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The Efficacy and Stress-Reducing Capacity of MS-222, Benzoak and Aqui-S for the Ornamental Cichlid Fish, Metriaclima estherae

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Marine Coastal Development
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Abstract

The financial crises and global change have caused setbacks to the improvements in many undeveloped countries. To be able to achieve the 'Millennium developmental goals' within 2015, it will require a higher effort. Sub-Saharan African countries show the slowest economic growth and have experienced the highest setbacks. Malawi is one of these countries and is struggling with a high share of poverty and poverty related issues. Trade is believed to be the most prominent tool to fight world poverty, as it will provide economic growth and employment.

Cichlid fishes from Lake Malawi are popular ornamentals for hobby aquarist's world wide, representing yearly turnover of 340 million US dollars. It is suggested that ornamental Malawi cichlids can be developed into an industry benefiting country and the local poor people. This can be achieved without pressure upon the lake biodiversity if the wild-collected fishes are used for breeding purpose and not for export.

To be able to deliver a high quality fish, it will be necessary to establish proper handling strategies that will ensure fish health and welfare. Stress from handling procedures is known to have negative impact on fish growth, reproduction, immune function and survival. Anesthetics may be a useful tool during handling procedures of the fish, as it can reduce the perception of the stressor and thus prevent activation of the hypothalamus-pituitary-interrenal (HPI) axis. In this study the three commercial anesthetics; MS-222, Benzoak® and Aqui-S™ were evaluated for; (1) anesthetic efficacy, (2) safety margin, (3) prolonged exposure and (4) stress-reducing capacity on the Malawi cichlid, red zebra (*Metriaclima estherae*). The overall results show that concentration of 150 mg/L MS-222, 120 mg/L Benzoak® and 50 mg/L Aqui-S™ gave satisfying introduction and recovery time for anesthesia. Both MS-222 and Aqui-S™ gave high safety margins as no fish mortality was recorded after anesthetic exposure for 30 minutes. Benzoak® gave a lower safety margin as there was recorded 50 % mortality following 10 minutes exposure. High mortality rate and sign of insufficient blockage upon the red zebra fish, suggested that prolonged exposure to the tested sedative dosages did not benefit the fish. MS-222 exposure reduced the stress response while Benzoak® and Aqui-S™ seemed to self-induce an increase in plasma cortisol concentration after anesthetic exposure. In conclusion; for short-term treatment on red zebra fish, a concentration of 150 mg/L MS-222 is recommended.

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Introduction

The International Ornamental Trade

The colorful ornamental fishes have become a popular “pet” for many hobby aquarists world wide (UNEP, 2007). The highest share of fishes for ornamental purpose originates from tropical freshwaters. Earlier the trade was based on wild fishes collected form their natural habitat, but as the interest and demand has increased the wild fishes has been exchanged with captive-bred fishes. Today 90 % of freshwater ornamental fish is bred in farming facilities (Watson and Moreau, 2006, UNEP, 2007).

Previously the ornamental fish trade involved only a small number of countries, but recently the industry has expanded (Figure 1) and today there are more than a hundred countries involved in the trade. The expansion has led to a multi-million dollar business world wide (Watson, 2000, UNEP, 2008, Rodriguez, 2006, Ploeg, 2007). Due to large irregularities and discrepancies, the true value of this industry is not accurately known (Ploeg, 2008), but FAO estimated an export value at 340 million US dollars for 2008.

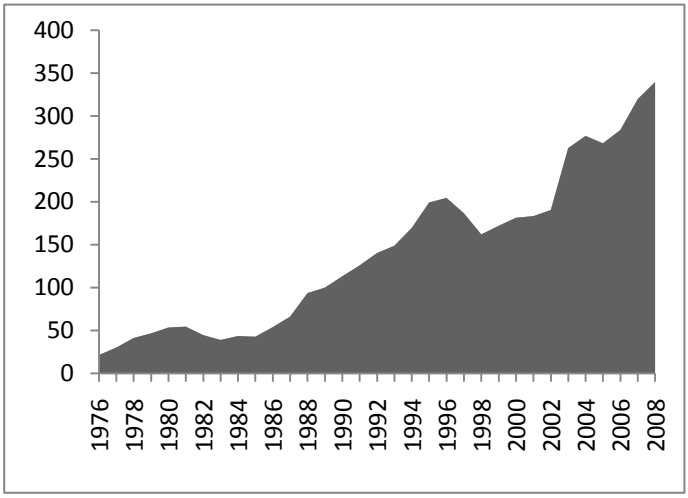

Figure 1: *World total export and re-export value of fish for ornamental purposes, giving in million USD (FAO, 2008).*

The highest share of wild collected fishes occur in the tropics where the biodiversity is highest (Watson and Moreau, 2006). A common problem in these areas is open access and no regulation (Konings, 2009). This has in many cases caused over-collection of fish stocks and environmental destruction. There has been raised concern about harvesting in these exposed areas, as well as on the methods and techniques used for catching the fish. The concern has caused ban on this kind of trade, as well as the promotion of the trade of captive-bred fishes (Watson and Moreau, 2006).

The price ratio for sale of ornamental fish compared to sale of food fish has been estimated at a level of 100:1 (Ploeg, 2007), and it is believed to have the potential to improve the living conditions in many poor and rural communities. Today, the wild collected ornamentals are collected for two main purposes; direct sale and renewal of the genetic material for captive-bred ornamentals. To increase the benefits for the poor at the same time reduce impact on the lake environment, the wild collected ornamentals should be used for local breeding purposes.

World poverty

Finance capital is extremely unevenly distributed throughout the world. According to Frienden (2007) and Aridas (2010), the wealthiest 10 % in the world control 85 % of global asset, while the world poorest 50 % only manage 1 % of global assets. Its estimated that 30 % of the world population is living below the national poverty on 1.25 US dollar a day (Figure 2)(UNDP, 2010b), and that half of the world population is living on less than 2.50 US dollar a day (Shah, 2011). 925 million people are hungry, and 16 000 children die from hunger-related causes every day (Bred-for-the-world, 2010a).

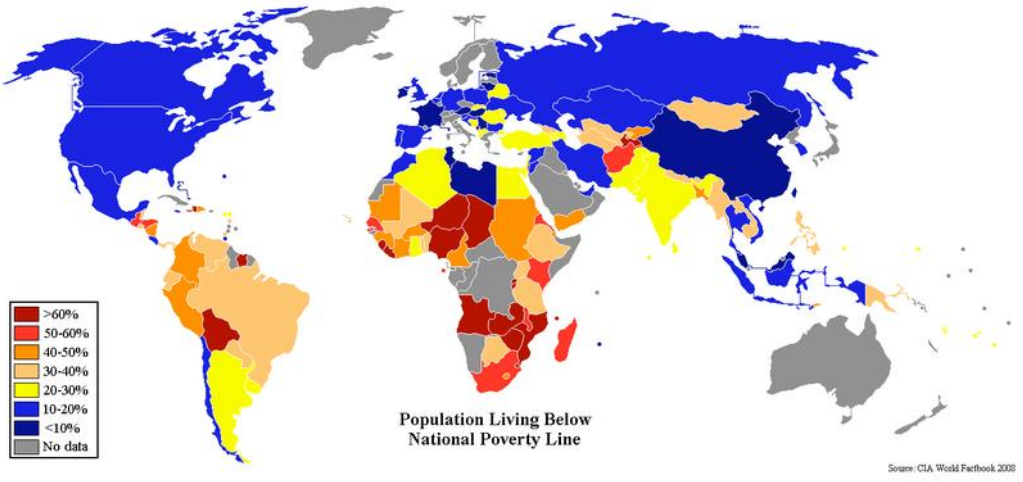

Figure 2: *Percentage of people living below the national poverty line defined as 1.25 USD a day (based on data from CIA, 2008) (websters-online-dictionary.org).*

To fight world poverty, the United Nation (UN) launched eight goals[1] in 2000. These were called the 'Millennium Development Goals' (MDG) and were set to be archived by 2015 by the world leaders. The aim of these goals is to save millions of peoples from poverty with giving them access to human needs and basic rights (UNDP, 2010b).

Since the announcement, livelihood improvements have been reported up till 2007. For this year the situation turned, as the global community was subjected to a financial crisis. The monetary breakdown affected the poorest countries (sub-Saharan Africa, South-Eastern Asia, Southern Asia and Oceania) the most. The financial crisis caused declines in trade and investment, and forced people to vulnerable employment or out of work. Some countries also experienced setbacks due to natural disasters like floods and earthquakes. As a cause of this, the World Bank has estimated that 53 million fewer people will be able to escape from poverty than projected in the MDG's (UNDP, 2010b).

[1] (1) Eradicate extreme poverty and hunger (2) achieve universal primary education (3) promote gender equality and empower women (4) Reduce child mortality (5) improve maternal health (6) combat HIV/AIDS, malaria and other diseases (7) ensure environmental sustainability (8) Develop a global partnership for equality and empower women (4) Reduce child mortality (5) improve maternal health (6) combat HIV/AIDS, malaria and other diseases (7) ensure environmental sustainability (8) Develop a global partnership for development (UNDP, 2010b).

Trade is believed to be the most prominent tool against poverty, and thus, trade of products on the international market will provide economic growth and jobs (Bene et al., 2010, Doney and Wroe, 2005, Frieden, 2007). Many of the Eastern-Asian countries have experienced fast economic growth due to exporting their goods abroad (Doney and Wroe, 2005). Since 1990 to 2009 the poverty percentage in Eastern-Asia has been reduced from 60 % to 13 % (UNDP, 2010b). As for sub-Saharan African countries there have been a slower growth, and since 1990, the trade of poverty has increased from 58 % to 64 % in 2009 (UNDP, 2010b). Heavy farm subsides in developing countries prevent African counties to take a bigger part at the world market, as their main export products are based on agriculture (UNDP, 2010a). In a UK campaign written by Doney and Wroe (2005) they declare that if the trade should be able to benefit poor countries, the global trading system should give everyone a fair chance to compete.

According to UNDP (2010b) are sub-Saharan African countries are growing slowly, and it is assumed that they will not archive the MDG targets within 2015. Even though there is progress, they still represent the highest percentage of extreme hunger and poverty, and the greatest incidence of people dying from water- and food-related diseases, malaria and HIV/AIDS. Sub-Saharan-African countries make up 22 out of 30 high-risk countries that are dependent on food assistance (Bred-for-the-world, 2010b). These countries are also expected to face big challenges in future, due to climate change and environmental degradation. The climate change is believed to increase extreme weather patterns, making agriculture production difficult (UNDP, 2010a). It's expected that climate change will cause a 50 % reduction in the agriculture production by 2020 (Bred-for-the-world, 2010b). The climate change will also cause water shortages, and it is assumed that 75-250 million people will be affected (UNDP, 2008).

Malawi and the cichlids

The Republic of Malawi is ranked as nr 172 out of 182 countries based on Gross Domestic Product (GDP)[2] (Aridas, 2010). Malawi is known as a landlocked and resource poor country, and is heavily dependent on financial support (CIA, 2010, NORAD, 2003). Today Malawi struggles with half of the population living under the national poverty line, rapid growth of HIV/AIDS, a population where 45 % is below the age of 15, undernourished children, low rates of education, environmental degradation, food scarcity and low availability of safe drinking water (FAO, 2010, CIA, 2010). Agriculture is the dominating employment and accounts for 90 % of the export revenues (FAO, 2010). Variable climatic seasons and climate change are challenging the agriculture production in Malawi. They are also believed to facing big challenges as tobacco accounts for half of the export (NORAD, 2003).

[2] GDP is defined as the market value of total good and service produced in a country in a given time (wikibooks.org, 2009).

Figure 3: *Topographic map of Malawi (junglephoto.com, 2005).*

Lake Malawi (Figure 3), one of the African Rift Valley lakes, has a surface area of 29,600 km^2 and makes up approximately 20 % of the land area of Malawi (CIA, 2010). The lake is most known for its enormous content of endemic cichlid fishes. There are over 450 described species (Genner et al., 2004), and it is believed to contain more than 1000 (Kocher, 2004). The cichlids represent the vertebrate group with most rapid radiation and highest speciation, and are considered an important model in evolutionary studies. Many of the cichlids are distinguished from each other only by minor differences and this makes it possible to explore the fundamental processes of speciation and diversification (Genner and Turner, 2005, Kocher, 2004, Stauffer et al., 2002, Kornfield and Smith, 2000). This enormous biodiversity is believed to have evolved over a short time scale, as studies indicate that the lake was 100 meters shallower only 200 – 300 years ago (Smith and Kornfield, 2002).

The wide variety of cichlid fishes in color and form has made them attractive for hobby aquarist world wide. In the 1970s and 80s the demand of wild-caught cichlid fishes from Lake Malawi was high. But subsequently the trade of wild-collected fish has declined as it became possible to buy cheaper captive-bred cichlids form south-east Asian countries and the United States (Watson, 2000). Today only a small trade connected to wild-collected cichlid fish from Lake Malawi is kept alive (Konings, 2009).

Habitat destruction, introduced species, pollution, population growth and overfishing are all factors affecting and threatening the enormous biodiversity in the three African Rift Valley lakes (Stiassny and Meyer, 1999). Open access and poor regulation in combination with widespread poverty constitute big challenges to the unique biodiversity Lake Malawi supports (Konings, 2009, Nyambose, 1997, Chafota et al., 2005).

Malawi cichlids provide a valuable resource that has the potential to improve the living conditions in Malawi. To increase the economic benefit, and at the same time, reduce the impact on the lake environment, the wild-collected fishes should be used for local breeding purpose. Fish production will, however, require proper handling procedures that will ensure fish health and welfare.

Stress tolerance and fish welfare

Stress is a well known concept and is defined by Wendelaar Bonga (1997) 'as a condition in which the dynamic equilibrium of animal organisms called homeostasis is threatened or disturbed as a result from actions of stressors'. The response is in itself beneficial to the fish, as behavioral and physiological changes allow it to avoid or cope with the challenges (Iwama, 2004). The stress response in fish is separated into two concepts defined as 'adaptive' or 'maladaptive'. Acute stress commonly caused by handling or abrupt environmental changes, are designated as adaptive as the fish most likely will be able to recover (Figure 4). When subjected to high density and bad quality water, stress response might become maladaptive as the fish are unable to escape. If the stress load gets chronic, the metabolic energy will be reallocated from the investment activity toward activity for restoring homeostasis (Bonga, 1997). The increased metabolic cost for coping with the stress, will eventually cost the fish its health and well-being (Barton, 2002, Davis, 2010). Overall the stress load will affect its physiological system, causing reduced growth, inhibit reproduction and suppress its immune function. Eventually the fish will be exhausted and is likely to incur disease and die (Barton, 2002, Bonga, 1997, Barton and Iwama, 1991, Portz et al., 2006, Davis, 2010, Adams, 1990, Crosby et al., 2006).

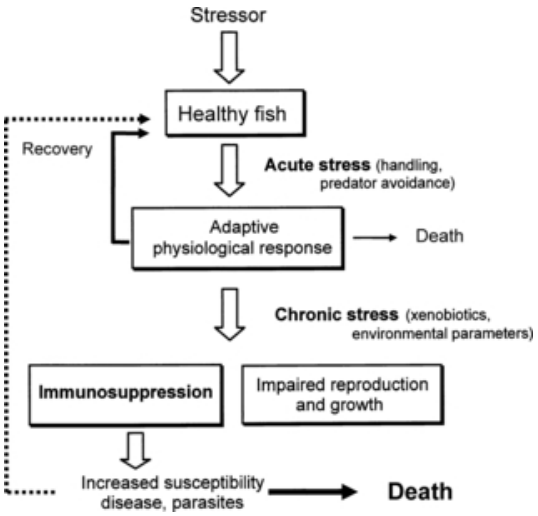

Figure 4: *A schematic illustration of the possible outcome for fish subjected to acute and/or chronic stress (Davis, 2010)*

Fish welfare is a concept that recently raised public concern, as aquaculture and scientific research have exploited (Ashley, 2007). There is still an ongoing discussion about fish as a sensing vertebrate, if it capable of suffering and feeling pain (Ross and Ross, 2008, Carter et al., 2010, Neiffer and Stamper, 2009). Iwama (2004) suggests that fish welfare should be based on its physiology rather than its capability to feeling pain or not. Lund and coworkers (2007) defined welfare as good when the animal's biological coping system is not being overloaded. This suggests that welfare status of animals can be identified or measured by using health and physiological indicators (Brattelid, 1999b, Conte, 2004). Its increased focus on stress physiology as studies show that stress has effect upon other hormones: in male songbird testosterone level was reduced by 37 - 52 % in response to acute stress (Deviche et al., 2010), in red-sided garter snake it was demonstrated that increased glucocorticoids inhibit melatonin synthesis (Lutterschmidt and Mason, 2010) and in rainbow trout prolactin levels were reduced up to 60 % when subjected to chronic stress (Pottinger et al., 1992). Considering fish welfare will not only benefit the fish, but also its owner, as a healthy fish wil increase profit (Lund et al., 2007, Crosby et al., 2006).

The stress response in fish

The physiological stress reaction follows the same basic pattern in all vertebrates; initiated with elevated levels of catecholamine's and corticosteroids. But the magnitude will vary according to duration and severity of the stressor, as well as species (Huntingford et al., 2007, Barton, 2002, Barcellos et al., 1999). In some species concentration of corticosteroids will fluctuate with diel and annual rhythms, as it may control other body processes (Davis and Parker, 1986). However, the physiological changes that occur during a stress reaction was first described in a non-specific way by Selye (1950) through the 'General Adaption Syndrome' (GAS). This GAS concept describes the stress reaction through three stages; the alarm reaction, the stage of resistance and the stage of exhaustion. In later studies, the stress response has been categorized into primary (neural and endocrine changes), secondary (the physiological changes) and tertiary response (changes on the whole individual and population level) (Iwama, 2004, Barton and Iwama, 1991, Bonga, 1997, Barton, 2002, Sigholt and Staurnes, 1992, Pickering, 1981).

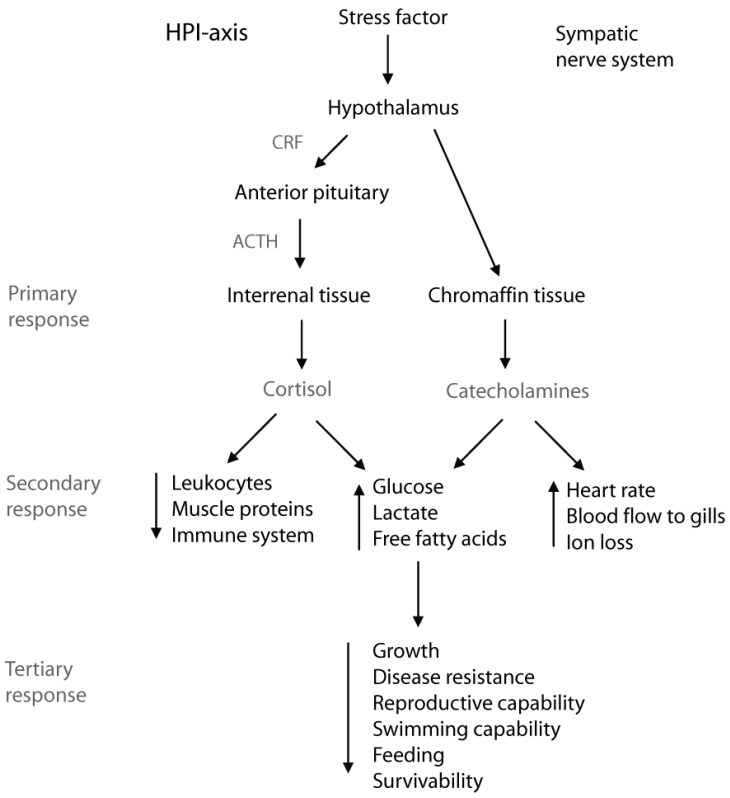

Figure 5: *Overview of the stress reaction, illustrating the two component systems; Hypothalamic-Sympathetic-Chromaffin axis and Hypothalamic-Pituitary-Interrenal (HPI) axis and the following physiological changes. Modified from (Sigholt and Staurnes, 1992, Brattelid, 1999a, Portz et al., 2006)*

The stressor activates a two component system (Figure 5) in the fish: the hypothalamic-sympathetic-chromaffin axis, release of catecholamine's (adrenalin and nor-adrenalin), and the hypothalamus-pituitary-interrenal (HPI) axis, producing and secreting corticosteroids (mainly cortisol in teleostean fish) (Pickering, 1992). The sympathic nerve fibers will stimulate the chromaffin cells in the head kidney to release their storage of catecholamines. The level of circulating catecholamines will increase immediately after stress exposure. On the other hand is the release and circulation elevation of cortisol a more delayed process. The initiation of cortisol production, by the interrenal cells in the head kidney, follows a three stage endocrine pathway. This pathway is initiated by external stimuli that will stimulate hypothalamus to release

corticotrophin-releasing-factor (CRF). CRF will stimulate corticotrophin cells in the anterior-pituitary to secrete adrenocorticotropin hormone (ACTH), which stimulates the interrenal cells to produce and secret corticosteroids, mainly cortisol. The elevated cortisol concentration in the circulatory system functioning as a control mechanism, giving negative feedback in all steps of HPI axis (Barton, 2002).

The increased concentration of circulating catecholamines and cortisol will in turn cause physiological changes on blood and tissue level, referred to as the secondary response. The increased hormone concentration will activate several metabolic pathways that will compensate for the changed condition (Iwama, 2004). Catecholamine's will cause increased ventilation rate and blood flow for increased oxygen uptake and consumption, as well as initiate glycogenolysis (Portz et al., 2006). The main function of cortisol is energy metabolism and hydromineral balance (Bonga, 1997). While catecholamines will cause a short and immediate change with a concentration peak in less than 10 minutes, cortisol will provide a prolonged and delayed effect. The concentration peak of circulating cortisol will occur 30 to 60 minutes after the stress stimulation, and the concentration will generally reach normal values within 5 to 10 hours after acute stress (Brattelid, 1999b). The function of cortisol is to maintain the hyperglycemia in addition to post-stress, after the catecholamine's have subsided (Iwama, 2004). Cortisol is also important for restoration of the osmotic balance and prevent overstimulation of the immune system (Bonga, 1997, Portz et al., 2006).

If the stress becomes chronic it may become maladaptive as the physiological changes will remain elevated over an extended period. This is known as the tertiary response. The physiological compensation for the stress reaction will cause suppression of the non-vital processes; growth, reproduction and immune function, affecting fish welfare and health (Iwama, 2004). The degree of the tertiary response is related to the intensity and duration of the stressor (Portz et al., 2006), but the increased metabolic cost will eventually go beyond its health and welfare. The fish is more susceptible to disease and survivability is reduced (Pickering, 1992).

Anesthesia

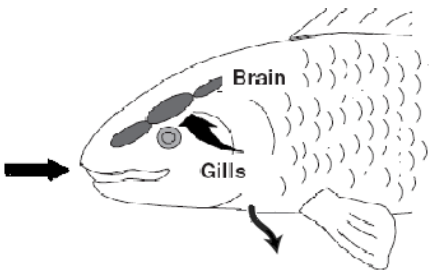

Figure 6: *Illustrates uptake and CNS effect from an active agent (Ross and Ross, 2008)*

Anesthetizing the fish is often useful during handling procedures to reduce trauma and injury (Neiffer and Stamper, 2009). 'Anesthesia' means loss of sensation or insensibility (Ross and Ross, 2008), and can be introduced to fish through physical or chemical techniques. Physical anesthetics are applied through electric tension or refrigeration (Brattelid, 1999c), while chemical anesthetics are based on immersing the fish in a water solution containing a chemical agent. These techniques will cause general anesthesia as they affect the fish sensitivity, equilibrium and consciousness. Mostly this is introduced through 'inhalation anesthesia' (Figure 6), where the active agent mixed in the water is ventilated through the

fish gills (minor through the skin). The agent will pass the blood-brain barrier and have an effect upon the fish central nerve system (CNS) (Brattelid, 1999c, Ross and Ross, 2008). The chemical agent interacts with membrane components and will causes blockage or depression of nerve impulses (Ross and Ross, 2008). This cause loss of mobility, equilibrium and muscle reflexes (Brattelid, 1999c).

Anesthetic treatment may reduce fish's perception of the stressor and thus prevent the nervous input to the CNS (Woods et al., 2008, Brattelid, 1999b). This is desirable because it will block or reduce the cortisol synthesis. Cortisol elevation is known to depend upon intensity and duration of the stressor, and may be detrimental to the fish as the cascade of physiological changes may persist for days or weeks. However, improper dosages and anesthetic drugs may have undesirable side effects upon the fish and may self induce unnecessary stress. It is therefore necessary to find the anesthetic and dosage that is appropriate and have desirable effects on the fish (Carter et al., 2010). An appropriate anesthetic dosage will provide a smooth and rapid anesthesia and recovery (Woods et al., 2008), and should not cause any undesirable side-effects. In addition, the anesthetic agent should provide a satisfying blockage upon the HPI-axis, in order to prevent cortisol elevation when anesthesia subsides (Brattelid, 1999b).

The degree of chemical blockage upon the nervous system varies according to chemical agent, dosage and duration (Burka et al., 1997, McFarland and Klontz, 1969). McFarland (1969) was the first to classify this chemical effect into stages based on behavioral signs (Table 1). The anesthetic effect is ranged from 'sedation', giving a calming effect, to 'surgical anesthesia', giving full immobilization. The analgesic effect of the anesthetics is, however, still unknown (Ross and Ross, 2008).

Table 1: *Stages of anesthesia; modified from (McFarland and Klontz, 1969, Burka et al., 1997)*

Stage	Description	Behavior sign
0	Normal	Active swimming patterns; reactive to external stimuli; normal equilibrium; normal muscle tone.
1	Light sedation	Reduced swimming activity; slight loss of reactivity to visual and tactile stimuli.
2	Light narcosis	Slightly loss of equilibrium
3a	Deep narcosis	Total loss of equilibrium; decreased muscle tone; reactivity to strong tactile stimuli; decreased respiratory rate
3b	Surgical anesthesia	Total loss of reactivity; total loss of muscle tone; low respiratory rate
4	Medullary collapse	Respiration creases, cardiac arrest; death ensures

The basic procedure for introducing anesthesia in fish is divided into three phases; *introduction*, *maintenance* and *recovery* (McFarland and Klontz, 1969, Ross and Ross, 2008). The dept of the introduced anesthesia will vary according to dosage and duration. In order

to not traumatize and stress the fish, the introduction phase should last for a few minutes. However, too rapid introduction is neither desirable as it will harm and kill the fish. The most desirable anesthesia is set to be achieved within 3 minutes (Ross and Ross, 2008, Marking and Meyer, 1985). In some procedures like transportation or surgery, it will be necessary to maintain anesthesia. It should be kept in mind, thought, that different species will have different tolerance to dosage and duration of anesthetic drugs. Maintenance of deep anesthesia for too few minutes is likely to cause death from ventilation and circulatory arrest. Flaring and spasms of the opercula function as warning signals to medullary collapse (McFarland and Klontz, 1969, Ross and Ross, 2008).

Recovery from anesthesia will occur when the fish is immersed in freshwater. The anesthetic agent is then excreted through the gills. As with the introduction of anesthesia, recovery is also divided into different stages based on behavioral sign (Table 2). The recovery should be attained within few minutes to prevent stress and harmful effects on the fish (Woods et al., 2008). The most desirable recovery is set to be retained within 5 minutes (Marking and Meyer, 1985, Ross and Ross, 2008). Higher concentrations and longer exposure time of the anesthetic correspond with longer recovery time (McFarland and Klontz, 1969). After attained an anesthetic procedure the fish is recommended to be under closer observation for 24-72 hours, as death can occur (Ross and Ross, 2008).

Table 2: *Stages of recovery; modified from (Hikasa et al., 1986)*

Stage	Behavior sign
1	Reappearance of opercula movement; weak muscle tone visible
2	Reappearance of swimming activity but still loss of equilibrium
3	Partial recovery of equilibrium
4	Full recovery of equilibrium; reaction in response to visual and tactile stimuli; still stolid behavioral response
5	Total behavior recovery; normal swimming activity

There are several different chemical drugs that can immobilize fish, but not all are described as safe and effective for use on fish. Marking and Meyer (1985) listed up six criteria for an ideal anesthetic; permit reasonable duration of exposure, produce anesthesia within 3 minutes or less, allow recovery within 5 minutes or less, cause no toxicity to fish at treatment levels, present no mammalian safety problems and leave no tissue residues after a withdrawal time of 1 hour or less.

Biological and environmental factors

The time to introduce anesthesia depends on both biotic and abiotic factors. Age, lipid content, size and metabolism are biological factors that will affect the anesthetic effect. The anesthetic can also have different effects within the same species due to biological differences between sex, life-stage and season (Brattelid, 1999c).

The chemical properties of anesthetics may depend upon environmental factors like temperature, pH, salinity, chemical additives and oxygen content (Burka et al., 1997). Lipid-soluble anesthetics may depend upon temperature or solvent for resolution, and some anesthetic will in turn have affect upon water parameters. Fish is a poikiloterm animal and temperature will affect its biological functions. Both temperature and pH will affect gill perfusion, which in turn affect uptake and clearance rate of the anesthetic agent (Ross and Ross, 2008, Burka et al., 1997). To avoid undesirable effects on the fish, the anesthetic treatment is recommended to be carried in water close to the fish biological optima (Brattelid, 1999c).

Anesthetics and ornamental fish

Use of anesthetic is well established within the aquaculture sector for food fish during handling, transport, confinement, vaccination, grading, etc. However, the wide variety in anatomy, physiology and behavior in the fishes, make the anesthetic treatment potential harmful (Neiffer and Stamper, 2009). Regarding tropical ornamentals there is insufficient information available for anesthetic use (Pramod et al., 2010, Crosby et al., 2006), however, there are some publication emphasize on the anesthetic efficacy on some species (Bircan-Yildirim et al., 2010, Young, 2009, Grush et al., 2004, Kaiser and Vine, 1998).

High post-transport mortality of ornamentals from rural communities is a common problem (Tlusty et al., 2005, Watson, 2000). Mortalities during and after transportation events are presumably caused by osmoregulatory dysfunction or stress-mediated diseases. A stressed fish will have increased metabolic rate, which gives increased metabolic load that in turn will give bad quality water. As a warranty for the buyer, the industry standard states that the exporter are expected to compensate for losses that exceeds 5 % death of arrival (DOA) (Lim et al., 2003). High DOA results in low quality products and low economical benefit for the exporter (Pramod et al., 2010). This is often the case in fish transported from rural communities due to absence of equipment, holding facilities and undeveloped infrastructure in combination with poor handling techniques. To supply a resistant and healthy fish, it is necessary to establish proper handling management that will avoid handling-related stress.

The anesthetics selected for this study were; MS-222, Benzoak® and Aqui-S™. MS-222 and Benzoak® are the most used within the Norwegian aquaculture sector, and are generally considered effective and safe in use (Hseu et al., 1998, Gilderhus and Marking, 1987). MS-222 (tricaine methane-sulphonate) is the most commonly anesthetic applied on fish, and is the only anesthetic verified by the U.S. Food and Drug Administration (FDA). It occurs as white crystalline powder directly applied to the water. The disadvantages with MS-222 include high cost, acidity and a required withdrawal period of 21 days for food fish due to lack of mammalian safety data. Benzocaine (the active compound in Benzoak®) is an isomer of tricaine methane-sulphonate. However, compared to MS-222 are benzocaine ≈ 250 times less soluble in water, neutral (Ross and Ross, 2008) and effective at lower concentrations due to hydrophobic properties (Hseu et al., 1998, McFarland and Klontz, 1969). Both MS-222 and benzocaine are classified as local anesthetic agents, as they block neuronal Na^+-

channels, which mean that transmission of action potential are prevented (Kiessling et al., 2009, Burka et al., 1997).

Aqui-S™ is a newer anesthetic developed in New Zealand. It is based on features from naturally produced clove oil that contains 70 – 90 % of the active substance, eugenol. Aqui-S™ contain the isomer, iso-eugenol (50 %), as it has been shown to give more effective and controlled anesthesia compared to clove oil. Aqui-S™ is considered an effective and "stress free" anesthetic, as well as having the advantages of being inexpensive and safe for humans (Ross and Ross, 2008, Kildea et al., 2004, Coyle et al., 2004). Aqui-S™ is approved as anesthetic in Australia, Chile, New Zealand, South Korea and Honduras with no withdrawal period before slaughter (AQUI-S, 2010). The U.S FDA has categorized Aqui-S™ as GRAS (generally regarded as safe) (Ross and Ross, 2008), but it still has not been approved as an anesthetic with no withdrawal period (Young, 2009). No required withdrawal period will mean that Aqui-S™ can be used for "rested-harvest" of fish ready for slaughter (Bosworth et al., 2007, Forgan and Forster, 2010) and field collected fish can be released back to their natural environment immediately after treatment (Stehly and Gingerich, 1999, Young, 2009).

Aim of study

The aim of this study is to assist development and improvement of living conditions in Malawi. To be able to deliver a healthy and resistant fish of high value, it is necessary to establish proper handling strategies that will take care of the fish. Anesthetics may be a useful tool, as they have the potential to both reduce physical injuries and perception of the stressor during handling and transport procedures. To find *the efficacy and stress-reducing capacity of MS-222, Benzoak® and Aqui-S™ on the ornamental cichlid fish, Metriaclima estherae,* the chosen study objectives are as follow

I. Which concentrations satisfy anesthesia and recovery?

II. What is the safety margin for the anesthetics and their chosen concentration?

III. What tolerance does the red zebra fish have for prolonged exposure to diluted concentrations?

IV. Will the anesthetic treatment reduce stress reaction in red zebra fish?

Materials and methods

Study site and object

The cichlid fish, red zebra (*Metriaclima estherae)*, was used as test animal for this study. The fish was supplied from a fish hobbyist in Levanger (Sør-Trøndelag). The fish were kept in 250 and 180 liter aquaria at NTNU (Figure 7). The room maintained a temperature at 23˚C and the light followed a photoperiod regime of 12L: 12D (turned on 8 o'clock in the morning and of 6 o'clock in the evening, giving one hour of dusk and dawn).

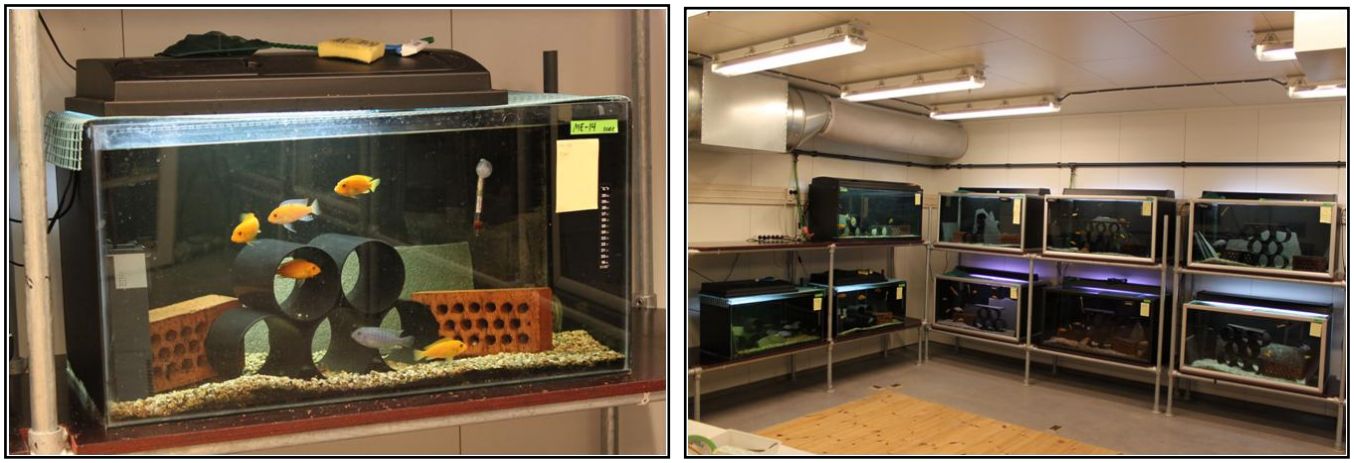

Figure 7: *The holding aquaria containing red zebra fish. The picture to the left shows four females and two males of juvenile-adult red zebra.*

All of the aquaria were equipped with a water pump, thermometer, magnetic algae scratch and aquaria lamp, as well as housing items such gravel substrate (calcareous sand and river gravel), stones, brick stones and polyethylene tubes. The sides of the aquaria were black-coated to prevent visual impact from neighboring aquaria. The first supply of fishes arrived to the animal room 14.05.2009, containing 15 adults (≈1 year) and 39 fry (≈2 months). The juveniles were divided into two aquaria, while the adults were placed in social groups consisting of one male and three to four females (male express territorial and aggressive behavior). The fish were a mixture of normal colored (orange) and color polymorphism (orange blotch) fish. The number of fishes increased during the study period, due to breeding in some of the aquaria.

The daily routine work was feeding and temperature recording. The fishes were fed once a day with commercial flake food (Tetra and Ocean nutrition™), while the fries were feed twice a day with a mix of commercial flake food and *Artemia*. Weekly routine include water change, while water pumps were cleaned monthly.

Red zebra fish

The red zebra fish, *Metriaclima estherae* (also called *Maylandia* or *Pseudotropeus*), used in this study is distributed throughout Lake Malawi. The red zebra is known as a rock-dwelling

mbuna[3], since they are found in the rocky areas in Lake Malawi. Their natural food consists of algae and invertebrates (Tornøe, 1992). The red zebra fish is a common aquarium fish where the regular females are orange red while the males are blue, but orange males and orange blotch (OB) color phenotype also appear within this specie. The orange males can be distinguished from the female by a light blue shade and the OB fish have orange and black spots for the females. The red zebra fish is a social fish where the females are attracted to dominant males. The dominant males differentiate from the subordinated males as they have a lighter and clearer blue color. The dominant males are also larger and more aggressive. The females are mouth-brooders and will keep the eggs and fry (Figure 8) in her mouth for three weeks before she releases them. The clutch size will depend upon the female size, but range from 15 to 40 eggs. The red zebra fish can have a life-span of 10 years (Pagan, 2008).

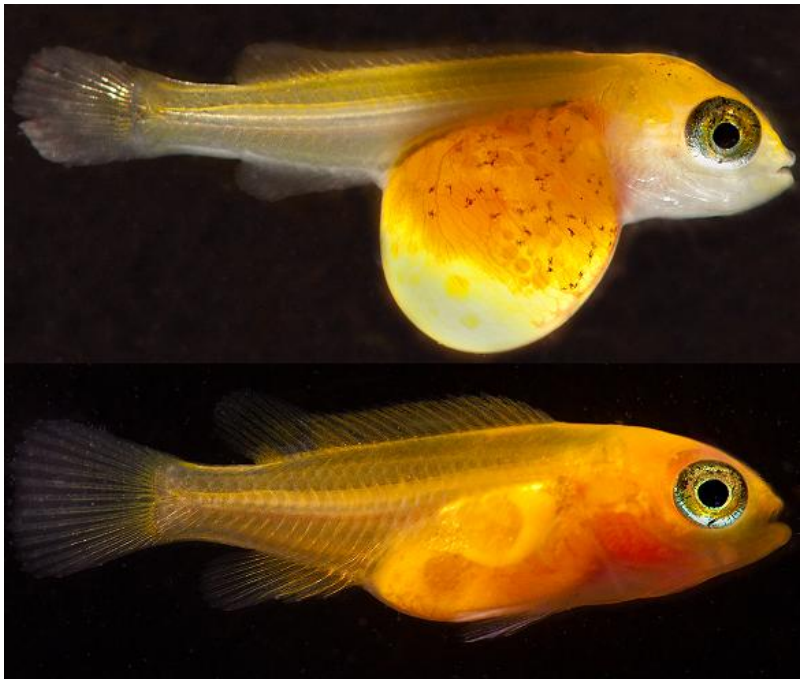

Figure 8: *Yolk sack fry of red zebra fish. Photo Per Olsen*

Natural environment vs. laboratory conditions

Seasonal rhythms in the tropics are not always clearly marked, and the growth and reproduction seem to be continuous year-round (Oduleye, 1982). This is also partly true for the lake environment the red zebra fish originate from. During the dry period (June to August), the water temperature is around 20˚C due to upwelling from deeper water. During the rain period (November to April) the water temperature can be as high as 30˚C. The annual mean water temperature (surface) is 25˚C. The pH varies between 7.8 – 8.6 and the hard water (carbonate) has a value at 3 dKH (Konings, 2003)

[3] Mbuna ("rockfish") makes up one out of three groups within the haplochromine group. In Lake Malawi the cichlids are divided into the two sub-groups; haplochromine (represents the ornamental fish) and tilapiine (represents the food fish) (Tornøe, 1992).

In the holding facilities the temperature was held at 24 (± 3) ˚C, pH ranged between 7.71 – 8.63 and water hardness was approximately 3 dKH (Tetratest kit, freshwater). The fishes showed vigorous feeding, breeding behavior and absence of diseases. Young (2009) refers to this as an indication that the fish thrives in the captive conditions.

Anesthesia

Anesthetics used

The anesthetic drugs selected for this study was: MS-222 (tricaine methane-sulphonate 100 % w/w; PHARMAQ Ltd. Fordingbride, United Kingdom), Benzoak® (200 mg/mL benzocaine, E.131 and additives; A. C. D PHARMACEUTICALS, Leknes, Norway) and Aqui-S™ (540 g/L isoeugenol + emulator; Scan Aqua, Årnes, Norway). All anesthetics are expressed in mg/L in relation to the active substance. MS-222 was measured by use of a gram-scale (Precisa 240A, NERLIENS, Oslo, Norway), while Benzoak® and Aqui-S™ was measured by use of a 50 mL glass cylinder. MS-222 and Benzoak® was applied directly to the temperate water, while Aqui-S™ was prepared as a stock solution at a ratio of 1 part Aqui-S™ solution to 10 parts temperate water.

Dose determination

The package recommended concentration was first tested on a smaller number of fish. These recommended dosages were based on introducing anesthesia on salmonids, which are generally more vulnerable for the anesthetics than tropical fishes. Due to little anesthetic effect on the red zebra fish, the package recommended concentration was chosen as the lowest (Table 3).

Table 3: *The three anesthetic drugs and the concentrations tested in experiment 1a and b upon fry and juvenile-adult red zebra fish.*

Anesthetics	***Concentrations (mg/L)***		
MS-222	*50*	*100*	*150*
Benzoak®	*40*	*80*	*120*
Aqui-S™	*10*	*25*	*50*

Experimental procedure

During the implementation of the different experimental procedures (described below), 80 liters glass aquaria were used, each equipped with aeration stone. The water in these aquaria was mixed (half from holding aquaria and half new freshwater stored on barrels). The fish was unfed for 24 hours prior to the experiments. The water temperature (aquatic mercury thermometer) and pH (MP220 pH-meter, Mettler Toledo) was recorded before and after the experiments.

Experiment 1a: Anesthetic efficacy of MS-222, Benzoak® and Aqui-S™

Groups of red zebra fish from two size classes (fry; 0.4 ± 0.3 gram, juvenile-adult; 6.1 ± 2.1 gram, n = 12) were exposed to one of three different concentrations of MS-222, Benzoak®

and Aqui-S™ (Table 3). The fish were transferred to an acclimation aquarium two hours prior to the experiment performance. When performing the experiments, single fish were quietly scooped and transferred from the acclimation aquaria and immersed to the anesthetic solution in the experimental aquarium. The time lapsed for introduction of the different anesthetic stages followed Table 1. After reaching the final stage, surgical anesthesia (stage 3b), the fish were weighed (Sartorius BP4100, Kebo-Lab) before transferred to the resuscitation aquaria containing only aerated freshwater. Total observation time was 10 minutes.

Experiment 1b: Recovery from MS-222, Benzoak® or Aqui-S™ exposure

The recovery tests were run independently from the anesthetic experiments, in order to give all fishes the same starting point. The exposure time was selected based on the average introduction time achieved from experiment 1a (Table 4). Groups of red zebra fish from two size classes were used (fry; 0.4 ± 0.3 gram, juvenile-adult; 6.1 ± 2.1 gram, n = 6). After the desirable exposure time of the chosen anesthetic and its concentration, the fish were transferred to the resuscitation aquarium for recovery. The time to intrigue the different recovery stages (according to Table 2) were recorded for each individual fish.

Table 4: *The anesthetic concentrations and the corresponding exposure time tested on fry and juvenile-adult red zebra fish.*

MS-222		***Benzoak®***		***Aqui-S™***	
Concentration (mg/L)	***Duration (min)***	***Concentration (mg/L)***	***Duration (min)***	***Concentration (mg/L)***	***Duration (min)***
50	*10*	*40*	*10*	*10*	*10*
100	*6*	*80*	*6*	*25*	*5*
150	*3*	*120*	*3*	*50*	*2.30*

Experiment 2: Safety margin; exposure to full anesthetic concentration of MS-222, Benzoak® and Aqui-S™ in time interval of 10, 20 and 30 minutes.

Groups of juvenile-adult (6.7 ± 1.3 grams, n = 6) red zebra fish were exposed to full anesthetic concentration of the three respective anesthetics in 10, 20 and 30 minutes (Table 5). Two hours prior to the experiment, the fishes were transferred to the acclimatization aquarium. Eighteen fishes were netted at a time to the anesthetic containing aquarium. After the desirable time intervals, six fishes were transferred to the resuscitation aquaria. Behavior was monitored.

Table 5: *The concentration and time interval used for testing safety margin of the three anesthetic dosages on juvenile-adult red zebra fish.*

Anesthetic	***Concentration***	***Duration (min)***		
MS-222	*150 mg/L*	*10*	*20*	*30*
Benzoak®	*120 mg/L*	*10*	*20*	*30*
Aqui-S™	*50 mg/L*	*10*	*20*	*30*

Experiment 3: Tolerance test; 48 hours exposure to diluted concentrations of MS-222, Benzoak® and Aqui-S™.

Groups of fry (0.4 ± 0.3 gram, n = 8) red zebra fish were exposed to one out of four sedative concentrations of MS-222, Benzoak® and Aqui-S™. The fishes were transferred to acclimation aquaria two hours prior to the experiment. Eight fishes were first transferred from the acclimatization aquarium to an aquarium containing full anesthetic dosage. After obtained surgical anesthesia, the fish were moved to the sedative maintenance solution (Table 6). During the exposure fish behavior were monitored and dead fish were removed. After 48 hours exposure the fish were transferred to the resuscitation aquaria. Fish observed with abnormal behavior still after 24 hours recovery was killed with an anesthetic overdose (300 mg/L). Fish that attained normal behavior were transferred back to their holding aquarium.

Table 6: *The anesthetic introduction concentration and subsequent sedative concentration for maintenance exposure on red zebra fry.*

Anesthetic	***Introduction (mg/L)***	***Diluted concentrations (mg/L)***			
MS-222	*125*	*75*	*50*	*25*	*12.5*
Benzoak®	*100*	*50*	*25*	*12.5*	*6*
Aqui-S™	*25*	*25*	*12*	*6*	*3*

Stress test

Cortisol is widely used as an endocrine stress indicator in fish (Martinez-Porchas et al., 2009, Bolasina, 2006, Crosby et al., 2006, Barton and Iwama, 1991). Cortisol is documented to have a delayed concentration peak 30 to 60 minutes after stress (Iwama, 2004), and as well the magnitude and extent of cortisol concentration usually reflect the intensity and duration of the stressor (Barton and Iwama, 1991). In order to elicit a cortisol elevation in red zebra fish, the fish were exposed to air for 1 minute. The same procedure has documented significant cortisol elevation for cobia (*Rachycentron canadum*) 30 minutes after stress (Trushenski et al., 2010).

Experiment 4: The stress-reducing capacity of MS-222, Benzoak® and Aqui-S™

Groups of adult (≈ 2-3 years) red zebra fish (22.08 ± 7.9 gram; 10.4 ± 1.3 cm, n = 7) of both sexes were exposed to MS-222 (150 mg/L), Benzoak® (120 mg/L) and Aqui-S™ (50 mg/L). Fish group of eight were transferred to the acclimation aquarium the day before. There was no human interaction with the fishes in the last 12 hours before the experiment. The anesthetic drug was applied through a plastic hose connected to the aquarium from the neighbor room. MS-222 was dissolved in 1 liter temperate water before applied. After the desired introduce time (MS-222: 3 minutes; Benzoak®: 3 minutes; Aqui-S™: 2.5 minutes) the fish were transferred to the resuscitation aquarium. 30 minutes from the anesthetic drug was applied, the fish were transferred to a bucket containing anesthetic overdose (300 mg/L MS-222). When equilibrium was lost, the fish was picked up and given a subsequent blow to

the head. Blood was sampled from the caudal vasculature by heparinized (Heparin LEO, 25 000 IE/mL) syringes (BD plastipak™: 1 mL, Madrid, Spain) cannula (BD Microlance™ 3: blue: 0.6 x 25 mm and yellow: 0.3 x 12 mm, Fraga, Spain). The blood samples were centrifuged (Mikro 22R, Hettich Zentrifugen) at 4500 rpm for 5 minutes, and the plasma were collected and frozen at -20 ˚C.

Analytic procedures

Cortisol concentration (nmol/L, nM) was quantified from the plasma samples by use of Radio Immunoassay kit (RIA) (Coat-A-Count Cortisol, Simens). This RIA kit used cortisol marked with radioactive I^{125} (iodine). The RIA method is based on a competition between labeled (Ag*, tracer) and unlabeled antigen (Ag, the hormone to be quantified) to a constant deficit concentration of a specified antibody (Ab). The kit supply pre-coated antibody tubes. The binding kinetics follows equilibrium equation and can be expressed by the following equation:

$$Ag + Ag^* + Ab \rightleftarrows AgAb + Ag^* \qquad Ag^*Ab + Ag \tag{1}$$

Ag*Ab and AgAb demonstrate the bound fraction, while Ag* and Ag demonstrate the free antigen fraction (Chard, 1995). In this study the Ag*Ab was counted by use of a gamma counter (Packard COBRA™II Auto-gamma). The unknown cortisol amount was quantified using a curve constricted from kit standards. The cortisol concentration was automatically calculated, and the counting time was 1 minute for each sample.

The samples were run as singles. In addition, control cortisol samples (Lyphochek Immunoassay Pluss Control, cortisol level 2, BioRad, France) were run in duplicates.

In order to evaluate the quality of the results obtained from RIA; *sensitivity, precision* and *accuracy* were defined. The detection limit (sensitivity) was defined by the gamma counter, while precision and accuracy of the assay was defined though calculating the coefficient of variation (CV = (SD/mean x 100 %)) for triplet samples. Preferably, the CV value should be ≤ 10%.

Conversion factor

Cortisol concentration given in ng/mL or µg/dL in other published papers was converted to nM

To convert ng/mL to µg/dL:

$$ng/mL \,/\, 10 = \mu g/dL \tag{2}$$

The convesion factor for µg/dL to nM is given as:

$$\mu g/dL = nM/27.95 \tag{3}$$

Graphic and statistics

All graphic presentations are presented as mean ± standard deviation (SD) and are produced by use of SigmaPlot 11.0 for Microsoft Windows. All statistical analysis are performed by use of SPSS 18.0.

Because of non-normalized data, Mann-Whitney U-test was used to run the statistical analyze for comparison of size classes (introduction and recovery time) and cortisol concentration between control/stress group and the anesthetic treated groups. $p < 0.05$ was designated as significant difference between groups.

Results

Anesthesia

Experiment 1a and b: Anesthetic efficacy and recovery from MS-222, Benzoak® and Aqui-S™
Figure 9 shows the average introduction and recovery times attained for the three different dosages tested of MS-222, Benzoak® and Aqui-S™ on two size classes of red zebra fish (fry; 0.4 ± 0.3 gram, juvenile-adults; 6.1 ± 2.1 gram). Temperature and pH had a mean value of 20.9 ˚C (± 0.8) and 8.0 (± 0.5) during the experiment.

The concentrations tested for MS-222 was 50, 100 and 150 mg/L (tricaine methane-sulphonate). Exposure to 50 mg/L MS-222 for 10 minutes was not sufficient to introduce red zebra fish to surgical anesthesia (stage 3b). Exposure to 100 mg/L MS-222 induced surgical anesthesia, but gave a long introduction time for both size classes (08:04 ± 01:20, 06:33 ± 1:43), while recovery was obtained within the desirable time (02:37 ± 00:23, 04:03 ± 01:52). Concentration of 150 mg/L MS-222 was chosen as the most satisfying concentration, as it gave the most suitable introduction and a desirable recovery time. For the fry, the introduction and recovery was within the desirable time (03:16 ± 00:34, 03:04 ± 00:31), while for juvenile-adults introduction time slightly extended the desirable criteria (04:32 ± 01:40). Recovery time was, however, suitable (04:03 ± 01:52).

The concentrations tested for Benzoak® was: 40, 80 and 120 mg/L (benzocaine). A concentration of 40 mg/L was not sufficient to introduce red zebra fish to surgical anesthesia. Concentration at 80 mg/L introduced surgical anesthesia, but extended the desirable time for both size classes (05:50 ± 00:51, 06:53 ± 00:58), while recovery remained within for the fry (04:03 ± 00:37) it slightly extended for juvenile-adults (05:32 ± 00:46). Concentration of 120 mg/L was chosen as the most satisfying concentration, as it induced surgical anesthesia within desired introduction time for both fries (1.93 ± 0.91) and juvenile-adults (2.83 ± 0.42). The fry obtain recovery within the desirable time (04:52 ± 01:37), while juvenile-adults slightly extended it (06:41 ± 00:33).

The concentrations tested for Aqui-S™ was: 10, 25 and 50 mg/L (iso-eugenol). The concentration of 10 mg/L Aqui-S™ was not sufficient to induce surgical anesthesia for juvenile-adults, while 41 % of the fry reached the stage. However, it was not observed any sedative traits and unbalance for the fry transferred to freshwater, while 67 % of the juvenile-adults struggled with the balance (stage 2 or 3). The concentration of 25 mg/L introduced surgical anesthesia for both fries (05:49 ± 00:55) and juvenile-adults (05:09 ± 01:10). While recovery time extended for juvenile-adults (07:52 ± 02:21), the fry recovered surprisingly fast (03:11 ± 00:31). Concentration of 50 mg/L was chosen as the most satisfying concentration, as it induce surgical anesthesia within the desirable time for both fries (02:38 ± 00:21) and juvenile-adults (02:31 ± 00:21). The recovery time for the fry (06:15 ± 01:40) exceeded slightly the desirable time, while it gave considerably longer recovery for juvenile-adults (09:23 ± 01:38).

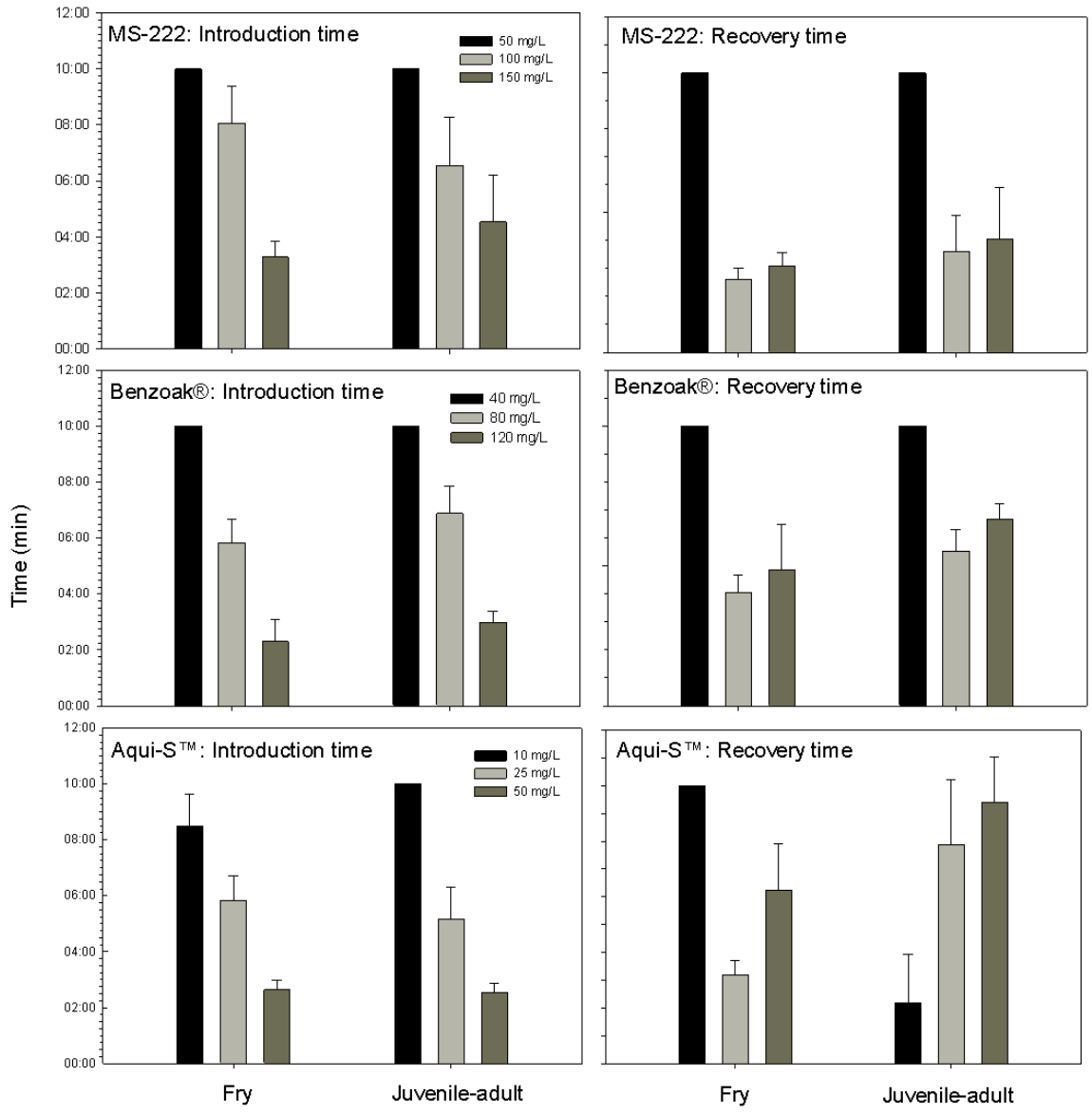

Figure 9: *Average time to introduction of surgical anesthesia (stage 3b) and time to retain full recovery (Stage 4) for two size classes (fry; 0.4 ± 0.3 gram, juvenile-adults; 6.1 ± 2.1 gram) red zebra fish exposed to three different dosages of MS-222, Benzoak® and Aqui-S™ (values are given as mean ± SD, introduction n = 12, recovery n = 6).*

Figure 10 shows the overall average introduction and recovery times for both size classes of red zebra fish at the chosen concentration of MS-222, Benzoak® and Aqui-S™. The efficient dosage of Aqui-S™ and Benzoak® gave the shortest introduction times (02:34 ± 00:21, 02:38 ± 00:43). MS-222 introduction time (03:54 ± 00:57) slightly exceeded the desirable time. Furthermore, Aqui-S™ gave the longest recovery time (07:49 ± 02:17) and MS-222 gave the shortest (03:34 ± 00:46). Recovery time for Benzoak® (05:46 ± 01:30) slightly exceeded the desirable time.

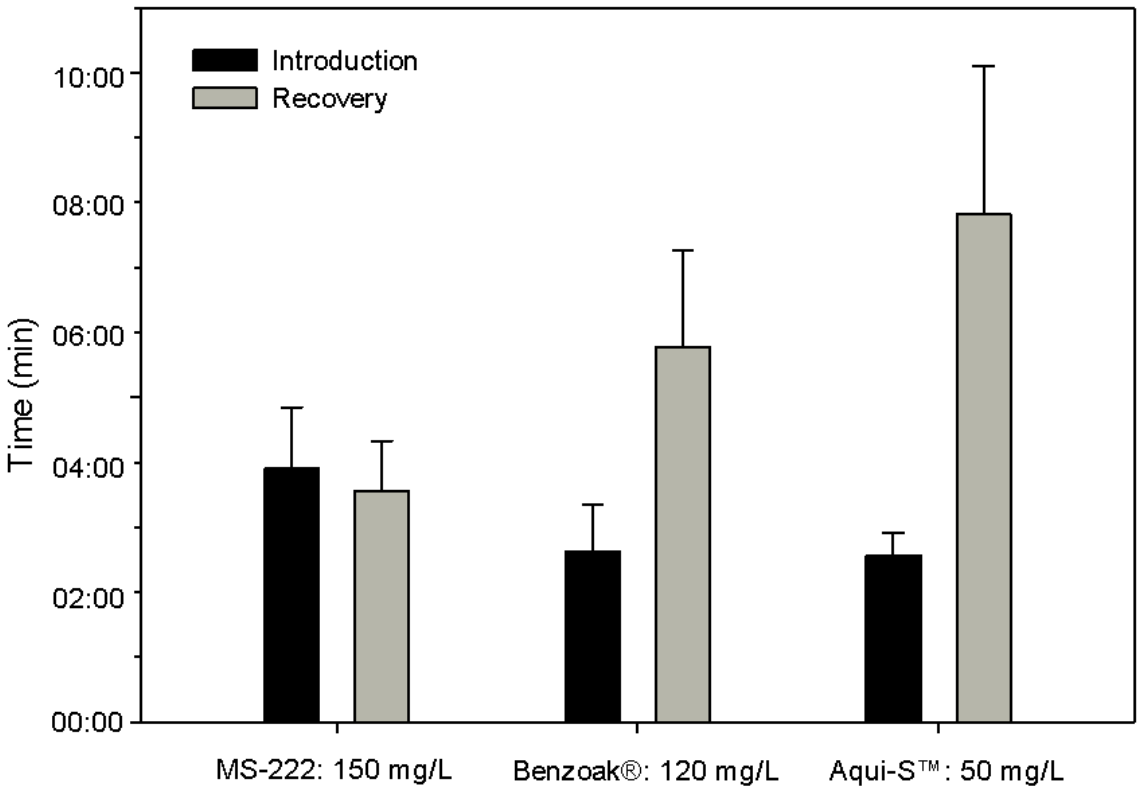

Figure 10: *Chosen dosages and resulting introduction and recovery time for three anesthetics used to anesthetize different size red zebra (fry; 0.4 ± 0.3 gram, juvenile-adults; 6.1 ± 2.1 gram); the overall average introduction and recovery time (values are given as mean ± SD, introduction n = 24, recovery n= 12).*

Experiment 2: Safety margin; exposure to full anesthetic concentration of MS-222, Benzoak® and Aqui-S™ in 10, 20 and 30 minutes

Table 7 shows the survival rate of juvenile-adults red zebra fish (6.7 ± 1.3 gram) exposed to the chosen anesthetic concentration of MS-222 (150 mg/L), Benzoak® (120 mg/L) and Aqui-S™ (50 mg/L) for 10, 20 and 30 minutes. The aim of this experiment was to determine the safety margin of the anesthetics for red zebra fish, as the chosen anesthetic concentration should not be toxic to the fish for extended exposure time. Temperature and pH had an average value of 21.8˚C (± 0.4) and 7.8 (± 0.2) during the experiment.

There were not recorded any mortalities for red zebra fish exposed to MS-222 (150 mg/L). For the fish exposed for 10 and 20 minutes the fish was observed to retain recovery (stage 4) within 6 minutes, while fish exposed for 30 minutes were observed to obtain recovery within 8 minutes. Based on these results and observation, it is suggested that MS-222 has high safety margin for red zebra fish.

Mortalities were recorded for red zebra fish exposed to Benzoak® (120 mg/L). There were recorded 30 % mortality for fish group exposed for 20 minutes and 70 % mortality in fish group exposed for 30 minutes. There was large variation in recovery time between individual fish in all three groups. For the fishes in the 10 minutes group, recovery was retained within

7 minutes, while this time extended and became more variable for the fish exposed for 20 and 30 minutes. Because of wide variation in recovery time and because of death occurrence, the safety margin of Benzoak® is considered to be low for red zebra fish.

There were not recorded any mortalities in fish groups exposed to Aqui-S™ (50 mg/L). But Aqui-S™ exposed fish had extended recovery time compared to MS-222 and Benzoak®. The fish laid motionless for several minutes in the resuscitation aquaria before any gill movement or muscle tone was observed. For the fish groups exposed for 10 and 20 minutes, regular gill movement was not observed before 3 minutes had past. Recovery seemed to be retained within 10 minutes, but in spite of their high swimming activity the fishes showed stolid behavior for a longer period. Stolid behavior means; swimming with its abdomen touching the ground and swimming on all obstacles. For the fish group exposed for 30 minutes, gill and muscle tone was not observed before 5 minutes had past, and recovery was retained within 13 minutes. Despite the long recovery time and the stolid behavior, is Aqui-S™ suggested to have high safety margin for red zebra fish.

Table 7: *Survival and mortality for juvenile-adult (6.7 ± 1.3 g) red zebra fish exposed to the selected dosages of MS-222, Benzoak® and Aqui-S™ for time intervals of: 10, 20 or 30 minutes (n = 6).*

Anesthetic	Time of exposure (min)	# surviving	# not surviving
MS-222: 150 mg/L			
	10	6	-
	20	6	-
	30	6	-
Benzoak®: 120 mg/L			
	10	6	-
	20	4	2
	30	2	4
Aqui-S™: 50 mg/L			
	10	6	-
	20	6	-
	30	6	-

Experiment 3: Tolerance test; 48 hours exposure to diluted concentrations of MS-222, Benzoak® and Aqui-S™

Table 8 shows the survival rate for groups of red zebra fry (0.4 ± 0.3 g) exposed to one out of four sedative concentrations (Table 6) for MS-222, Benzoak® and Aqui-S™ for a period of 48 hours. Temperature and pH had an average value of 20.9˚C (± 0.4) and 8.2 (± 0.3) during the experiment.

In all three anesthetics, the two least diluted concentrations caused 100 % mortality (or in the case of Benzoak® the whole group was killed after the exposure). For the second most sedated concentrations, there were recorded 100 % mortality overall for MS-222, 25 % mortality for Benzoak® and 75 % in total for Aqui-S™. The most sedated concentrations gave considerably low mortality rate for all three anesthetics. For MS-222 (12 mg/L), 25 % was

killed in retrospect due to abnormal behavior still observed after 24 hours. Benzoak® (12 mg/L) corresponds with the result obtained from same concentration of MS-222. For Benzoak® (6 mg/L) there was no mortality recorded, and during the exposure the fish seemed not to be affected by the anesthetic. For Aqui-S™ (3 mg/L) there was one death (12.5 %) recorded between 24 - 48 hours. During the exposure period the fish seemed to be sedated, as some lost equilibrium while others were lying on their abdomen. The fish also showed hyperactive response to visual stimuli. Red zebra fry showed little tolerance to prolonged exposure of diluted anesthetic concentrations. In this experiment they seemed to be most sensitive to MS-222 and Aqui-S™.

Table 8: *Survival and mortality of red zebra fish (0.4 ± 0.3 gram) exposed to sedative concentration of MS-222, Benzoak® and Aqui-S™ for 48 hours. The column "killed after" refers to fry that not recovered back to normal behavior within 24 hours from end of exposure (n = 8).*

Concentration (mg/L)	# mortality (0 - 24 h)	# mortality (24 - 48 h)	# killed after
MS-222: 125 mg/L			
12	0	0	2
25	0	6	2
50	4	4	-
75	8	-	-
Benzoak®: 100 mg/L			
6	0	0	0
12	0	0	2
25	0	0	8
50	8	-	-
Aqui-S™: 25 mg/L			
3	0	1	0
6	2	1	3
12	8	-	-
25	8	-	-

Experiment 4: The stress-reducing capacity of MS-222, Benzoak® and Aqui-S™

Figure 11 shows the plasma levels of cortisol for adult fish (22.0 ± 8.0 gram, 10.4 ± 1.3 cm) 30 minutes after treatment with MS-222 (150 mg/L), Benzoak® (120 mg/L) and Aqui-S™ (50 mg/L). Temperature and pH had an average value of 22.2 (± 1.3) and 7.6 (± 0.2) during the experiment.

The plasma cortisol level from control fish (523.40 ± 303.06 nM) appear to be significant ($p < 0.05$) different from the stress group (1130.33 ± 339.79 nM), Benzoak® exposed group (1146.11 ± 372.63 nM) and Aqui-S™ exposed group (1391.25 ± 569.06 nM). MS-222 exposed group (789.23 ± 159.33 nM) was not significant different from the control group. None of the anesthetic treated groups was significantly different from the stress group.

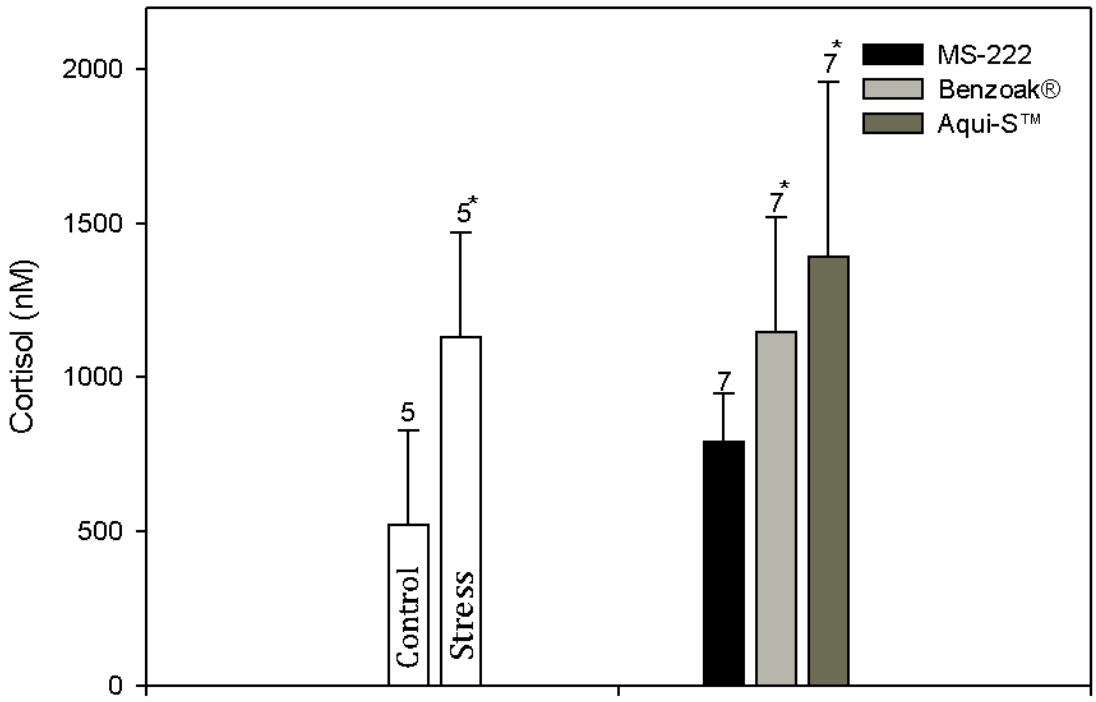

Figure 11: *Plasma cortisol levels in adult red zebra fish (22 ± 8.0 gram, 10.4 ± 1.3 cm) 30 minutes after inflicted anesthesia (and subsequent recovery) with 150 mg/L MS-222, 120 mg/L Benzoak® and 50 mg/L Aqui-S™(values are given as mean (± SD), * mark significance (p < 0.05) from the control group).*

Quality parameters

The sensitivity (detection limit) of the assay was 0.4 nM, the precision had a CV value of 3 % (from triplicate with a concentration of 130.55 ± 3.93 nM) and the accuracy of the known control sample (Lyphochek Immunoassay Pluss Control) was within the defined range (CV at 4.5 % overall from the stated mean value).

Because of high values in the first run, RIA was run twice. In the second run the plasma was diluted 10 times. In all samples from the second run, cortisol concentration had fallen and the inter-assay CV value was estimated at 24 %. There are, however, two important differences to account for the two assay runs; plasma values was thawed for the second time (cortisol may have been degraded by enzymes present in the plasma) and in the second run the values were present in the steep part of the curve while in the flat end of the curve in the first run (the accuracy of the values are different).

Discussion

The cichlid fishes of Lake Malawi are considered to be a “world living treasure” due to their importance within evolutionary and behavioral studies. However, today their biodiversity is in danger due to poor peoples overfishing and pollution (Chafota et al., 2005, Nyambose, 1997). The Malawi fishes are popular as ornamentals on the world basis, but sale of wild-collected cichlids has leveled off due to strong competition of cheaper farmed raised cichlids from other countries (Watson, 2000). In order to preserve the biodiversity and at the same time utilize this valuable resource, the wild-collected fishes should be used for breeding purposes instead of export. Breeding will, however, require procedures for proper keeping, handling and also transport to be able to deliver a high quality ornamental fish at the world market. Today there is little knowledge about the effect of stress on the cichlid fish physiology. Accordingly, this thesis has studied how different anesthetics can reduce stress handling reactions on the cichlid fish, *Metriaclima estherae*.

The endocrine stress response

Selye (1950) described the general response in the following three stages; the alarm reaction (change at the endocrine level), stage of resistance (change at blood and tissue level) and stage of exhaustion (appear if the stress persists). Cortisol and catecholamine’s represent the endocrine change when subjected to a stressor. These stress hormones will subsequently cause a cascade of metabolic and physiological changes, making the animal ready and capable to cope with the undesirable situation. If the stressor persists and the stress hormones remain elevated, the increased energy demand by the life-vital organs will go beyond non-life vital processes like growth and reproduction (Martinez-Porchas et al., 2009). If this continues, the stage of exhaustion will eventually occur and the animal is more susceptible to disease and survivability will be reduced (Pickering, 1992).

Cortisol concentration is often used as a stress indicator to the degree of stress in fish (Martinez-Porchas et al., 2009, Barton, 2002). Air-exposure has in other studies proven to elicit cortisol elevation (Trushenski et al., 2010), and was chosen as the stress test for red zebra fish. The air-exposed fish demonstrated a doubling of cortisol concentration compared to the control group. This proves that the red zebra fish also elicit a physiological stress reaction.

Acute stressor has also proven to elicit increased cortisol concentration for nil tilapia (*Oreochromis niloticus*) (Vijayan et al., 1997), Mozambique tilapia (*Oreochromis mossambicus*) (Foo and Lam, 1993, Galhardo et al., 2011, Galhardo et al., 2008) and *N. pulcher* (Mileva et al., 2009). While the red zebra fish gave a doubling in cortisol concentration, gave *Neolamprologus pulcher* a fourteen-fold increase (Mileva et al., 2009) and Mozambique tilapia a twelve-fold increase after confinement stress (Foo and Lam, 1993).

The control group of red zebra fish represents an extremely high resting level of cortisol concentration compared to other related species. For Mozambique tilapia basal level of

cortisol was reported to be less than 27 nM (Foo and Lam, 1993). For the same specie in another study, the basal level was reported at 70 nM (Vijayan et al., 1997). Also for nil tilapia basal level around 60 – 70 nM is reported (Barcellos et al., 1999). One possible suggestion for the high basal concentration measured in red zebra fish may be connected to a rapid cortisol response. For Mozambique tilapia, Foo and Lam (1993) reported a significant cortisol rise already 4 minutes after sampled by netting. A rapid and pronounced cortisol elevation has also been reported for *N. pulcher*, where cortisol concentration raised to 1372 nM after 10 minutes confinement stress (Mileva et al., 2009). For salmonids the plasma cortisol concentration are characteristically reported between 110 – 560 nM after stressor (Barton and Iwama, 1991). In this experiment, the blood sampling procedure took about 8 minutes to finish, and where the cortisol concentration increase following the individual fish collected (except for one). This suggests that the physiological change has been induced before the blood sampling procedure was finished.

Another factor that may explain the high cortisol concentration is the consequence of changed environment. The cichlid fishes social system is regulated by aggressive interactions (Clement et al., 2005). Dominant males are territorial and aggressive toward other fish in the group (Galhardo et al., 2008), and it is therefore believed that replacement of the red zebra fish from their holding aquaria to a new aquaria have caused changes in the internal social rankings. At this point the exact cause of the high basal concentration of cortisol measured for red zebra fish is not clear, and it is suggested to be reinvestigated.

Anesthetic efficacy

Affecting factors

The efficacy and toxicological effect of an anesthetic will depend upon species and its body size, fat deposits and age. These biological features may also vary according to season, life stage and maturation (Burka et al., 1997, Brattelid, 1999c, McFarland, 1959).

Environmental factors like temperature, pH, salinity, chemical additives and oxygen content may also have affect upon anesthetic efficacy. Fish is a poikilothermic animal, and its biological functions will in most cases depend upon ambient temperature. Temperature and pH is also known to affect gill perfusion area (Burka et al., 1997). Water quality can have affect on the agent's chemical properties (McFarland, 1959), and on the other hand may some anesthetic have affect on the water quality (Ferreira et al., 1979). Accordingly, testing of anesthetic efficacy should ideally be excluded at the fish's known physiological optima (Sylvester, 1975).

Introduced anesthesia

Behavioral observation out of the three concentrations (50, 100 and 150 mg/L) tested for MS-222, showed that 150 mg/L gave the most satisfying introduction time of 4 minutes for both size classes of red zebra fish. This result correspond with Smith and Hattingh (1979) study on *Sarotherodon mossambicus* (tilapia) reporting that 150 mg/L MS-222 also gave a

mean introduction time of 4.50 minutes. Concentration at 100 mg/L MS-222 is reported to give a suitable introduction time for both *S. mossambicus*, *Cyprinus carpi* (carp) (Ferreira et al., 1979) and goldlined sea bream (*Sparus sarba*) (Hseu et al., 1998). Warm water species seems to require higher concentration to get anesthetized compared to cold water species. This correlates with the higher metabolic rate expressed in warm water species, causing a faster metabolic clearance (Young, 2009). Typically concentration of MS-222 is recommended between 60 – 80 mg/L for salmonids (steelhead-trout, *Oncorhynchus mykiss* (Pirhonen and Schreck, 2003), rainbow trout, *Oncorhynchus mykiss Walbaum* (Wagner et al., 2003), arctic charr, *Salvelinus alpinus* (Andersen, 2005), snow-trout, *Schizothorax plagiostomus* (Hveding, 2008) and Atlantic salmon, *Salmo salar* (Kiessling et al., 2009)) while 100 – 200 mg/L for warm water species *C. carpio* (Hikasa et al., 1986), *S. mossambicus* (Smit and Hattingh, 1979) and goldlined sea bream (Hseu et al., 1998)).

Behavioral observation out of the three concentrations (40, 80 and 120 mg/L) tested for Benzoak®, showed that 120 mg/L gave the most satisfying introduction time at 2.50 minutes for both size classes of red zebra fish. This is consistent with results from a study on nil tilapia, where 120 mg/L benzocaine gave an introduction time at approximately 2 minutes (Okamura et al., 2010). For *O. mossambicus* gave 100 mg/L benzocaine hydrochloride anesthesia within 3 minutes (Ferreira et al., 1984). For *S. mossambicus* and *C. carpi* concentration > 80 mg/L benzocaine was reported to be sufficient (Ferreira et al., 1979). Concentration at 100 mg/L benzocaine was also reported for both tambaqi (*Colossoma macropomum*) (Gomes et al., 2001) and Crucian carp (*Carassium carassium*) (Heo and Shin, 2010), while concentration down to 50 mg/L was reported as sufficient to anesthetize the goldlined sea bream (Hseu et al., 1998). As for MS-222, tropical species generally require higher dosages of Benzoak® compared to species adapted to lower temperatures. For temperate species like rainbow trout (*Salmo gairdneri*) (Gilderhus and Marking, 1987) and Atlantic salmon (Iversen et al., 2003), concentration of 35 and 40 mg/L benzocaine is recommended. In comparable studies, benzocaine has shown to be more effective at lower concentrations compared to MS-222 (Andersen, 2005, Hveding, 2008, Hseu et al., 1998, Ferreira et al., 1979). Ferreira and coworkers (1979) found that 50 mg/L benzocaine hydrochloride corresponded to the effect of 80 mg/L MS-222, with study based on carp and tilapia. This correlates with observations in this study, as 80 mg/L Benzoak® and 100 mg/L MS-222 both induce surgical anesthesia of juvenile-adults between 6 - 7 minutes. Benzocaine is more lipid-soluble than MS-222 and will penetrate the blood-brain barrier easier, giving a faster and more pronounced effect. However, it was observed that fish immersed in Benzoak® seemed to attain stage 2 (slightly loss of equilibrium) before stage 1 (slightly loss reactivity to visual and tactile stimuli), and the fish seemed to be more conscious even though equilibrium was lost. This is in contrast to Kiessling and coworkers (2009) study, as they report quicker visual effect in fish (Atlantic salmon) treated with benzocaine compared to MS-222. They relate this observation to the easier penetration of benzocaine through the blood-brain barrier. Why the opposite were observed in this study is

not known, but may be a consequence of the physiological difference between red zebra fish and Atlantic salmon.

Behavioral observation out of the three concentrations (10, 25 and 50 mg/L) tested for Aqui-S™, showed that 50 mg/L gave the most satisfying introduction time of 2.50 minutes for both size classes of red zebra fish. There were not found any studies testing Aqui-S™ on related species in the literature. However, a study on nil tilapia found that 250 mg/L clove oil was the most suitable concentration (Simoes et al., 2010). For zebra fish (*Danio rerio*) 100 mg/L clove oil was reported as most suitable concentration (Grush et al., 2004). For the small freshwater fish, *Melanotaenia australis,* concentration of 80 mg/L Aqui-S™ caused introduction in less than 3 minutes, while a concentration of 40 mg/L gave extended introduction time for 10 minutes (Young, 2009). A concentration of 20 mg/L Aqui-S™ is reported to be satisfying for bluegill (*Lepomis macrochirus*), channel catfish (*Ictalurus punctatus*), lake-trout (*Salvelinus namaycush*), rainbow trout and yellow perch (*Perca flavescens*), while concentration at 50 mg/L Aqui-S™ was required for walleye (*Stizostedion vitreum*) (Stehly and Gingerich, 1999). Even though there are fewer comparable studies with Aqui-S™, it seems like red zebra fish requires lower concentration of iso-eugenol/eugenol compared to other tropical species. The concentration satisfying red zebra fish seems to correspond more with species like rainbow trout (Wagner et al., 2003) and Chinook salmon (Hill and Forster, 2004) where concentration at 40 mg/L clove oil and 60 mg/L Aqui-S™ has been reported as satisfying concentrations. In the sections above, studies on salmonids report lower dosages for MS-222 and benzocaine compared to red zebra fish (Strange and Schreck, 1978, Hill and Forster, 2004, Gilderhus and Marking, 1987). This “lower” optimal dosage found for Aqui-S™ may be related to the irritating effect it seemed to have on the red zebra fish. When the red zebra fish was immersed in the Aqui-S™ solution, it showed frequently “coughing” reflex and expressed frantic swimming behavior. These behavioral signs are also observed for zebra fish exposed to clove oil (Grush et al., 2004) and for tambaqui immersed in higher than optimal concentrations of benzocaine (> 200 mg/L) (Gomes et al., 2001). For tambaqui this behavioral observation corresponded with increased plasma glucose. Ross and Ross (2008) defined the coughing behavior as an external stress indicator, where the purpose of the coughing reflex is to reverse the water flow over the gills. One suggestion for the rapid anesthesia introduction obtained at the 50 mg/L Aqui-S™ concentration may therefore be related to an inflicted stress reaction. Ventilation and heart rate will change immediately during stress, which in turn may facilitate the uptake and affect of the anesthetic. Furthermore, iso-eugenol has another mode of action compared to MS-222 and benzocaine, where it is described to block nerve signals by disturbing membrane function, while MS-222 and benzocaine will block on specific ion-shuttles (Kiessling et al., 2009). The rapid and pronounced anesthetic effect upon the red zebra fish is believed to be a combination of iso-eugenol mode of action, highly lipid-soluble features and stress impact upon the fish.

For both MS-222 and Benzoak®, introduction time for fry was significantly lower than for juvenile-adult. For Aqui-S™ exposure, there was no significant difference between the two size classes. The quicker introduction time for fry in MS-222 and Benzoak® exposure, is related to the larger gill surface in relation to body mass present in smaller fish. A larger surface area will cause faster uptake and therefore faster effect (Ross and Ross, 2008, Brattelid, 1999c). This has also been observed for channel catfish (Stehly and Gingerich, 1999) and common carp (Basavaraju et al., 1998). However, Durve (1975) reports that fish size has little relation to introduction time for mullet (*Liza tade*) exposed to MS-222. The opposite has also been reported for bluegill (*Lepomis macrochirus*) where juvenile-young succumbed faster to Aqui-S™ exposure than fry-fingerlings. This is suggested to be in relation to disproportionate changes in gill area present in the juvenile-young fish (Stehly and Gingerich, 1999). This is unknown for the red zebra fish, but it is suggested that Aqui-S™ different mode of action may be one explanation for no significant difference recorded between the two size classes.

Recovery from anesthesia

Higher concentrations will introduce faster anesthesia than lower concentrations, but will hence correspond with longer recovery time (Hveding, 2008, Gomes et al., 2001, Hoskonen and Pirhonen, 2004). McFarland and Klontz (1969) argued that the recovery time was proportional to the concentration and exposure time of the anesthetic. This is connected to the increased drug accumulation, which has shown to be in accordance with the study on mullet fingerlings (Durve, 1975). Recovery time will, however, depend upon chemical, dosages and exposure time.

Exposure of 150 mg/L MS-222 for 3 minutes gave desirable recovery time of 3.50 minutes for both size classes of red zebra fish. This correlates with Smith and Hattingh (1979) study on the cichlid fish, *S. massambicus* that obtained recovery within 2.50 minutes exposed to same concentration. The same study show that *C. carpi* and *Salmo gairdneri* (trout) retained recovery within less than 3 minutes. A concentration of 100 mg/L MS222 gave recovery time of 2.50 minutes for tilapia (Ferreira et al., 1979), while goldliner sea bream retained recovery within 1 minute for the same concentration (Hseu et al., 1998). MS-222 consist of both non-polar and polar metabolites, and where the non-polar are rapidly metabolized and excreted trough the gills. This rapid elimination gives a quick recovery (Carter et al., 2010, Burka et al., 1997).

Exposure of 120 mg/L Benzoak® for 3 minutes gave recovery time at approximately 6 minutes for both size classes of red zebra fish. For nil tilapia the same concentration gave a recovery time of 4.50 (Okamura et al., 2010). *S. mossambicus* and *C. carpio* introduced to anesthesia with concentration of 100 mg/L benzocaine, retained recovery within 2.50 and 3 minutes (Ferreira et al., 1984). Also tambaqui subjected to 100 mg/L benzocaine retained recovery within desirable time of 5 minutes, while concentration of 150 mg/L gave extended recovery time of 9 minutes. The slightly longer recovery time for fish exposed to benzocaine

solution seems to correspond with other related studies. This was also expected due to the more lipid-soluble properties of benzocaine compared to MS-222.

The concentration of 50 mg/L Aqui-S™ gave a more extended recovery time of approximately 8 minutes for both size classes of red zebra fish. This longer recovery phase has also been reported in other studies (Kiessling et al., 2009, Hveding, 2008, Keene et al., 1998), and is probably related to iso-eugenol highly lipid-soluble characteristics. However, Young (2009) report satisfying short recovery time at 3 minutes for the small freshwater fish *M. australis* exposed to 80 mg/L Aqui-S™. Stehly and Gringerich (1999) report recovery time at ≤ 7.3 minutes for all five species (bluegill, channel catfish, lake-trout, rainbow-trout and yellow perch) subjected to 20 mg/L Aqui-S™. Also Aqui-S™ exposure on snow-trout (Hveding, 2008) and Arctic charr (Andersen, 2005) gave recovery time of 6-7 minutes. In other studies, the criteria for sufficient recovery have been set to be attained within 10 minutes (Stehly and Gingerich, 1999, Gilderhus and Marking, 1987, Hveding, 2008). Kiessling and coworkers (2009) even suggest that too rapid clearance from anesthesia may make the fish more subjected to stress during the recovery phase. The slow clearance rate and rapid accumulation of iso-eugenol is also demonstrated in the lower concentrations. From the result on red zebra fish, juvenile-adults got anesthetized even though they didn't attain surgical anesthesia. Young (2009) demonstrated that under-dosing of Aqui-S™ caused extensive introduction and recovery times.

There was no significant difference found in recovery time between the two size classes for any of the three anesthetics. There is, however, a tendency of shorter recovery time for fry in all three anesthetics. As with the introduction time, the quicker recovery time is likely a cause of larger gill surface present in smaller fish. As the larger fish often struggled long to attain equilibrium, the fry seemed to attain recovery surprisingly quickly. The fry was also calmer after attained equilibrium, unlike the juvenile-adult fish which was more restless. Sedative signs were more visible in juvenile-adults compared to the fry, which made it more difficult to ascertain recovery stage 4 for fry. This represents some uncertainties about the recovery time.

Overall, Benzoak® and Aqui-S™ gave the most satisfying introduction time, while MS-222 gave the fastest recovery. Furthermore, Aqui-S™ gave the longest recovery time, in addition to have an unpleasant effect on the fish. Based on these observations Aqui-S™ is suggested to be less suitable for red zebra fish. Due to sufficient efficacy and no abnormal behavioral signs, MS-222 and Benzoak® is suggested to comply with the demand as an efficient anesthetic on red zebra fish.

Toxicological effect of the anesthetics

The toxicity of the anesthetic is a function of waterborne concentration and time of exposure (Stehly and Gingerich, 1999). A high safety margin is designated when the effective dosage are lower than the toxic dosage (Ross and Ross, 2008). Gilderhus and Marking (1987)

had a third criteria for the efficacy dosage; giving no mortality after 15 minutes in the anesthetic solution.

Safety margin

In this experiment, neither full exposure to MS-222 (150 mg/L for 30 minutes) nor Aqui-S™ (50 mg/L in 30 minutes) caused any mortality for juvenile-adult red zebra fish. Exposure to full dosage of Benzoak® (120 mg/L) gave mortalities for fish exposed longer than 10 minutes. For Benzoak® there were recorded 30 % mortality for fish exposed for 20 minutes, and 70 % mortality for fish exposed for 30 minutes. For juvenile tambaqui there were not recorded any mortalities for fish exposed to 100 mg/L benzocaine in 10, 20 and 30 minutes (Gomes et al., 2001). Hence, the safety margin for benzocaine appears to vary between studies and species, where some report about its effective properties (Gilderhus and Marking, 1987, Iversen et al., 2003), while other refers to its negative effects (Carneiro et al., 2002, Robertson et al., 1988, Basavaraju et al., 1998). The death occurrence in this study may be a consequence of too high effective concentration chosen for Benzoak®, giving an overdose. Medullary collapse is described as a combination of the agent affect upon the CNS and hypoxia in the ventilation musculature (Brattelid, 1999c). The high mortality rate recorded for Benzoak® exposure indicates a lower safety margin on red zebra fish.

MS-222 seems to have high safety margin for red zebra fish, as there were no mortality recorded. In contrast, it was reported 100 % mortality for mullet fingerlings exposed to 150 mg/L MS-222 for 10 minutes, while no mortality for mullet exposed to 100 mg/L MS-222 for 30 minutes (Durve, 1975). Typically, MS-222 is considered to have low safety for fish, as the effective and toxic concentrations tend to be close (Ross and Ross, 2008, Gilderhus and Marking, 1987, Burka et al., 1997). The rapid elimination of the non-polar metabolites implies that it requires a higher concentration in order to immobilize the fish (Burka et al., 1997). In this study it is believed that MS-222 has high safety margin for red zebra fish.

Due to the rapid and irritating effect Aqui-S™ seems to have on red zebra fish, the high survival rate came more as a surprise. In other studies, Aqui-S™ has been reported to have high safety margin for fish. Stehly and Gingerich (1999) found that the toxic dosage of Aqui-S™ concentration was at least 2.5 times the selected efficacious concentration for young-adult, while 1.4 times the selected efficacious for fingerlings of; bluegill, channel catfish, lake-trout, rainbow-trout, walleye and yellow perch. Exposure to concentration < 50 mg/L Aqui-S™ for 60 minutes gave no mortalities for channel catfish (Bosworth et al., 2007). For *M. australis* there were reported 10 % mortality after 15 minutes exposure to 80 mg/L Aqui-S™ (Young, 2009). In contrast, Sladky and coworkers (2001) found the opposite to be true for red pacu (*Piaractus brachypomus*) exposed to different concentration of eugenol. They discussed the low safety margin of eugenol to be a result of its oily characteristics, as it may coat the fish's gill epithelium and further inhibit gas exchange. In accordance with this study, it was observed that the exposure gave irregular and long recovery. Even when equilibrium was fully obtained and the swimming activity increased, the fishes seemed confused as they constantly swam on to each other and other objects. Aqui-S™ is believed to have a high

safety margin for red zebra fish, but should be used with care due to irregular recovery and lasting effect on the red zebra fish.

The high survival rate for juvenile-adult red zebra fish was unforeseen as the fish was observed to have no gill movement (or only occasionally) through the exposure. Cardiac contractions has been documented to continue for 3 to 5 minutes after opercula movement has ceased (McFarland, 1959). For the red zebra fish, the opercula movement was observed to cease when exposure exceeded 10 to 15 minutes. Despite this, most of the fishes recovered within few minutes in resuscitation aquarium. It is suggested that the high survival for red zebra fish may be related to its high tolerance to hypoxia. From previous experiment (unreported) oxygen level has been measured down to 3 mg/L for red zebra fish without showing any visible effect on the fish. Study on *Astonotus Ocellatus* (Amazonian cichlid fish) demonstrate survival up to 16 hours exposed for hypoxia (Muusze et al., 1998).

Tolerance test

Lower concentrations of the anesthetic can be used to induce sedation in fish, which may be beneficial during longer handling procedures and transportation. Sedation can induce a calming effect on the fish causing reduced metabolism, oxygen consumption and metabolic waste (Coyle et al., 2004, Ross and Ross, 2008, Forgan and Forster, 2010). In this regard it would therefore be interesting to see if prolonged exposure to sedative anesthetic concentrations would benefit the red zebra fish.

The prolonged sedative treatment for 48 hours did not seem to benefit the fry of red zebra fish, due to the high mortality rate recorded. It was recorded 100 % mortality in the two highest concentrations for all three anesthetics. This was evidently early as the fish did not wake up when it was placed in the sedative concentration. During the exposure, fading of ventilation and irregular opercula movement was observed, which is referred to as warning signs for medullary collapse (Hikasa et al., 1986, Hajek et al., 2006). The second highest concentration of Benzoak® (25 mg/L) did not, however, cause any death during the exposure, but seemed to have incurred damaging effect on the fish in retrospect. The statement of dysfunctional effect on the fish is based on the abnormal behavior observed, still expressed after 24 hours recovery. Abnormal behavior was observed as low swimming activity (with the abdomen on the bottom), stolid behavior and frequently shifting between upright and lying position. This is most likely to be a consequence of prolonged hypoxia. Thomas and Robertson (1991) report that anesthetics can act as asphyxiate on fish, due to their possible depressive effect on respiration and autonomous function. Prolonged oxygen depletion will cause physiological and hematological changes, and cause arrhythmia that is damaging to the heart (Brattelid, 1999c). A number of fish in the lower concentration was also killed after the anesthetic exposure, as some of them seemed to have incurred damaging effect as well. Hence, most of these fishes were observed to be in imbalance both during the exposure and recovery. It is therefore believed that fish had low tolerance to prolonged anesthetic exposure, since the fishes that were most affected was the ones that incurred damaging effect in retrospect.

In this experiment, MS-222 and Aqui-S™ exposed fish tend to have higher mortality rate compared to Benzoak® exposed fish, which is in contrast to the safety margin experiment. The concentration of 25 mg/L MS-222 caused mortality during the exposure, while 25 mg/L Benzoak® exposed fish was killed after due to incomplete recovery. Hence, gave concentration of 12 mg/L, for both of the anesthetics, 25 % mortality. The lower mortality rate represented by Benzoak® may be related to its neutral properties. MS-222 has a sulphonate side-chain that makes it acidic (Iversen et al., 2003, Ross and Ross, 2008). In MS-222 exposure, it was observed that the pH value increased from start to end of exposure. In the two lowest concentrations for MS-222 (12 and 25 mg/L) the pH value increased from 7.95 to 8.35 and 7.64 to 8.18, respectively. This is most likely because of rapid degradation on MS-222. McFarland (1959) reported that *Fundulus parvipinnis* sedated by MS-222 seemed not to be affected by the anesthetic, and the rapid breakdown of MS-222 was suggested to be the cause. Furthermore, this means that there should be expected a lower mortality rate for MS-222 exposed red zebra fish. Change in pH during the exposure might be a reason for the higher mortality recorded in MS-222, but a pH change of 0.5 and 1.0 should normally not give any adverse affect on fish (Alpharma, 2001). It is therefore believed that the pH change had little effect on fish survivability. The higher death represented for MS-222 exposed fish are still unknown. Nevertheless, for *Puntius filamentosus* (ornamental freshwater fish) it was reported more pronounced mortality for fish exposed to benzocaine in 48 hours compared to MS-222 (Pramod et al., 2010).

Aqui-S™ concentration at 3 mg/L caused one death after 24 hour exposure, while concentration ≥ 12 mg/L gave 100 % mortality for red zebra fish. This correlates with the study on zebra fish, where no mortality was recoded in 2 mg/L clove oil, one death in 5 mg/L clove oil and 100 % mortality in 30 mg/L clove oil for 96 hours exposure (Grush et al., 2004). From previous experiment, Aqui-S™ treatment is believed to not benefit red zebra fish. During the exposure in 3 mg/l Aqui-S™ the fish was observed to be sedated, due to low swimming activity. The sedation on red zebra fish seemed, however, to be inappropriate as the fish showed hyperactive reaction to visual stimuli. The hyperactive response was observed as a “jumpy” reaction, and where the fish seemed exhausted after the incident. The same observation was made for fish exposed to 12 mg/L Benzoak®. This hyperactive response observed for the red zebra fish is likely to be a consequence of improper blocking on the CNS. Burka and coworkers (1997) explain stage 2 of anesthesia as a level of hyperexcitability as the agent first will block the inhibitory neurons. Both 3 mg/L Aqui-S™ and 12 mg/L Benzoak® seem to induce an undesirable sedation stage on the red zebra fish.

The prolonged exposure to sedative concentrations did not seem to benefit the fry of red zebra fish, due to high mortality rate either during or after exposure. The reason for the high mortality is likely to be a consequence of too high concentrations combined with long exposure time. Benzoak® concentration at 6 mg/L was the only sedative concentration that gave no fish mortality. From the observations it is unknown if the concentration had any tranquilizing effect on the fish, as the fish seemed to have normal swimming activity and

behavior throughout the exposure. All the lower concentrations of the anesthetics (12 mg/L MS-222, 12 mg/L Benzoak® and 3 mg/L Aqui-S™) seem to induce sedation on the fish, as sedative traits were observed during the exposure. Furthermore, both Benzoak® and Aqui-S™ exposed fish showed hyperactive reaction to external stimuli. Hyperactivity is termed as a external signal for stress on fish (Ross and Ross, 2008). Stress during prolonged periods of oxygen starvation is also reported to have detrimental effect (Basavaraju et al., 1998). It has also been reported incidents were anesthetics self-induce physiological changes (Davis and Griffin, 2004, Ross and Ross, 2008, Carneiro et al., 2002). Physiological changes were not demonstrated in this experiment, but underlying changes may have affected fish survivability.

In overall, for the toxicological experiment, MS-222 and Aqui-S™ seems to have high safety margin for short time treatment on red zebra fish. The safety margin for Benzoak® is believed to be lower, due to mortality occurrence. The red zebra fish seem to have low tolerance for long-term exposure.

The physiological effect of anesthetics

In order to establish correct dosage regimes and thereby promote optimal use, physiological and pharmacokinetic analyzes are important (Kiessling et al., 2009). The magnitude of stress is also known to depend upon duration and intensity of stressor, and cortisol is known as the principal endocrine stress response (Molinero and Gonzalez, 1995, Barton and Iwama, 1991, Martinez-Porchas et al., 2009, Thomas and Robertson, 1991). Anesthetics might be a useful tool mitigating fish handling by immobilization of fish and by reduce/block cortisol release (Crosby et al., 2006). In this study it was tested if recovery was accompanied by an increased in stress response.

The anesthetics stress-reducing capacity

All the anesthetic treated groups surpassed the plasma cortisol concentration for the control group. MS-222 with the lowest cortisol concentration was neither significant different from the control nor the stress group. Benzoak® and Aqui-S™ treated groups appear to be significant different from the control group and exceeded the mean cortisol concentration for the stress inflicted group. From the result obtained it seems like Benzoak® and Aqui-S™ self-induce an increased cortisol concentration, when recovering from the anesthetic treatment. The same anesthetics showed to increase cortisol concentration also for cannulated Atlantic salmon 30 minutes after treatment, but where MS-222 gave the most pronounced cortisol elevation (Kiessling et al., 2009). A more pronounced cortisol effect has also been reported for channel catfish, where 100 mg/L MS-222 gave a eight-fold increase while 100 mg/L clove oil gave no significant increase (Small, 2003). Also Bosworth and coworkers (2007) reported lower cortisol concentration in channel catfish 30 minutes after Aqui-S™ treatment compared to the same procedure for MS-222. For fathead minnows a six-fold increase in cortisol concentration was reported 30 minutes after treatment with MS-222, while eugenol gave a smaller increase (Palic et al., 2006). Also other published papers

support the theory of MS-222 lacking stress-reducing capacity upon fish (Thomas and Robertson, 1991, Wagner et al., 2003, Olsen et al., 1995, Molinero and Gonzalez, 1995). The cortisol increase during MS-222 exposure has been suggested as a consequence of reduced ventilation causing hypoxia and increased release of hematocrit, which in turn cause activation upon the HPI-axis (Kiessling et al., 2009, Bolasina, 2006, Molinero and Gonzalez, 1995, Brattelid, 1999c). However, it has been demonstrated on *Astronotus ocellatus* (cichlid fish) that hypoxia exposure did not elicit cortisol elevation (Muusze et al., 1998). This might explain the lower plasma concentration of cortisol present in red zebra fish after treatment with MS-222. Pickering (1993, cited Ross and Ross (2008)) demonstrated that MS-222 blocked the increase of plasma cortisol concentration for rainbow trout, but that the plasma concentration of cortisol increased significantly when anesthesia subsided. Also Strange and Schreck (1978) report that MS-222 abolish cortisol elevation in Chinook salmon.

The high cortisol mean measured for red zebra fish treated with Benzoak® indicate that the treatment was accompanied by cortisol elevation. This correlates with a study on Brazilian codling (*Urophycis brasiliensis)*, reporting that benzocaine anesthesia induce an acute stress response in the fish (Bolasina, 2006). Also a study on Atlantic salmon document increased plasma concentration of cortisol after recovered from benzocaine anesthesia (Iversen et al., 2003). In this study the high cortisol concentration present may be connected to incomplete dissolution of Benzoak® present in the aquarium water. It was observed that the fish was not fully immobilized after 3 minutes from addition of Benzoak®. The incomplete dissolution caused a less effective concentration which in turn caused an extended introduction time at 7 minutes in total to induce surgical anesthesia. The result obtained form Benzoak® exposure should therefore be interpreted with caution.

The highest cortisol mean was measured from Aqui-S™ treated fish. This corresponds with the previous observation of external stress sign during exposure to Aqui-S™. However, Aqui-S™ is reported to be a good alternative anesthetic for MS-222, due to documented stress-reducing effect upon many fish species. Aqui-S™ showed to reduce cortisol concentration for Atlantic salmon exposed for concentration > 20 mg/L (Iversen et al., 2003). Both Small and Chatakondi (2005) and Bosworth and coworkers (2007) reported stress reducing effect of Aqui-S™ upon channel catfish. As explained in the previous section, the similar compound, clove oil, has also shown to have positive effect upon different fish species. In accordance with this study, Davis and Griffin (2004) reported that Aqui-S™ gave significant higher cortisol concentration compared to control fish. The higher cortisol concentration for Aqui-S™ treated fish may also be a result of too high dosage chosen for the red zebra fish. A study on juvenile tambaqui showed significant increased cortisol concentration when anesthetized with higher than optimal dosages of benzocaine (Gomes et al., 2001). Also for gilthead sea bream it is reported that increasing dosages of MS-222 gave an increase in plasma cortisol concentration (Molinero and Gonzalez, 1995).

This study investigates if the anesthetic treatment is accompanied by an increase in cortisol concentration after anesthesia has subsided, and the blocking effect on red zebra fish is therefore not known. However, the high cortisol concentration measured in Benzoak® and Aqui-S™ treated fish, may be a consequence of insufficient blocking upon the HPI-axis. An insufficient blocking may occur at the interrenal level, where this means that anterior pituitary will continue secreting and producing ACTH. When the anesthesia subsides and the blocking effect is removed, this may give a cortisol boost (Nilssen K. J., pers. com.). Olsen and coworkers (1995) demonstrated that the hypnotic drug, metomidate, block at the interrenal level on Atlantic salmon. Further research will be necessary to verify or reject this.

In overall, Benzoak® and Aqui-S™ exposure did not seem to benefit red zebra fish, due to high cortisol concentration measured after the exposure. Both anesthetics surpassed the cortisol mean represented for the stress group, indicating that the treatment was more stressful than air-exposure for 1 minute. The high cortisol concentration for Aqui-S™ did, however, correspond with the behavioral observation done in experiment 1. The result obtained form Benzoak® exposed fish should, however, be interpreted with caution as the longer introduction time might have contributed to the high plasma cortisol. MS-222 was not significantly different from the control group, and it is believed to reduce or alleviate the cortisol elevation in red zebra fish.

The most satisfying anesthetic drug

Based on behavioral observation and the physiological response, MS-222 is believed to be the most suitable anesthetics for short-term treatment on red zebra fish. Table 9 shows an overall evaluation over the three anesthetics versatility based on the different experiments. Although MS-222 came out as the best anesthetic for red zebra fish, the disadvantages of high cost and lack of human safety (Pirhonen and Schreck, 2003, Ross and Ross, 2008) raise the question if it is suitable for use in Malawi.

Aqui-S™ is described as inexpensive and totally safe for humans, and is therefore considered as the most desirable anesthetic for use in Malawi. Based on the result observed in this study, it is believed that Aqui-S™ treatment will not be beneficial for treatment on red zebra fish. Benzoak® has also the advantage that it is inexpensive in use (Ross and Ross, 2008), but gave low safety margin and failed to reduce cortisol elevation for red zebra fish.

To ensure the production of cichlid fish in the future, it is suggested that stress management with use of anesthetics is further investigated. Fish welfare and health will be essential to obtain good results for production and profit.

Table 9: *An overall overview of the three anesthetics and the effective concentration for red zebra fish. Their versatility is categorized according to experimental result obtained in this study.*

Anesthetic	***Suitable concentration***	***Introduction***	***Recovery***	***Safety margin***	***Stress-reducing***	***Cost***
MS-222	*150 mg/L*	*Moderate*	*Short*	*High*	*Moderate*	*High*
Benzoak®	*120 mg/L*	*Short*	*Moderate*	*Low*	*No*	*Low*
Aqui-S™	*50 mg/L*	*Short*	*Long*	*High*	*No*	*Low*

As a final comment to the study, the findings should be used as a guideline rather than as a manual. Different conditions, fish strain and water quality is all factors that will affect the outcome of the anesthetic efficacy. It is therefore recommended to test the anesthetic and its corresponding dosage on a small number of fishes prior to the full anesthetic treatment, as adjustments may be necessary.

The importance of sustainable cichlid production in Malawi

This thesis started with introducing that 'trade is the most prominent tool against poverty'. An increased trade of farm raised cichlids for ornamental purpose is believed to provide a poor and undeveloped country like Malawi economic growth and employment. In order to reduce poverty, local people from rural communities should be involved in the fish production operation. To achieve a sustainable and cost-effective production, the locals should be trained in how to (1) collect and keep the wild cichlid fish, (2) farm raise eggs and fry, (3) handle the fish and (4) transport the fish. Adequate production of high quality fish will provide high profit.

Sale of farmed raised Malawi cichlids is believed to provide development and improvements of living conditions in Malawi. In social terms this means development of schools, health care center, infrastructure, and other necessary goods and services. Employment will also provide livelihood for poor local families, allowing more children to attend school. Education is considered as fundamental for long-term sustainable development, where training local poor people in sustainable production can contribute as a model for food production and production of other goods. Education is said to 'empower the peoples to help themselves' (IIASA, 2008).

Summary

The most efficient dosage of the anesthetic drugs tested was chosen to be 150 mg/L MS-222, 120 mg/L Benzoak® and 50 mg/L Aqui-S™ for both size classes of red zebra fish. Under the introduction treatment of Aqui-S™ the fish expressed external stress signs. There was no such observation for fish during MS-222 and Benzoak® treatment.

The chosen anesthetic dosage of MS-222 and Aqui-S™ for 10, 20 and 30 minutes caused no mortality, indicating high safety margin for red zebra fish. Benzoak® gave a mortality rate of 50 % for red zebra fish exposed for longer than 10 minutes, giving Benzoak® a lower safety margin for red zebra fish.

None of the three anesthetics seem to satisfy needs for prolonged sedation of fry because of high mortality rate recorded and dysfunctional signs observed for some of the surviving fish. Observation during the exposure indicates insufficient blockage on the CNS as the fry (especially treated with the lower dosages of Benzoak® and Aqui-S™) showed hyperactive response to external stimuli.

Both Benzoak® and Aqui-S™ treatment is believed to self-induce an increased cortisol concentration. Whether MS-222 block at any level in the HPI-axis is still unknown, but it is believed that MS-222 reduces or alleviates the stress response in red zebra fish.

Conclusions

I. The concentration that gave satisfying introduction and recovery time for anesthesia on red zebra fish was 150 mg/L MS-222, 120 mg/L Benzoak® and 50 mg/L Aqui-S™.

II. The safety margin was considered as high for MS-222 and Aqui-S™, while low for Benzoak®.

III. The tolerance for prolonged sedation was considered to be low for red zebra fish fry.

IV. The anesthetic treatment of MS-222 seems to reduce the stress response, while Benzoak® and Aqui-S™ seems to self-induce an increase in plasma cortisol concentration.

Perspectives

A continuation of this study may be to document the true basal cortisol concentration for red zebra fish. Furthermore, it should be investigated if the cortisol concentration will vary in fishes with different social rankings, and if the red zebra fish has a quicker and more pronounced cortisol elevation compared to other fish species.

In this study it was tested if recovery from anesthetic treatment was accompanied by an increased cortisol response. As a further step, the anesthetic could be investigated for their blocking effect upon the HPI-axis during surgical anesthesia, and whether it blocks at the right level in the HPI-axis.

Transportation and water quality management is a subject that needs to be investigated. High mortality during transportation is a consequence of oxygen depletion, accumulated ammonia, reduced pH, increased temperature and bacteria buildups. Overall this gives bad transport water. It should be investigated if anesthetic will facilitate transportation of cichlids, and whether it reduces occurrence of stress and hyperactivity. Other additives like zeolite, diluted salt solution and lower water temperature may also be useful alternatives.

There is insufficient knowledge when it comes to cichlid fish physiology. To optimize production it should be examined if reproduction and growth rate is affected by environmental factors. It will also be necessary to examine how to incubate eggs and raise fry. Furthermore, it may be interesting to investigate if the mother only functions as a shelter for the eggs and fry, or if there is other physiological and endocrine factors involved.

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