed than in wild Atlantic cod is 50-70% of liver wet weight and the hepatosomatic index (HSI) is approximately 10% (dos Santos et al., 1993; Grant et al., 1998; Jobling, 1988; Lie et al., 1986; Morais et al., 2001). In comparison, the HSI of Atlantic halibut and Atlantic salmon is approximately 1% and the lipid content of the livers approximately 20 and 5% respectively (Aursand et al., 1994; Martins et al., 2007; Nortvedt and Tuene, 1998). In addition to varying with diet, both the weight and the lipid content of the liver change with age, sexual maturity, body size, and water temperature. In striped bass acclimated to different temperatures for four weeks it was found that the liver was twice as large in fish acclimated to 5°C compared to 25°C and the liver lipid content was 40% compared to 25% in the 25°C acclimated fish (Stone and Sidell, 1981). Similar trends have also been observed during seasonal temperature changes in channel catfish (Kent et al., 1992). In channel catfish both the HSI, liver glycogen and total lipid were low during the high summer temperatures, intermediate during winter and high during spring (HSI and total lipid) and fall (liver glycogen) (Kent et al., 1992). In rainbow trout the blood flow to the liver was found to increase at lower water temperatures (Barron et al., 1987). If the relative weight and the fat content of the liver of Atlantic cod is higher at lower temperatures, and if the blood supply to the liver increases as the water temperature decreases, the potential for the

liver to serve as a reservoir for lipid soluble substances increases with decreasing temperatures. As anaesthesia sets in during exposure the depressant effect on the circulation would possibly result in a delayed clearance. Liver accumulation has been observed in channel catfish anaesthetised with benzocaine (Hayton et al., 1996), and in the elasmobranch dogfish anaesthetised with MS-222 (Stenger and Maren, 1974). Data regarding the importance of water temperature on HSI values for Atlantic cod show no uniform trend. Perez-Casanova et al. (2009) found no differences in the HSI of Atlantic cod examined at water temperatures of 2, 6 and 11°C. Levesque et al. (2005) concluded that changes in HSI of Atlantic cod were influenced by photoperiod rather than by temperature and metabolic demand, as no differences were observed in HSI, liver lipid or fatty acid content, between fish held at 9°C and fish held at ambient water temperatures varying from 0°C during the winter season to 16°C during the summer season. In a study by Purchase and Brown (2001) increased HSI at lower temperatures was observed in Atlantic cod from the Grand Banks while the opposite was found in Atlantic cod from the Gulf of Maine.

The uptake of fat from the diet and storage in adipose tissue, as well as incorporation of lipids into membranes vary with temperature. There is an increase of incorporation of unsaturated fatty acids and an increase of aggregation of hydrophobic substances in membranes with decreasing temperature (Berge et al., 1980; Cossins, 1983; Ruyter et al., 2006; Sellner and Hazel, 1982). Substances with higher lipid solubility such as benzocaine, isoeugenol, and metomidate may therefore penetrate, and possibly also accumulate, in the lipid bilayer of cell membranes more readily at lower temperatures than more hydrophilic substances such as MS-222. Such an accumulation would alter the characteristic of the membranes and affect normal cell function by influencing transport mechanisms, stabilising the membranes and possibly inhibit propagation of nerve signals.

Anaesthetic depth has been associated with the concentration of anaesthetic agent in the brain. This has been reported for snapper (*Pagrus auratus)* and several fresh water species anaesthetised with MS-222, as well as sting ray (*Dasyatis sabina*) and lemon shark (*Negaprion breviostris*) anaesthetised with quinaldine (Brown et al., 1972; Hunn, 1970; Ryan, 1992). The brain receives a large supply of oxygen rich blood from the gills and the blood brain-barrier is permeable to anaesthetic agents. Accumulation in the brain, will probably lead to an enhanced central effect, with depression of the brain centres controlling

respiratory and cardiovascular function as a consequence. The study of Atlantic cod (Paper II) indicates that both lipid soluble agents, as well as lower water temperature may result in an enhanced central effect, especially during anaesthesia with benzocaine. In order to determine whether an accumulation of anaesthetic agent in lipid bilayers of cell membranes or whether differences in distribution of anaesthetic in relation to temperature are involved, further investigation is necessary.

4.6 Combination anaesthesia

The main incentive for combining anaesthetic agents is to produce a better and safer anaesthesia. Combinations of anaesthetics that have different effects may produce a more complete anaesthesia than individual agents. Furthermore, synergistic effects between different agents may offer a potential for reducing the dosage of each agent thereby possibly minimising adverse effects and expanding safety margins. In the current study the trends following combination anaesthesia were shorter induction and recovery times and fish that were more responsive to handling in comparison with individual agents (Paper II and Paper III). As the dosages of all agents used in combinations were lower compared to agents administrated individually both for Atlantic cod and Atlantic halibut, the shorter induction times indicate synergistic effects. While the dosages were almost halved for Atlantic halibut only small reductions were made for Atlantic cod. In Atlantic cod dosage adjustments were necessary both in relation to water temperature and body weight (increased dosages with increasing temperature and body weight). So, while Atlantic halibut tolerated, and also required higher dosages of individual administered anaesthetic agents compared to Atlantic cod, the synergistic effects of the combinations allowed large dosage reductions. Atlantic cod was more sensitive to the anaesthetics and tolerated lower dosages only, and although there was a synergistic effect of the drug combinations a reduction of the already low dosages was not possible if sufficient anaesthetic depth was to be obtained. However, the safety margins were increased during combination anaesthesia. While benzocaine administered individually was found unsuitable for anaesthetising Atlantic cod of 1000 g at 8°C, anaesthesia was obtained with no mortalities when benzocaine was administered following pre-anaesthetic sedative treatment with either metomidate or 2-phenoxyethanol. Since one of the aims of the pre-anaesthetic sedation was to prevent a stress induced rapid

and excessive uptake of anaesthetic the fish should preferably have been sedated prior to being netted. Under the prevailing experimental conditions this was not possible and although an effort was made to net the fish as gently as possible the fish were in a stressed state upon entering the sedation bath. However, in the stress experiment with Atlantic cod it was found that the progress of induction during MS-222 anaesthesia following netting and pre-anaesthetic metomidate sedation resembled that of MS-222 anaesthesia without any stress from netting or handling, indicating that the pre-anaesthetic sedative treatment would reduce the acute stress caused by the netting (Paper II). All fish were calm following the 5 minutes of sedation when they were carefully lifted out and transferred to the anaesthesia bath. Fish anaesthetised with combinations generally showed an increased responsiveness and a higher presence of reflexes than fish anaesthetised with individual agents, indicating insufficient anaesthetic effect and an inadequate blockage of peripheral nerves. By increasing the dosages that were used here deeper anaesthesia would be obtained, and the requirements of rapid induction and recovery would probably still be met. In situations where anaesthesia has been produced by combining agents of different properties, improved recovery and fish that quickly resume normal behaviour have been observed (Eliason et al., 2007; Karlsson et al., 2006; Kiessling et al., 2003). The combinations obviously offer an opportunity to optimise the anaesthetic protocol and thereby ensure the welfare of the fish.

4.7 Anaesthesia and stress

Handling in form of netting or crowding is the normal practice in connection to anaesthesia of fish. The fish are therefore most likely in a stressed state upon exposure to the anaesthetics. Increased ventilation due to increased activity immediately following handling will, accompanied by the cascade of the physiological stress response (see section 1.6.5), facilitate the uptake of anaesthetic by stimulating respiration and circulation and increasing the blood:water interface. The result will be an immediate and excessive uptake of anaesthetic agent leading to faster induction and possibly also deeper anaesthesia. A doubling of oxygen consumption rates have been observed both in coho salmon (*Oncorhynchus kisutch*), rainbow trout and Atlantic cod following handling, returning to basal levels within one and three to five hours in coho salmon and Atlantic cod respectively (Barton and Schreck, 1987; Davis and Schreck, 1997; Saunders, 1963). Stress prior to

sedation may have marked practical consequences, as demonstrated by Kiessling and co-workers in a series of studies on feeding and growth following vaccination of salmon smolts anaesthetised with and without pre-anaesthetic sedation (Kiessling et al., 2001; Oppedal et al., 2000). Kiessling and co-workers found that fish vaccinated following combination anaesthesia resumed normal feeding behaviour within one week while fish subjected to the normal procedure of anaesthesia (netting directly into the anaesthesia bath) required more than three weeks. At that time the body weight of the fish anaesthetised with combination anaesthesia was 22% higher than the fish anaesthetised without pre-anaesthetic sedation.

In the current investigation, Atlantic cod subjected to stress in the form of 30 sec in air in a dip-net prior to anaesthesia had shorter induction times, became deeper anaesthetised and recovered more slowly compared to unstressed fish (Paper II). The dosage of MS-222 had to be reduced in order to avoid mortalities in the stressed group but was too low to provide sufficient anaesthetic effect in the unstressed group.

Anaesthetic agents may be effective in reducing handling stress (Davis and Griffin, 2004; Iversen et al., 2009; Iversen et al., 2003; Olsen et al., 1995; Sink et al., 2007; Small, 2004; Small and Chatakondi, 2005; Thomas and Robertson, 1991), but the exposure may also induce a stress response displayed by increased levels of cortisol (Barton and Peter, 1982; Davidson et al., 2000; Davis and Griffin, 2004; Molinero and Gonzalez, 1995; Thomas and Robertson, 1991). Exposure to all anaesthetic agents tested in the current study resulted in increased plasma cortisol levels (Paper I) and a release of cortisol into the water (Paper IV). The differences between the agents were more evident when the response was assessed by cortisol released to water than by plasma cortisol. In the assessment of water cortisol the stress response was induced in the absence of any concomitant stress from handling or confinement in connection to anaesthesia or sample collection (Paper IV). MS-222 induced most profound release of cortisol, rapidly returning to basal levels. The initial response to benzocaine was less profound but followed a bimodal pattern where there was a second increase in the release and an elevated cortisol level lasting towards the end of the trial (6 hours). The response to isoeugenol, which was only tested in Atlantic salmon, resembled that of MS-222 although without reaching the same elevated level. While the temporal dynamics, with a maximum peak in cortisol levels occurring within 0.5-1.5 h post stress, was in line with previous studies of the stress response in fish, both of plasma cortisol

and water cortisol measurements, the magnitude of the response, which is related to the nature of the stressor, was low following exposure to anaesthetics compared to stress from handling and confinement (Barton, 2002; Barton and Iwama, 1991; Fanouraki et al., 2008; Scott and Ellis, 2007; Scott et al., 2008). Several factors may affect the cortisol response to anaesthetics, including the method of administration, chemical properties of the agents, the mode of action, effects and adverse effects, induction time, the dosage used etc. It may seem that the temporal dynamics of the response may be associated to pharmacokinetic properties, MS-222 being quickly distributed and eliminated while benzocaine and isoeugenol being eliminated more slowly (Paper I). Metomidate, which is reported to inhibit cortisol synthesis in fish (Olsen et al., 1995; Thomas and Robertson, 1991) did not block the cortisol stress response in the current experiments. In Atlantic cod and Atlantic halibut only a minor elevation in cortisol release was observed however, whereas in Atlantic salmon the initial elevation was followed by a second and more profound elevation, a bimodal pattern similar to that of benzocaine. In general, Atlantic salmon responded with the highest cortisol release rates, followed by Atlantic halibut and Atlantic cod. This is in agreement with previous reports indicating that the amount of cortisol released in response to stress is lower in Atlantic cod than in Atlantic salmon (Brown et al., 2010; King et al., 2006; Olsen et al., 2002; Olsen et al., 2008; Olsen et al., 1995). Similar data were not found for Atlantic halibut. Differences in the magnitude of the cortisol response exist both between and within species, both in plasma cortisol levels and in the amount of cortisol released to water (Barton, 2000; Barton and Iwama, 1991; Pottinger and Moran, 1993; Pottinger et al., 1994; Scott and Ellis, 2007). Since cortisol is released over the gills (Ellis et al., 2005) factors influencing gill blood flow, respiration rate and diffusion area will also influence cortisol release. The Atlantic halibut of the current experiments were of smaller body size than the Atlantic salmon and the Atlantic cod. Atlantic halibut of larger body size would possibly have a lower cortisol release rate than found here. Although the amount of cortisol released by all three species in response to the exposure was low, indicating a minor level of stress, it may represent an additional burden under otherwise stressful conditions.

4.8 Anaesthesia and nociception

Studies of rainbow trout and goldfish have demonstrated that fish possess the basic neural system necessary for nociception, i.e. perception of painful stimuli (Dunlop and Laming, 2005; Sneddon, 2002; 2003b; Sneddon et al., 2003). Thus, in order to ensure the welfare of fish subjected to procedures that might inflict pain anaesthetic agents with the ability to block signals of nociceptive pathways are necessary. The two local anaesthetics benzocaine and MS-222 tested in the current study possess such effect, in addition to isoeugenol. However, the route of administration may be of importance for the effect. Systemic administration of local anaesthetics has been found inadequate for blocking peripheral nociceptive pathways in higher vertebrates (mice, rat, cat, and man) (Boas et al., 1982; Haegerstam, 1979; Rigon and Takahashi, 1996; Wiesenfeld-Hallin and Lindblom, 1985; Woolf and Wiesenfeld-Hallin, 1985). Haegerstam (1979) concluded that it seemed unlikely that peripheral pain pathways could be blocked by systemic i.v. injections of local anaesthetics. Based on these data it is unclear whether it is possible to obtain sufficient analgesic effect peripherally in fish when anaesthetic agents are administered via inhalation, even though the agents have proven local anaesthetic effect. As the whole body surface of the fish is exposed to anaesthetics during bath immersions, i.e. topical administration in addition to inhalation, the agents will also exert an effect peripherally. Absorption of small amounts of anaesthetic through the skin of the fish has been observed (Ferreira et al., 1984), but whether this amount produces the peripheral analgesia necessary during surgical procedures is not shown. It is therefore important to determine whether a lack of response to nociceptive stimuli in fish is due to a blockage of nociceptive afferent pathways or due to a general inhibition of CNS activity. The former yields a true analgesic effect without either peripheral or central hypersensitivisation, while the latter may have severe welfare consequences as all afferent nociceptive pathways may be activated while the lack of response is the result of a blockage of the central nerves. The data in Paper II and III indicate that neither of the tested agents administered individually or in combination produced a true blockage of afferent signals normally associated with injections of local anaesthetics in the target tissue, as shown by Kiessling al. (2003; 1995) during combination anaesthesia with metomidate, MS-222, and local injections of lidocaine. In the current study, the lower dosages used in combination anaesthesia resulted in similar induction times as individually

administrated agents but fish that displayed increased reactions to external stimuli (Paper II and III). This is possibly due to an enhanced CNS effect without a parallel enhanced effect in peripheral nociceptive pathways. All surgical procedures are associated with nociception, and should therefore not be conducted without local injections of anaesthetics.

4.9 Concluding remarks and future perspectives

The overall objective of the current study was to gain more knowledge regarding anaesthesia of farmed fish, with special emphasis on Atlantic cod and Atlantic halibut. Large variations in effect were observed between the different anaesthetic agents that were tested, and within each agent there were large variations both between and within the fish species. In general, Atlantic cod was far more sensitive to the anaesthetic agents than Atlantic halibut. In order to understand the basis for the differences between and within species and between agents a thorough pharmacokinetic investigation of each anaesthetic agent in each species is necessary. Measurements of absorption, distribution, clearance and elimination should be conducted with minimum stress to avoid invalid results. With regards to the high sensitivity of Atlantic cod an investigation of liver enzymes and metabolism might be needed. In both Atlantic cod and Atlantic halibut there was a tendency that the more lipophilic agents, such as metomidate and isoeugenol resulted in prolonged recovery. This complies with pharmacokinetic data that indicate that the clearance rates are slower following anaesthesia with agents of higher lipid solubility. Measurements of accumulation in fat rich tissues may contribute in explaining these differences both between the various agents and between the species.

Factors such as body weight and water temperature are important for the effect. Higher water temperature resulted in shorter induction times in both species. Atlantic cod recovered more quickly at high water temperature and the therapeutic window was wider. Atlantic halibut on the other hand recovered more slowly at the higher water temperature, and the high temperature seemed to result in stronger central effect and reduced peripheral effect. Although no uniform relationship was found in the effect of anaesthesia in relation to body weight a trend of longer induction times and shorter recovery times was observed in Atlantic halibut of large body size. In Atlantic cod of large body size a narrower therapeutic

window was observed. Pharmacokinetic studies where factors such as body weight and water temperature are included will be useful in order to understand the mechanisms behind the observed differences.

A protocol consisting of a combination of two anaesthetic agents, one agent to induce sedation followed by one agent to induce anaesthesia, showed promising results. Both Atlantic cod and Atlantic halibut were anaesthetised at lower anaesthetic dosages. Shorter or similar induction times indicated a synergistic effect between the agents. Recovery was improved by reduced recovery times. The anaesthetic agent benzocaine, which caused mortalities in Atlantic cod of high body weight when administered individually, was used successfully in combination anaesthesia. The main incentive for combining agents is to attain a more complete anaesthesia, reduce adverse effects, and improve recovery. Combinations are standard in anaesthetic protocols of both veterinary and human medicine, and the results of the current investigation show that combinations may improve the anaesthetic protocols also for fish. Further work should include a more thorough investigation of agents and dosages so that the optimal combinations are found, both according to species and to the kind of procedure the fish are being subjected to.

Anaesthesia during procedures that may inflict pain (i.e. nociception), such as surgery, should always include agents that block peripheral nociceptive nerve signals. Anaesthetics which only have an effect centrally should not be used as the only agent during these kinds of procedures. The results of the current study indicate that neither of the tested agents administered individually or in combinations, produces a true blockage of peripheral nerves. Responses to handling and reflex reactions to the caudal peduncle pinch were observed in all groups of anaesthetised fish. As local anaesthetics administered through i.v. injections have been found inadequate for blocking peripheral nociceptive pathways in higher vertebrates it is important to determine whether this also applies for fish anaesthetised by local anaesthetics administered through inhalation. Future studies are needed in order to clarify whether the lack of response to different stimuli is due to a blockage of peripheral nerves or due to a general inhibition of CNS activity.

Anaesthetic agents are used in various situations to reduce stress from handling and confinement. The assessment of stress in response to exposure to anaesthetics, measured as

the amount of cortisol released to the water and as increases in plasma cortisol, showed however that all the agents that were tested induced a stress response. Metomidate, which is known to inhibit the synthesis of cortisol, did not block the cortisol response. This might be due to the low dosage that was used, or to species differences as the observed response was much lower in Atlantic cod and Atlantic halibut than in Atlantic salmon. Inhibition or blockage of the cortisol response to stress minimises or blocks adverse effects caused by increased levels of cortisol, but as metomidate is not reported to affect the increased levels of catecholamines associated with the physiological stress response the effects of increased levels of catecholamines will remain.

In general, the cortisol release rates in response to exposure to anaesthetic agents were higher in Atlantic salmon than in Atlantic halibut and Atlantic cod. As all the tested agents seemed to induce a stress response, the basis for this response needs to be examined further before anaesthetics are administrated to the different species of fish with the aim of reducing stress. Anaesthetics may induce stress in fish by being sensed through taste and smell or by irritating the body surface, by affecting the endocrine system directly, by influencing the capacity to sustain normal posture, or by other reasons not yet known. Although the amount of cortisol released in response to the agents and dosages chosen in the current investigation was low compared to what is reported following strong stressors, such as handling and confinement, the stress induced by anaesthetic agents may represent an extra load during otherwise stressful circumstances.

Anaesthetic protocols should be tailored both to the specific requirements of each fish species and to the different kinds of procedures the fish are being subjected to. Further, protocols should always be tested on a few fish under prevailing conditions in order to ensure an adequate level of depth while avoiding overdosing. This recommendation applies whether the anaesthetic protocol comprises one single agent or a combination of agents.

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