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The Physiological Stress Response of Bighead Carp *Hypophthalmichthys Nobilis*, *and the Efficacy of Three Chemical Anaesthetics in Bighead Carp Fingerlings*

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Abstract

By increasing the knowledge of physiological responses in different fish species and improving handling protocols by making them more species specific, one can potentially increase production in aquaculture. Bighead carp *Hypophthalmichthys nobilis* is central in Asian aquaculture, but there is limited knowledge regarding physiology, and lack of species specific protocols for anaesthetics. Hence the choice of this species as study species.

The present study examined the stress response in bighead carp by measuring plasma cortisol and glucose in fish after temporary water level reduction. High plasma cortisol (226 $nmol^{-1}$) and glucose (3.4 $mmol^{-1}$) levels in control fish, and lack of significant changes in in cortisol and glucose levels after exposure to a stressor indicate that control fish were stressed. This may be due to the fish not being fully acclimatized after the change in environment, thus, more than 24 hours of acclimatization is recommended for bighead carp. There is also a possibility that the anaesthetic MS-222 may have induced the stress response. Further studies must be conducted to determine the stress profile of bighead carp.

One way to avoid handling related stress is to subject fish to temporary anaesthesia. Accordingly, efficacy of three anaesthetics, MS-222, Benzoak and Aqui-S, were evaluated for bighead carp fingerlings. Induction time, recovery time, and tolerance to prolonged exposure were tested for each of the sedatives. The observations in this study show that of the tested anesthetic agents and concentrations, 75 mgL^{-1} MS-222 gave the most satisfactory results for bighead carp. Induction and recovery times were short, and there was a good safety margin.

In rural areas of developing countries piscicidal plants are used for traditional ethnical fishing. Such plants may be of interest for aquaculture and fish management since they have known biological effects which can potentially have qualities for the development of future commercial use. A pilot study was conducted to observe piscicidal effects from the plant mauwa *Engelhardia spicata,* when applied in rivers (by local fishermen) and under controlled exposure in aquariums. The plant extract proved to be toxic to fish. The extract caused bleeding from the gills, which is most likely a non-reversible damage. Based upon this it is believed that the use of mauwa plant extracts for fishing in river systems may have detrimental ecological consequences.

Sammendrag

Ved å øke kunnskapen om fysiologisk respons i ulike fiskearter, og å forbedre håndteringsprotokoller ved å gjøre dem mer artsspesifikke, kan man potensielt øke produksjonen i akvakulturer. *Hypophthalmichthys nobilis* er sentral i asiatisk oppdrett, men det er mangel på kunnskap om artens fysiologi, og mangel på arts spesifikke prosedyrer for bedøving, på grunnlag av dette ble denne arten valgt til dette studiet.

Dette studiet undersøkte stressrespons i *H. nobilis* ved å måle plasmakortisol og plasmaglukose i fisk etter en reduksjon av vannstanden i vanntanker. Høye plasmakortisol- (226 $nmol^{-1}$) og plasmaglukosenivåer (3.4 $mmol^{-1}$) i kontrollfisken, og mangel på signifikante endringer i kortisol- og glukosenivå etter eksponering for en stressor tyder på at kontrollfisken i dette studiet var allerede stresset. Dette kan skyldes at fisken ikke ble fullt akklimatisert etter endringen i miljøet, og dermed anbefales mer enn 24 timer akklimatisering for *H. nobilis*. Det er også mulig at MS-222 kan ha indusert stressrespons. Videre studier bør gjennomføres for å fastslå stressprofilen til *H. nobilis*.

En måte å unngå håndteringsrelatert stress er å bedøve fisken. Følgelig ble effekten av tre bedøvelsesmidler, MS-222, Benzoak og Aqui-S, for settefisk av *H. nobilis* evaluert. Induksjonstid, oppvåkningstid, og toleranse for langvarig eksponering ble testet for hver av de tre bedøvelsesmidlene. Observasjonene i dette studiet viser at av de testede bedøvelsesmidlene og de tilhørende konsentrasjonene, ga 75 mgL^{-1} MS-222 de mest tilfredsstillende resultatene for *H. nobilis*. Induksjons- og oppvåkningstidene var korte, og det var en god sikkerhetsmargin.

I rural områder i utviklingsland brukes planter med fiskedrepende effekter (piscicidale) i tradisjonell fiske. Slike planter kan være interessante for akvakultur og fiskeforvaltning siden den har kjente biologiske effekter som kan ha kvaliteter som potensielt kan bli utviklet for kommersielle bruk. Et pilotstudie ble gjennomført for å observere piscicidale effekter fra planten *Engelhardia spicata* når den brukes i elver (av lokale fiskere) og under kontrollert eksponering i akvarier. Ekstrakt fra planten viste seg å være giftig for fisk. Ekstraktet forårsaket blødning fra gjellene, hvilket er mest sannsynlig en ikke-reversibel skade. Basert på dette er det antatt at bruk av *E. spicata* planteekstrakter for fiske i vassdrag kan ha store økologiske konsekvenser.

Acknowledgements

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Trondheim, December 2013

Rosemary A. Juell

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1 Introduction

1.1 Stress

Hans Selye (1950) defined stress as "the nonspecific responses of the body to any demand made upon it". He termed the overall reaction the General Adaption Syndrome (GAS), and described it as having three reaction stages; *(i)* the alarm reaction, *(ii)* the stage of resistance, and *(iii)* the stage of exhaustion.

A conceptual framework, dividing the stress response in vertebrates into primary, secondary and tertiary levels was then developed. The primary level involves mainly the endocrine release of catecholamines and glucocorticoids, which induces the secondary response level. The secondary level includes changes in metabolism, hydromineral balance and cardiovascular system. If the stressor becomes chronic, a tertiary response level affects body growth, reallocation of energy, resistance to disease, and survival (Barton, 2002). Thus, it is important to note that impacts may relocate metabolic energy away from investment activities, and may ultimately lead to death if restoration of homeostasis is not achieved. This framework has further been developed to include fish (Wendelaar Bogna, 1997, Barton, 2002) (**Figure 1**).

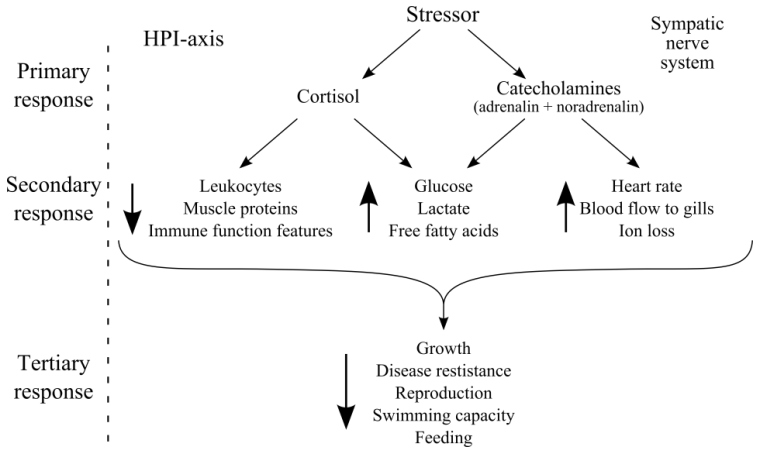

Figure 1 Stressors act on fish and evoke biochemical and physiological effects. These can be grouped as primary, secondary, and tertiary response levels. Arrows indicate induction (↑) and reduction (↓) of physiological factors (modified from Barton 2002 and Barton and Iwama 1991).

1.2 The endocrine stress response in teleost fish

Stress in fish produces many neural and endocrine responses, with those related to the hypothalamus-sympathetic-chromaffin cell (HSC) axis and hypothalamus-pituitary-interrenal

(HPI) axis being best defined. Stimulating the HSC axis causes release of catecholamines (adrenalin and noradrenalin). This response is normally rapid and short lived, affecting respiration, the cardiovascular system, blood oxygen transport capacity, and the mobilization of energy substrates. The activation of the HPI axis produces an increase in glucocorticoids (cortisol and corticosterone). The stressor stimulates the secretion of corticotrophin-releasing hormone (CRH) from CRH-producing neurons in the hypothalamic *nucleus preopticus* (NPO). In fish the CRH is released in vicinity of the corticotrope cells in the pituitary *pars distalis*. Consequently the corticotrope cells secrete and release adrenocorticotropic hormone (ACTH) into general circulation, which in turn stimulates the interrenal cells in the head kidney to synthesise and release cortisol. The cortisol elevation, for acute stressors, may last for one or more hours and affects energy metabolism and hydromineral balance (Papoutsoglou, 2012a, Wendelaar Bogna, 1997; **Figure 2**).

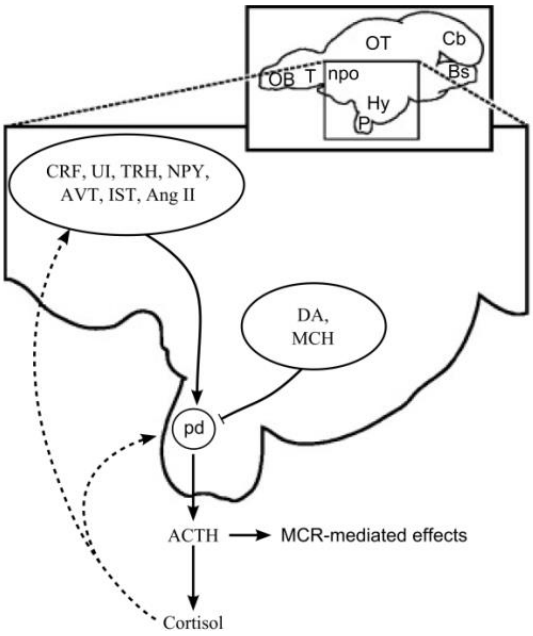

Figure 2 Schematic overview of major factors affecting the hypothalamus-pituitary interrenal (HPI) axis in teleost fish. The mid-sagittal section at the top shows the major divisions of the brain. The preoptic area and the hypothalamus, the primary areas involved in the hypophysiotropic regulation of the HPI-axis, are enlarged. ACTH is recognized as the primary stimulator of cortisol secretion, but several secondary factors may play a role. The actions of both ACTH and cortisol are mediated by different receptor subtypes and cortisol can exert negative feedback on the corticotropes of the pituitary, as well as on some of the hypophysiotropic factors. Solid arrows indicate stimulation, T-line indicates inhibition and dashed arrows indicate negative feedback. Ang II: angiotensinII, AVT: arginine vasotocin, BS: brainstem, Cb: cerebellum, CRF: corticotrophin releasing factor, DA: dopamine, NPY: Neuropeptide Y, Hy: hypothalamus, IST: isotocin, MCH: melanin-concentrating hormone, MCR: melanocortin receptors, npo: *nucleus preopticus*, OB: olfactory bulb, OT: optic tectum, P: pituitary, pd: *pars distalis*, T: telecephalon, TRH: thyrotropin-releasing hormone, UI: urotensin I (modified from Bernier et al. 2009), NPY has been added by the author).

1.3 Factors affecting the release of CRH in teleost fish

1.3.1 Stimulatory factors

Several studies have shown that CRH nerve fibres in fish project into the pituitary *pars distalis*, and are found in particular close proximity to ACTH cells (Cerdá-Reverter and Canosa, 2009). The CRH signal is determined through CRH-receptor subtypes, CRH-R1 or CRH-R2, where CRH-R1 has the highest affinity for CRH. *In vitro* dose-dependent stimulation of ACTH secretion by CRH has been documented in common carp *Cyprinus carpio carpio* (Metz et al., 2004), goldfish *Carassius auratus auratus* (Fryer et al., 1983), and in rainbow trout *Oncorhynchus mykiss* (Baker et al., 1996).

Other hypothalamic factors may also be involved in the regulation of the HPI axis (Wendelaar Bogna, 1997, Bernier et al., 2009). Urotensin-I (UI), a member of the CRF family of peptides, also stimulates ACTH secretion in fish. In a study with UI from white sucker *Catostomus commersonii,* UI was significantly more potent than ovine CRH in stimulating ACTH secretion from superfused goldfish *pars distalis* cells (Fryer et al., 1983). While some uncertainty remains, Neuropeptide Y (NPY) seems also to be indirectly involved in regulating ACTH. A study on chronic stress in rainbow trout suggests that CRH is involved in maintaining the activation of the HPI axis during chronic social stress, and that NPY could also participate in this activation, possibly through interactions with CRH (Doyon et al., 2003).

There is evidence that neurohypophysial hormones also participate in the regulation of ACTH secretion in fish. Isotocin and arginine vasotocin have been reported to stimulate the release of ATCH from the pituitary of goldfish (Fryer et al., 1985) and rainbow trout (Baker et al., 1996). Isotocin and arginine vasotocin produce additive actions (Baker et al., 1996), but do not appear to potentiate the ACTH response to CRH or UI in goldfish (Fryer et al., 1985).

Angiotensins and thyrotropin-releasing hormone (TRH) are additional factors stimulating the release of ACTH. Both angiotensin I and II (Ang I and II) have been shown to stimulate ACTH release from goldfish pituitary *in vitro* (Weld and Fryer, 1987). There is uncertainty regarding TRH interactions with the release of ACTH. No clear evidence that THR-fibres innervate *pars distalis* has been found (Cerdá-Reverter and Canosa, 2009), giving further support to Fryer et al. (1983) who found that in superfused goldfish *pars distalis* no effect was observed on the secretion of ATCH. Contradicting this is a study by Rotllant et al. (2000)

with *in vitro* TRH perfusion of gilthead sea bream *Sparus aurata* pituitary demonstrating ACTH secretion. Alternatively, there could be some major species differences in the secretory control of pituitary endocrine cells.

In summary, regulation of teleost ACTH release is complex involving multiple factors, where CRH seems to be the most significant factor, followed by impacts from UI, AVT, and IST.

1.3.2 Inhibitory factors

Melanin-concentrating hormone (MCH) has been found to be a potent inhibitor of ACTH secretion *in vitro* in rainbow trout (Baker et al., 1985, Baker et al., 1986). Although the majority of MCH axons innervating the teleost pituitary terminate in the posterior area of the neural lobe, some fibres also innervate the *pars distalis*, thus giving further evidence of MCH involvement in the control of synthesis and release of adenohypophyseal hormones (Cerdá-Reverter and Canosa, 2009).

Evidence from an *in vitro* study with common carp suggested ACTH to be under inhibitory control of DA. In addition, CRF can only stimulate ACTH secretion under mild DA inhibitory stimuli (Metz et al., 2004). The innervation of dopaminergic neurons into the pituitary gland has been documented in several species of teleost fish (Cerdá-Reverter and Canosa, 2009).

Cortisol is self-suppressing by negative feedback, both at the pituitary level and at the hypothalamic level (Wendelaar Bogna, 1997). In an *in vitro* study with coho salmon *Oncorhynchus kisutch* cortisol was also found to exert negative feedback directly at the level of the interrenal gland (Bradford et al., 1992).

1.4 Release of ACTH in teleost fish

ACTH is recognized as the primary stimulator of cortisol secretion during the acute phase of the stress response (Flik et al., 2006). Also factors such as sympathetic nerve fibers (Arends et al., 1999), opioid β-endorphin (βEND) (Bernier et al., 2009), Ang II (Perrott and Balment, 1990), UI (Kelsall and Balment, 1998), atrial natriuretic peptide (Arnold-Reed and Balment, 1991) and α-melanocyte-stimulating hormone (αMSH) (Lamers et al., 1992) have been suggested to have indirect corticotropic activity by enhancing the steroidogenic action of ACTH in response to specific stressors and during chronic stress in teleost fish (Bernier et al., 2009). Binding of ACTH to the melanocortin receptors (MCR) in the head kidney activates the enzymatic pathway that converts cholesterol to cortisol (Aluru and Vijayan, 2008, Hagen

et al., 2006). ACTH does so by stimulating steroidogenic acute regulatory protein (StAR) (Aluru and Vijayan, 2008, Hagen et al., 2006), P450 side chain cleavage ($P450_{scc}$) (Aluru and Vijayan, 2008) and 11β-hydroxylase (P45011β) (Hagen et al., 2006). StAR facilitates the transport of cholesterol across the mitochondrial membrane where cholesterol is initially converted to pregnenolone by $P450_{scc}$. Pregnenolone is then subjected to isomerization and hydroxylation by several steroidogenic enzymes and is converted to 11-deoxcortisol. Finally, P45011β converts 11-deoxycortisol to cortisol ((Mommsen et al., 1999; **Figure 3**).

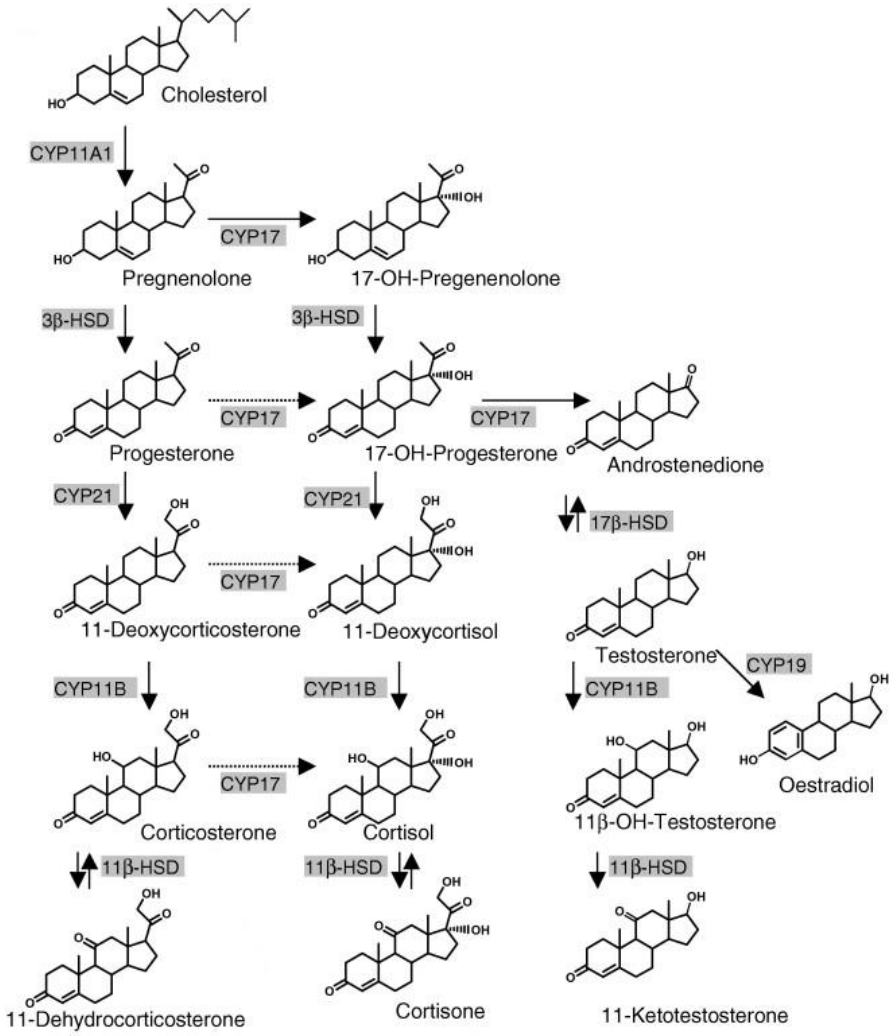

Figure 3 Simplified biosynthesis of adrenocortical and sexual steroids in fish. The steroidogenic enzymes are indicated on arrow labels. The fish corticosteroid synthesis pathway has a number of distinct features: CYP17 catalyses reactions that convert products of corticosterone synthesis to those in the cortisol biosynthesis pathway; teleost fish probably lack aldosterone synthase CYP11B2 and the dominant corticosteroid is cortisol (modified from Bury and Sturm, 2007).

1.5 Cortisol in teleost fish

An increase in plasma cortisol is the most commonly used indicator of stress in fish (Barton and Iwama, 1991). Usually plasma cortisol levels rise rapidly a few minutes after exposure to an acute stressor, and return to normal levels after one or more hours. When the stressor is chronic, the cortisol level remains elevated, but below peak levels (Pickering et al., 1987, Wendelaar Bogna, 1997). Cortisol is then circulated in the bloodstream either as free fraction or attached to transporter proteins as a bound fraction. The bioactive form of the hormone is the free fraction, which due to its lipophilic properties can easily penetrate cell membranes. (Burton and Westphal, 1972, Papoutsoglou, 2012b).

In fish, cortisol has a widespread spectrum of activities in target organs including the gills, intestines, and liver (Wendelaar Bogna, 1997; **Figure 1**). In the classic model for steroid action, free steroids diffuse into the target cell where they bind to intracellular receptors resulting in activation, dimerization and translocation into the nucleus. Activated nuclear receptors subsequently bind to the hormone response elements on appropriate genes causing alterations in their transcription rates (Beato and Sanchez-Pacheco, 1996, Rousseau, 1984). However, some actions of steroid hormones are too rapid to be mediated by this classic genomic mechanism. Accumulating results rather suggest that steroid hormones, including glucocorticoids, may also have non-genomic effects which can rapidly alter physiological processes through membrane-associated mechanisms typically ascribed to the fast effects of neurotransmitters and peptide hormones (Borski, 2000). Comparable phenomena have also been described in teleost fish for cortisol (Mommsen et al., 1999).

1.6 Sedatives in aquaculture

Farmed fish may be subjected to handling and confinement through netting, weighing, sorting, transport and finally slaughter (Zahl et al., 2012). Several reports show that stress associated with handling can be reduced by use of anaesthetic agents. Thus, to ensure welfare, sedation may be necessary in such situations to reduce stress (Neiffer and Stamper, 2009, Ross and Ross, 2008). Sedation is a reduction in sensitivity, and is an introductory stage of anaesthesia. Anaesthesia is a reversible and generalised depression of the CNS, involving hypnosis, analgesia, and subdual of reflex activity, and relaxation of voluntary muscles (Zahl et al., 2012). The depression of the CNS caused by anaesthesia is due to actions on nerve axons, transmitter release, membrane excitability or a combination of these factors. In fish the

anaesthetic is generally administered through inhalation, by dispersing the agent into the water where it may be absorbed across the gills. The effect following sedation/anaesthesia is then usually assessed by monitoring the induction and recovery time, and by the fish reaction to external stimuli (Ross and Ross, 2008).

1.6.1 Stages of anaesthesia and recovery

The progression of the induction and the depth of the anaesthesia are usually divided into distinct and observable stages, first described by McFarland (1959) and later modified by Zahl et al. (2012). The stages are usually described by changes in swimming activity, balance and respiration (Zahl et al., 2012) (**Table 1**).

Table 1 Stages of anaesthesia in fish (Zahl et al., 2012)*.

Stages	Plane	Description stage/ plane	Appearance	Swimming activity	Equilibrium	Responsiveness	Respiration
0		Normal	Normal	Normal	Normal	Normal	Normal
I		Light sedation	Disoriented	Reduced	Normal	Slightly reduced	Normal
II		Excitatory stage	Excited	Increased	Struggles to maintain balance	Normal or exaggerated	Irregular or increased
III	a	Light anaesthesia	Anaesthetised	Stopped	Lost	Reacts to strong tactile stimuli	Normal or decreased
	b	Surgical anaesthesia	Anaesthetised	Stopped	Lost	None	Shallow
	c	Deep narcosis	Anaesthetised	Stopped	Lost	None	Nearly absent
IV		Impending death	Moribund	Stopped	Lost	None	Stopped

* In this study anaesthesia is considered a distinct stage different from sedation.

To ensure good control of the anaesthetic procedure it is important to understand the relationship between concentration, exposure time and the achieved anaesthetic stage. An optimal anaesthetic agent should induce sedation or anaesthesia rapidly, preferably within three to five minutes. Recovery should preferably be obtained within five minutes.

Furthermore the agent is effective at a low dose, and its toxic dose should be considerably higher, giving a large margin of safety (Marking and Meyer, 1985).

1.6.2 Factors affecting the efficacy of anaesthetics

Teleost fish represent a highly diverse group within vertebrates. According to recent estimates the number of accepted species is more than 30 000, which accounts for more than half of all known vertebrates species (Papoutsoglou, 2012b). There are large species variations in reaction to anaesthetics due to heterogeneous pharmacokinetics (i.e. how the drug is absorbed, distributed, metabolised and eliminated) and pharmacodynamics (i.e. the consequences of the interactions between the drug and the site of action). Both pharmacokinetics and pharmacodynamics are affected by biotic and abiotic factors. Important biotic factors include age, sex, life stage, body weight, growth rate, body composition and physiological state. Important abiotic factors are water temperature, salinity, pH, and oxygen level. The anaesthetics' water-solubility, ionisation and stability are physicochemical properties that also influences pharmacokinetics (Zahl et al., 2012).

1.6.3 Anaesthetic drugs - MS-222, Benzoak and Aqui-S

The most frequently used anaesthetics for fish in Norway are MS-222 and Benzoak (Zahl et al., 2012, Kiessling et al., 2009). Both MS-222 and Benzoak act by blocking voltage sensitive sodium channels, thus inhibiting the voltage-dependent increase in sodium conductance. This prevents the initial propagation of action potentials in excitable cells, thereby blocking most neurons and muscle cells (Frazier and Narahashi, 1975, Neumcke et al., 1981; **Table 2**).

MS-222 has a rapid induction and recovery time. Although a good safety margin has been reported, it has also been reported as narrower at higher temperatures (Ross and Ross, 2008). However, both Burka et al. (1997) and Gilderhus and Marking (1987) report a narrow safety margin for MS-222 in salmonids. MS-222 is highly water soluble both in fresh and salt water. MS-222 may reduce pH by 0.5–1.0, and has therefore been described as a potential irritant for fish. However, a short lived environmental reduction of pH by this amount should have a minimal effect in most fish (Ross and Ross, 2008, Burka et al., 1997; **Figure 4** (c)).

Benzoak has a rapid induction and recovery time, and has a good safety margin in cold water (Ross and Ross, 2008). Although Benzoak is chemically very similar to MS-222, it is less acidic and much less water soluble. Thus, stock solutions, including ethanol, aceton or

propylglycol must be made, which, in turn may be irritants for the fish (Burka et al., 1997) (**Figure 4** (b)).

Aqui-S has similar induction time to MS-222, but a longer recovery time. It is particularly useful for harvesting since it has no withdrawal period. It has so far been approved in New Zealand, Australia and Chile (Ross and Ross, 2008; **Figure 4** (a)).The active ingredient in the anaesthetic Aqui-S is isoeugenol which is one of the constituents found in natural clove oil. It is structurally similar to eugenole, also found in clove oil, which has been used as an analgesic in dentistry. Isoeugenol and eugenol are believed to have similar modes of action, inhibiting sodium, potassium and calcium channels, as well as inhibiting N-methyl-D-aspartate receptor (NMDA) receptors and potentiating gamma amino butyric acid $_A$ ($GABA_A$) receptors (Zahl et al., 2012; **Table 2**).

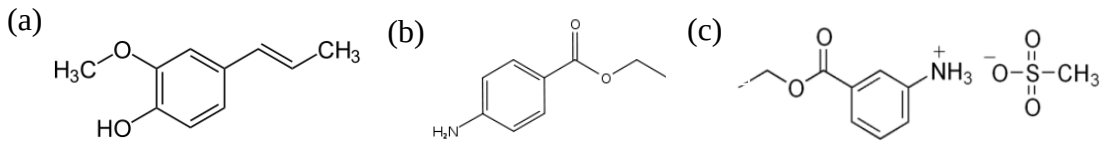

Figure 4 Chemical structure of; isoeugenol (a) the active ingredient in Aqui-S, benzocaine (b) the active ingredient in Benzoak, and tricaine methane-sulphate (c) the active ingredient in MS-222

Table 2 Effects and mechanism of action for tricaine methane- sulphate, benzocaine and isoeugenol,, (modified from Zahl et al. 2012)

Anaesthetic agent	**Mechanism of action**	**Effects**
Tricaine methane-sulphate and benzocaine	Blocks Na^+ channels	Blocks most neurons, glands, and muscle cells (including striated, cardiac and smooth). May cause paralysis and respiratory depression, as well as nociception. In fish, adverse effects include; depression of cardiovascular and respiratory function, increased lactate and elevated catecholamine levels.
Isoeugenol	Inhibits Na^+, K^+, and Ca^{2+} channels Potentiates $GABA_A$ receptors Inhibits NMDA receptors	Produces sedation and hypnosis. No nociception blockage. In fish adverse effects include respiratory and cardiovascular depression as well as inhibition of cortisol synthesis.

1.7 Piscicidal plants

To avoid animal predation many plants produce poisonous substances (secondary metabolites) as a defence mechanism (Levin, 1976). For poor rural living ethnic groups such plants have become important tools in river fishing. Typically, the plant material is thoroughly

pounded using a rock and the crushed material is thrown into a shallow pool of slow moving or stagnant water. The resulting sedated fish are then collected for food. Possible impact of plant chemicals on human health or river ecology is generally not known. Most countries prohibit this traditional form of fishing. However it is still practised in remote areas of the world (Cannon et al., 2004, Neuwinger, 2004).

Some chemicals used in aquaculture are derived from plant extracts. Therefore plant extract from known piscicidal plants may have a future commercial value and potential for use in aquaculture (as is the case of isoeugenol).

1.8 Aquaculture and food security

Undernourishment and malnutrition are still global problems, above all in the world's poorest countries. This affects particularly women and children. To increase food security, and combat starvation and malnutrition it is necessary to increase food production by 75% within year 2050 (UNEP, 2013). Fish are rich in proteins, essential fatty acids, micronutrients and minerals, and are therefore a good food source to improve human nutrition (Kawarazuka and Bene, 2010). Aquaculture, in particular small scale aquaculture, is considered to have great potential in contributing to increased food and nutritional security on a global scale (Ahmed and Lorica, 2002). Furthermore, fish farming industries are more efficient and environmentally friendly compared to other animal protein industries, both when it comes to amount of water and feed consumed (Verdegem et al., 2006).

In 2010, global production of farmed fish for consumption was 59.9 million tonnes; this was 7.5 percent more than in 2009. Over the last three decades (1980–2010) the amount of fish produced for food consumption has increased almost twelvefold (FAO, 2012). Grass carp *Ctenopharyngodon idella*, silver carp *Hypophthalmichthys molitrix*, common carp and bighead carp *Hypophthalmichthys nobilis* contribute to approximately half of the global finfish production (Dey et al., 2006). In 2011, total output from Nepalese inland aquaculture was 30 950 tonnes. In comparison Bangladesh, another developing country in Asia, had an inland production of 1 523 759 tonnes (FAO, 2013b). This illustrates that the productivity in Nepalese fish farming is low.

The Sustainable Poverty Reduction in Nepal (SPRN) is a program initiated by the Norwegian University of Science and Technology (NTNU). The program aims to reduce poverty and

increase food availability for the poor people in Nepal by combining ecological friendly hydropower with development of fish farming and education (K. J. Nilssen, pers. comm.).

Since most of its production is done within extensive systems, the stress and growth related impacts from handling is not known. Bighead carp represents an important species in Asian aquaculture. Thus, this study was designed to document the stress response of bighead carp and to investigate the sedative/anaesthetic efficacy of selected chemicals.

1.9 Aim of this study

- Determine the stress response of bighead carp.
- Test the anaesthetic efficacy of the three chemicals Aqui-S, Benzoak and MS-222 in bighead carp fingerlings.
- Observe piscicidal effects from the mauwa plant *Engelhardia spicata*, when applied on rivers (by local fishermen) or in aquariums.

2 Material and methods

2.1 Study site

Experiments were performed in Nepal, at the Nepal Agricultural Research Council (NARC) research centre in Begnas, Kaski District, Gandaki Zone, North-Central Nepal (**Figure 5**). NARC was established in 1991 "to conduct agricultural research in the country to uplift the economic level of the people" (NARC, 2007). Field work was conducted in Khimti Khola River, in Ramechhap District, Janakpur Zone, in the North-Eastern part of Nepal. Nepal is a climatically and topographically diverse country. There are three main climatic zones; the Himalayan range, Hilly midlands and the Terai plain. In Terai the climate is subtropical, whereas the Himalayan range is cold, and snow and ice are present throughout the year. Most of the annual rain falls in the summer months, during monsoon (Haugan, 2012, CIA, 2013). Both Begnas and Khimti are situated in the Hilly midlands. All lab work and field work carried out in Nepal was done through late March and April 2012.

Figure 5 To the left: map of the world, Nepal is coloured green, positioned between India and China (wikimedia, 2013). To the right: map of Nepal. Study sites are indicated with red, to the left lies Begnas and to the right Khimti (GoogleMaps, 2013).

2.2 Study species

Bighead carp (**Figure 6**) were used for experiments 1-5. Bighead carp belong to the family *Cyprinidae*. It is a freshwater fish native to China, inhabiting both rivers and lakes. Bighead carp normally dwell in the upper part of the water column. It feeds on zooplankton in its natural habitat, but in captivity it will also feed on by-products from grain processing and organic detritus. Bighead carp is fast growing, and can reach a maximum weight of 40 kg (FAO, 2013a).

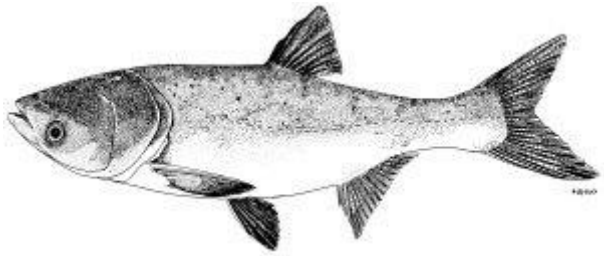

Figure 6 Bighead carp *Hypophthalmichthys nobilis* (FAO, 2013a).

2.3 Experimental design-experiment 1-4

For experiment 1, six large black barrels containing approximately 90 litres of water were provided. The barrels were designed in such a way that the water could easily be temporarily drained. There was a continuous flow of fresh water into the barrels, thus ensuring stable oxygen levels throughout the experiment.

Six large aquaria (30 cm x 30 cm x 50 cm) containing approximately 45 litres were used for observation during experiment 2-4. Since assessment of induction and recovery from surgical anaesthesia (s.a.) can be somewhat subjective, bias was minimized by having the same observer assessing when fish were sedated and recovered. All aquaria were kept aerated at all times, and three out of four sides were covered by black material to reduce disturbance of the fish.

Water temperatures for all experiment was $23.5 \pm 0.5^{\circ}C$. Fish were not used in more than one experiment per day (minimum 24 hours of recovery after being used in an experiment).

2.3.1 Experiment 1: Changes in plasma cortisol and glucose levels after exposure to a stressor

The purpose of experiment 1 was to investigate changes in plasma glucose and cortisol levels in bighead carp after exposure to a stressor. The fish were put in black tanks filled with water overnight (approximately 24 hours). The experiment was performed the following day (approximately 9am-5pm). They were then stressed by temporarily draining the water in the tank for approximately one minute. Blood samples were taken of groups (n= 6 or 7) of fish at different time interval after exposure to the stressor, as well as of a control group which were not exposed to the stressor. Blood samples were taken at time intervals of 15, 30, 60, 120, and 240 minutes. Anaesthetics were added directly to the tank (approximately 75 mgL^{-1} of MS-222) prior to taking blood samples. After the fish had reached surgical anaesthesia, the water was drained before the fish were netted, and transferred to a bucket with the same concentration of anaesthetic.

The fish were kept in the bucket until the blood samples were taken; no more than six minutes. The blood was collected from the caudal vein by use of heparinized syringes (Heparin LEO 5000 IE/mL). All fish were weighed and total length was measured. All fish where euthanized after sampling. After sampling, the blood was centrifuged with a hand centrifuge for approximately five minutes. The plasma was frozen (minimum -10° C) immediately and kept frozen until further analyses. Due to rationing of electricity in Nepal, ice was made, and packed tightly around the samples to avoid thawing.

2.3.1.1 Plasma analyses

Plasma samples were analysed to determine concentrations of cortisol and glucose. This was done using radioimmunoassay (RIA) and colorimetric assay. The analyses were performed at NTNU, Trondheim, Norway.

2.3.1.2 RIA Cortisol

The concentration of plasma total cortisol was determined by use of a commercial RIA kit (Coat-A-Count Cortisol, Siemens). Coat-A-Count is a solid-phase radioimmunoassay. ^{125}I labelled cortisol compete with free plasma cortisol for limited binding sites on cortisol antibody in the precoated tubes provided with the kit. The antibodies are immobilized to the wall of the tube, so by decanting the supernatant terminates the competition, and isolate the antibody-body fraction of the radiolabeled cortisol. The bound fraction was counted for one

minute by a gamma counter. All samples were run as singles, and were diluted 1:3 with cortisol calibrator 0 (containing no cortisol). See appendix A.1.1 for standard curve.

2.3.1.3 Principle of RIA

The basic components of a radioimmunoassay are the antibody (Ab), the unlabeled antigen (Ag) and the radioactive labeled antigen (Ag*). RIA is based on a reversible competitive reaction between known concentrations of Ag* and unknown concentrations of Ag. Ag* and Ag compete for binding sites to the Ab and Ab is thus the limiting component **(1)**. The ratio between bound and free Ag and Ag* is linked to the total concentration of Ag in the sample. Thus this can be used to determine the concentration of Ag. A higher concentration of Ag in the solution gives fewer Ag*-Ab complexes, and therefore more free Ag* (Berson and Yalow, 1968, Davies, 2013a, Davies, 2013b).

$$\begin{array}{lll} [Ag] & & [Ag\text{-}Ab] + [Ag^*] \\ + \quad [Ab] & \leftrightarrow & \\ [Ag^*] & & [Ag^*\text{-}Ab] + [Ag] \end{array} \qquad \textbf{(1)}$$

Bound and free fractions of Ag* in the samples are separated by decanting the supernatant. By measuring radioactivity in the bound fraction with a gamma counter one can determine [Ag*-Ab] and thus indirectly the concentration Ag. To do this a standard curve made from solutions of known concentrations of Ag, expressing percentage bound Ag* at different concentrations of Ag, is made (**Figure 7**). Thus, by comparing percentage of bound Ag* in a mixture of unknown Ag, concentration can be determined with the help of the standard curve (Berson and Yalow, 1968, Davies, 2013a, Davies, 2013b).

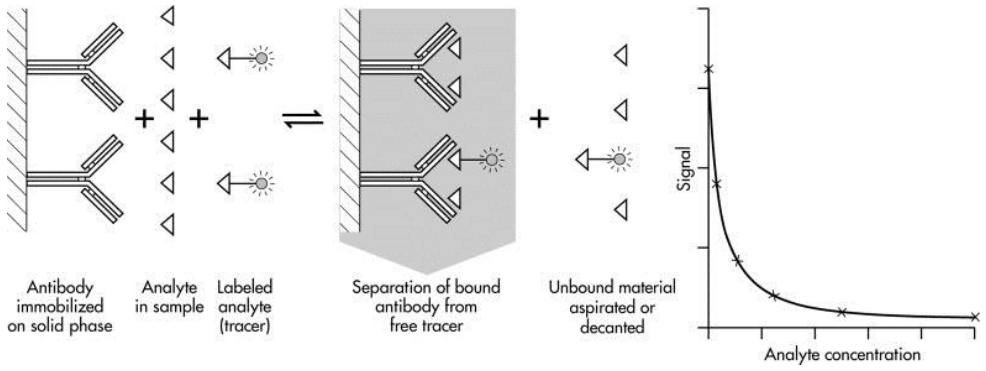

Figure 7 Schematic presentation of the principle of a competitive immunoassay, such as Coat-A-Count Cortisol by Siemens (Wild, 2013).

2.3.1.4 Validation of RIA

To test for any interference in the assay affecting the results, commercial RIA kits require validation.

Sensitivity is the lowest measurable concentration of the analyte different from zero (Davies, 2013a).

Precision examines the variation between measurements, and is measured between duplicates. Precision of the assay was defined through calculating the coefficient of variation (CV = (SD/mean) x 100 %) for triplet samples. Preferably, the CV value should be ≤ 10% (Davies, 2013a).

Specificity tests if the components in the kit respond to other closely-related molecular structures (Davies, 2013a). Specificity is evaluated with a non-specific-binding sample (NSB). Low NSB value indicates that the amount of cross-reaction with other elements is low. NSB was used to normalize data, thus reducing the effect of background noise.

2.3.1.5 Glucose colorimetric assay

The concentration of plasma glucose was determined by use of a commercial kit (Glucose Colorimetric Assay Kit, Cayman Chemical Company). The kit uses the glucose oxidase-peroxide reaction to quantify the glucose in the sample. All samples were run as duplicates. The plate was read by a plate reader measuring absorbance at 514 nm. The samples were diluted 1:10. This was done because a test run with a dilution of 1:5 (as recommended by the producers) resulted in the samples having higher concentrations (up to twice as high) than the standard curve. See appendix A.1.2 for standard curves.

2.3.1.6 Conversion factors

Glucose and cortisol concentrations are given in SI-units. Conversion of values to conventional units used in the literature is described for cortisol **(2)** and for glucose **(3)**. Values from literature used in the discussion have been converted to SI-units in this study.

Cortisol:

$$nmolL^{-1} = ng/ml * 2.75 \quad \textbf{(2)}$$

Glucose:

$$mmolL^{-1} = mg/dl * 0.0555 \quad \textbf{(3)}$$

2.3.2 Experiment 2: Surgical anesthesia, induction time for bighead carp fingerlings subjected to Aqui-S, Benzoak or MS-222.

The purpose of experiment 2 was to determine optimal concentration of MS-222, Aqui-S and Benzoak for induction of surgical anaesthesia in bighead carp fingerlings. The fish were collected from the tank in which they had been kept overnight and transferred to an aquarium with the anaesthetic. The concentrations of the anaesthetics are given in **Table 3**. Six fish were exposed for each of the four different concentrations of MS-222, Benzoak and Aqui-S. The time from when the fish were put into the tank and until state of s.a. was reached was noted for each individual fish. If this took more than fifteen minutes, the fish where noted as "not reaching s.a.".

Table 3 Anaesthetics and concentrations used for experiment 1

Anaesthetic	MS-222	Benzoak	Aqui-S
Concentration [mgL^{-1}]	25	25	10
	50	50	20
	75	75	50
	100	100	100

2.3.3 Experiment 3: Recovery time from surgical anaesthesia for bighead carp fingerlings

The purpose of experiment 3 was to determine recovery time from s.a. induced by MS-222, Benzoak and Aqui-S for bighead carp fingerlings. The fish were collected from the tank in which they had been kept overnight, and transferred to an aquarium with anaesthetic. The concentration for the anaesthetic was selected based on the results from experiment 2. Six fish were exposed to each of the anaesthetics for five minutes, before they were moved to a restitution aquarium (without anaesthetic). The time until full recovery from the anaesthetic was recorded. The fish were classified as dead if no movement was observed after fifteen minutes in the restitution aquarium.

2.3.4 Experiment 4: Tolerance to prolonged anaesthetic exposure for bighead carp fingerlings

The purpose of experiment 4 was to determine the effects of prolonged exposure to MS-222, Benzoak and Aqui-S for bighead carp fingerlings. The fish were exposed to selected optimal

concentrations based on results from experiment 2. The fish where exposed for ten, twenty, thirty and forty minutes. After exposure they were transferred to a restitution aquarium (without anaesthetics) where the time until full recovery from the anaesthetic was recorded. The fish were classified as dead if no movement was observed after fifteen minutes in the restitution aquarium.

2.4 Experimental design – pilot study

Ten small aquaria (15cm x15cm x 15 cm), containing about 0.33 litres of water, were used. All aquaria were kept aerated at all times, and three out of four sides were covered by black material to reduce disturbance of the fish. Observations in experiment 5 were of single fish. The temperature in the water was measured at 23˚ C. All aquarium were kept constantly aerated.

2.4.1 Experiment 5: controlled exposure to plant extract from mauwa, pilot study

The purpose of experiment 5 was to investigate the effects of plant extract from the plant mauwa in bighead carp. To make the plant extract 40 g ± 5 g (twenty-two frozen leafs with stalks) from the mauwa plant were added to 1500 mL of fresh water. Due to difficulties in accurately weighing the leaves, there is some inaccuracy as to the exact weight of plant material. After being added to the water, the leaves were crushed as much as possible. Different amounts of this solution were added to 2 L of fresh water in a small aquarium. (**Table 4**). One fish for each concentration was transferred to the aquarium. The fish was then exposed for 15 minutes before being transferred to a clean aquarium. The fish was observed during exposure and up to an hour after exposure. To determine lethal concentration, low concentrations were used first, and gradually increased until lethal dose was reached.

Table 4 Concentration used in experiments investigating the effects of plant extract from the mauwa *Engelhardia spicata* on bighead carp *Hypophthalmichthys nobilis*.

Experiment	Concentration of solution (plan extract /water)
1	2,5 ml / L water
2	7,5 ml / L water
3	12,5 ml L water
4	15 ml / L water
5	18 ml / L water
6	22,5 ml/ L water
7	30 ml L water
8	32,5 ml /L water
9	16,25 ml / L water

2.4.2 Field exposure; Demonstration of how the plant mauwa is used by local fishermen - pilot study

The purpose of the field work was to observe how local fishermen use mauwa to fish, and thus acquire more knowledge of herbicides used by locals for fishing, and potential ecological consequences. Field work was conducted in Khimti valley, Nepal. The fisherman used rocks, wood, and leaves to cut off a part of the river. This area was approximately 10 m x 10 m, but with many large rocks making the effective area smaller. Approximately five kg of fresh mauwa shoots were collected from surrounding areas. The mauwa was then put in an empty rice bag, and thoroughly crushed with rocks. This bag was then submerged into the water. The local fishermen then waded around in the constructed dam with the bag, and picked up fish with their bare hands. A total of twelve fish where caught. After being caught all the fish were put into a bucket with fresh water for further observation.

2.5 Statistical analyses

The statistical software IBM SPSS Statistics 21.0 (SPSS Inc. 2013) was used for all statistical analyses. Statistical significance was set at $p < 0.05$. Values are given as mean ± standard deviation (± SD). Graphs were made using Microsoft Excel 2010.

To determine any significant differences between groups the data was analysed using parametric One-Way Analysis of Variance (ANOVA). Normal distribution was tested by a Shapiro-Wilk test. Natural logarithmic (ln) transformation of data was done to meet underlying assumptions of normal distribution for One-way ANOVA when necessary. If significant differences were detected by the ANOVA test a Tukey test was performed. Data which did not meet underlying assumptions of One-Way ANOVA (normal distribution and homogeneity of variances) were analysed using a nonparametric analysis of variance (ANOVA on ranks), the Kruskal-Wallis One-Way Analysis of Variance on Ranks test. If significant differences were detected by the the Kruskal-Wallis test pairwise Multiple Comparisons was performed. A Univariate Analysis of Variance (ANCOVA) test was performed to determine if total length and/or weight affected glucose and/or cortisol levels in experiment 4. See appendix and A.2 and A.3.3 for statistical tables.

3 Results

3.1 Experiment 1: Changes in plasma cortisol and glucose levels after exposure to a stressor

Average cortisol concentration in plasma samples from bighead carp at 15, 30, 60, 120 and 240 minutes after being exposed to a temporary stressor are presented in **Figure 8**. Plasma cortisol levels varied between 254.9 $nmolL^{-1}$ and 126.1 $nmolL^{-1}$. Levels in control (0 min) fish were high (mean; 225.5 ± 70.9 $nmolL^{-1}$). Plasma cortisol concentrations 30 minutes after exposure to the stressor were the lowest (mean; 126.1 ± 49.2 $nmolL^{-1}$). One can see a slight increase in cortisol concentration after the exposure to the stressors, when excluding results for 30 minutes, that gradually drops reaching a low at 240 minutes (mean;194.4 ± 66. 6 $nmol \cdot L^{-1}$) after exposure. No significant difference (Kruskal-Wallis: H (5) = 6.020, p = 0.304) in cortisol level between the groups was found.

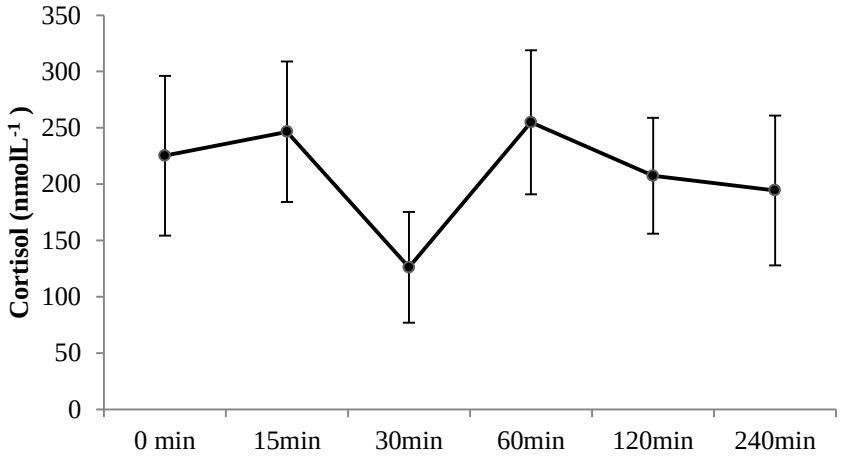

Figure 8 Average cortisol concentrations ($nmolL^{-1}$; ± SD) in plasma for bighead carp *Hypophthalmichthys nobilis* 15 (n = 7), 30 (n = 7), 60 (n = 7), 120 (n = 6) and 240 (n = 7) min after exposure to stressor (temporary draining of the water in the tank).

Average glucose concentration in plasma samples from bighead carp at 15, 30, 60, 120 and 240 minutes after exposure to a temporary stressor are presented in **Figure 9**. Plasma glucose levels varied between 2.9 $mmolL^{-1}$ and 3.8 $mmolL^{-1}$. Levels in the control fish were high compared to fish exposed to stressor (mean; 3.43 ± 1.13 $mmolL^{-1}$). The glucose plasma concentration was slightly higher in samples taken 30 minutes after exposure to the stressor (3.84 ± 0.79 $mmolL^{-1}$). The glucose level in the plasma samples gradually drops as time since

exposure increases. The glucose level is at its lowest in samples taken 240 minutes (2.58 ± 1.15 $mmolL^{-1}$) after exposure. No significant difference was detected (ANOVA: F (5) = 0.999, p = 0.433) among groups.

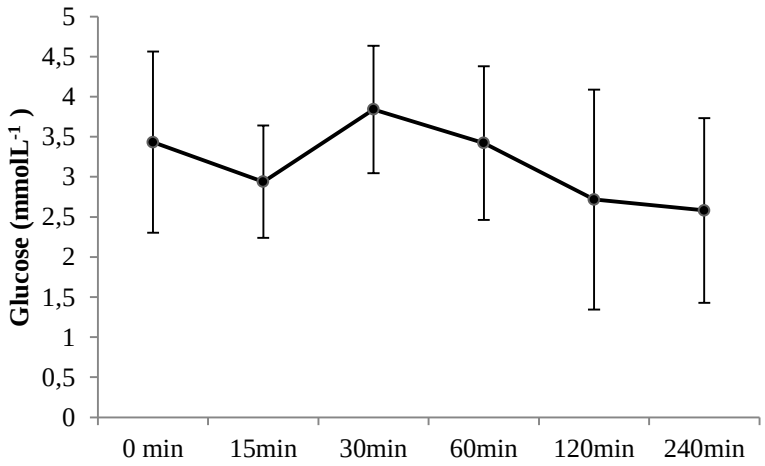

Figure 9 Average glucose concentrations ($mmolL^{-1}$; ± SD) in plasma for bighead carp *Hypophthalmichthys nobilis* 15 (n = 7), 30 (n = 7), 60 (n = 7), 120 (n = 6) and 240 (n = 7) min after exposure to stressor (temporary draining of the water in the tank).

3.1.1.1 Weight and length of fish used in experiment 1

The total length and weight for fish used in experiment 1(mean; 19.3 ± 2.2 cm, and 54.7 ± 21.4 g) are presented in the appendix A.3.No significant difference in length (ANOVA: F (5) = 0.366, p = 0.869) or weight (ANOVA: F (5) = 0.662, p = 0.655) was found among the different groups of fish used in experiment 1.

An ANCOVA test indicted that neither length nor weight had a significant effect on glucose or cortisol levels.

3.1.1.2 Validation of RIA

Detection limit (sensitivity) for the assay, calculated by the gamma counter, was 2.08 $mmolL^{-1}$ cortisol. The precision had a CV value of 6.9 % and 19.9 % (from triplicate with a concentration of 516.0 ± 35.9 $mmolL^{-1}$and 981.5 ± 196.1 $mmolL^{-1}$). NSB was measured as 589 CPM. Samples have all been NSB-corrected.

3.2 Experiment 2: Surgical anaesthesia, induction time for bighead carp fingerlings subjected to MS-222, Benzoak or Aqui-S.

Average induction time for s.a. for bighead carp fingerlings subjected MS-222 is presented in **Figure 10**. None of the fish exposed to the lowest concentration (25 mgL^{-1}) reached s.a. within 15 minutes of exposure. Only two fish exposed to 50 mgL^{-1} reached s.a. within 15 minutes of exposure (mean; 14.3 ± 1.3 min). Fish exposed to 75 mgL^{-1} MS-222 reached s.a. rapidly (mean; 3.4 ± 1.3 min). The fish exposed to 100 mgL^{-1}, used slightly longer time before reaching s.a. (mean; 3.9 ± 0.9 min). A significant difference in induction time among was detected (Kruskal-Wallis: $H(3) = 19.142$, $p < 0.001$). Induction time between fish exposed to 25 $mgL^{-1}$and fish exposed to 75 ($p = 0.002$) and 100 mgL^{-1} ($p = 0.014$) was significantly different. There was also a significant difference in induction time between fish exposed to 50 and 75 mgL^{-1} ($p = 0.014$).

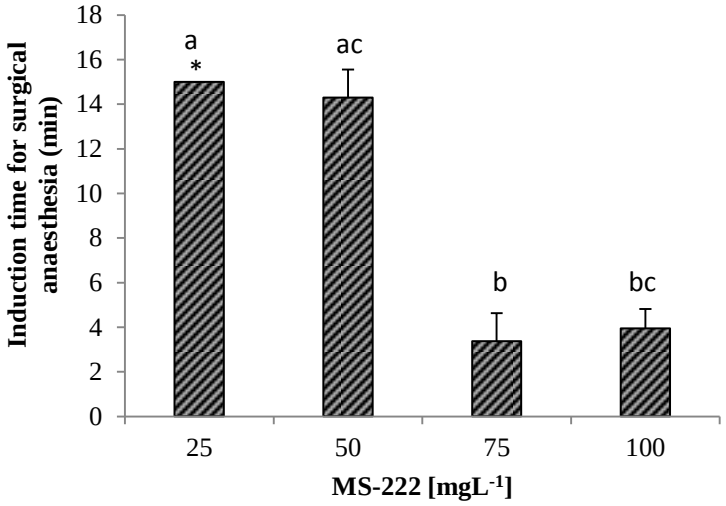

Figure 10 Average time (min + SD) (n = 6) until s.a. for bighead carp *Hypophthalmichthys nobilis* exposed to MS-222, was reached. Experiment was aborted if s. a. was not reached within 15 min of exposure (*). Statistical significant differences between groups are indicated by different letters.

Average induction time for s.a. for bighead carp fingerlings subjected to Benzoak is presented in **Figure 11**. None of the fish exposed to the lowest concentration (25 mgL^{-1}) reached s.a.. All fish exposed to 50 mgL^{-1} reached s.a. (mean; 10.6 ± 3.4 min). Fish exposed to 75 mgL^{-1} reached s.a. rapidly (mean; 2.5 ± 0.4 min). For fish exposed to 100 mgL^{-1} induction time was even shorter (mean; 1.3 ± 0.2 min). A significant difference in induction time was detected (Kruskal-Wallis: H (3) = 21.943, $p < 0.001$). Induction time for fish exposed to 25 mgL^{-1} was significantly different from induction time for 75 mgL^{-1} ($p = 0.018$) and 100mgL^{-1} ($p < 0.001$). Induction time for fish exposed to 50 mgL^{-1} was significantly different from induction time for fish exposed to 100 mgL^{-1} ($p = 0.018$)

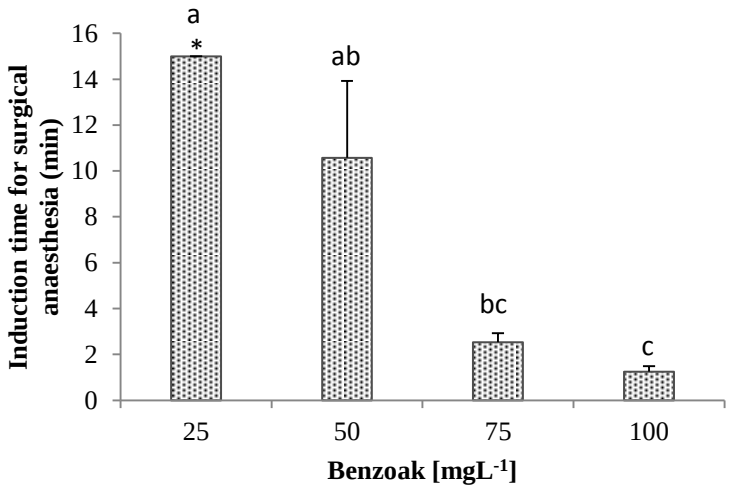

Figure 11 Average time (min + SD) (n = 6) until s.a. for bighead carp *Hypophthalmichthys nobilis* exposed to Benzoak, was reached. Experiment was aborted if s. a. was not reached within 15 min of exposure (*). Statistical significant differences between groups are indicated by different letters.

Average induction time for s. a. for bighead carp fingerlings subjected to Aqui-S is presented in **Figure 12**. None of the fish exposed to the lowest concentration (10 mgL^{-1}) reached s.a.. With one exception (s.a. reached after 14.6 min), the same absence of s.a. was found in fish exposed to 20 mgL^{-1}. All fish exposed to 50 mgL^{-1} reached s.a. (mean; 4.6 ± 4.1 min). For fish exposed to 100 mgL^{-1} of AQUI-S induction time was short (mean 1.6 ± 0.2 min). A significant difference in induction time between the different concentrations was detected (Kruskal-Wallis: H (3) = 21.583, $p < 0.001$), induction time was significantly different for fish exposed to 10 and 20 mgL^{-1} opposed to 100 mgL^{-1}($p \leq 0.001$).

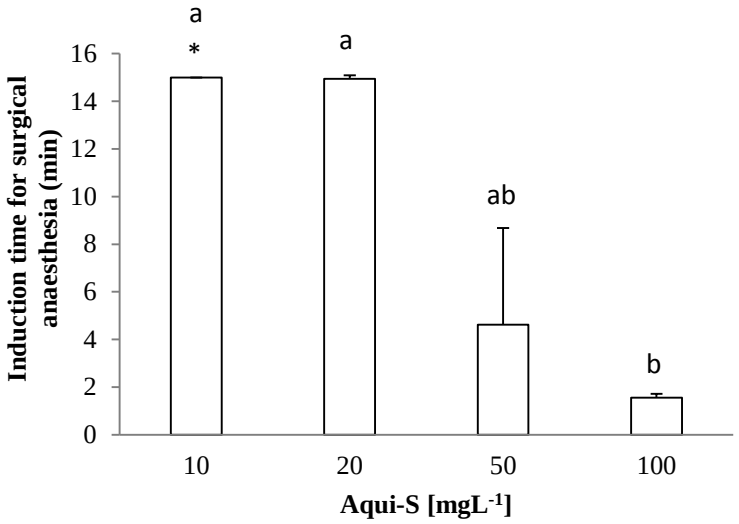

Figure 12 Average time (min + SD) (n = 6) until s.a., for bighead carp *Hypophthalmichthys nobilis*, exposed to Aqui-S, was reached. Experiment was aborted if s. a. was not reached within 15 min of exposure (*). Statistical significant differences between groups are indicated by different letters.

3.3 Experiment 3: Recovery time from surgical anaesthesia for bighead carp fingerlings

Average recovery time for bighead carp fingerlings after five minutes of exposure to selected concentrations of MS-222, Benzoak and Aqui-S are presented in **Figure 13**. Recovery time was shortest for fish exposed to 75 mgL^{-1} of MS-222 (mean; 2.7 ± 0.7 min). The longest recovery time was found for fish exposed to 50 mgL^{-1} of Aqui-S (mean; 10.2 ± 3.0 min). Fish exposed to 75 mgL^{-1} Benzoak had an average recovery time of 7.4 ± 2.4 min. A significant difference in recovery time for the different anaesthetic agents was detected (Kruskal-Wallis: H (2) = 13.414, p = 0.001). Recovery time for fish exposed to MS-222 was significantly different from recovery time for fish exposed to Aqui-S (p = 0.001). **Figure 14** illustrates differences in induction time and recovery time for selected concentrations of Aqui-S, Benzoak and MS-222 used in experiment 2.

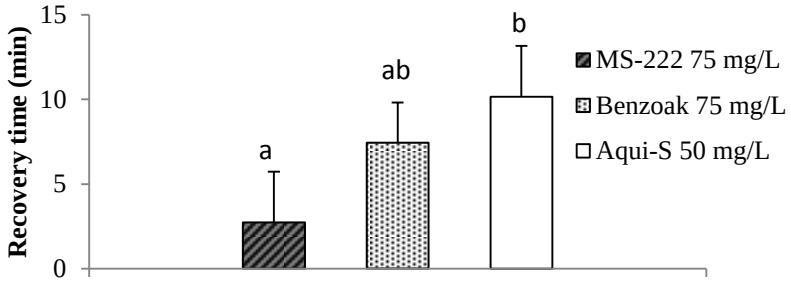

Figure 13 Average recovery time (min +SD) (n =6.) for bighead carp *Hypophthalmichthys nobilis* after being exposed for 5 minutes to selected optimal concentrations of Aqui-S (50 mgL^{-1}), Benzoak (75 mgL^{-1}), and MS-222(75 mgL^{-1}). Statistical significant differences between groups are indicated by different letters.

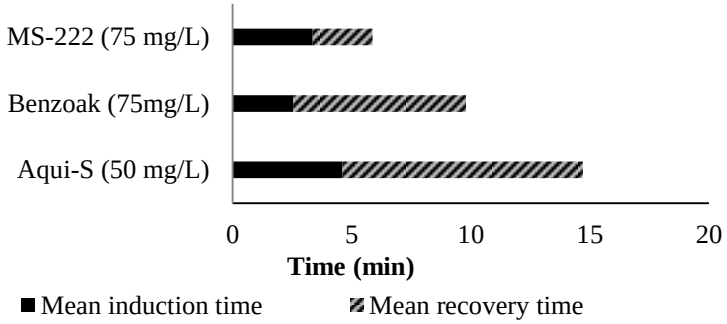

Figure 14 Schematic illustration of induction and recovery time of bighead carp *Hypophthalmichthys nobilis* sedated to a state of surgical anaesthesia using different chemical sedatives: MS-222 (75 mgL^{-1}), Benzoak (75 mgL^{-1}) or Aqui-S (50 mgL^{-1}). Identical exposure time (5 minutes) was carried out for all fish.

3.4 Experiment 4: Tolerance to prolonged anaesthetic exposure for bighead carp fingerlings

The average recovery time for bighead carp fingerlings subjected to prolonged exposure to 75 mgL^{-1} of MS-222, 75 mgL^{-1} of Benzoak and 50 mgL^{-1} of Aqui-S is presented in **Figure 15**.

For fish exposed to 75 mgL^{-1} MS-222, a clear increase in recovery time was first seen in fish exposed for 40 minutes. Recovery time was longer for fish exposed for fifteen minutes (mean; 8.4 ± 5.4 min) than for fish exposed for both twenty (mean; 5.2 ± 4.9 min) and thirty (mean; 5.9 ± 4.5 minutes) minutes. Moreover, fewer fish survived exposure for 15 minutes (four) than both 20 (five) and 30 minutes (five). Recovery time after 40 minutes was the longest (mean; 11.4 ± 5.6 min). A significant difference was detected (Kruskal-Wallis: H (4) = 13.476, p = 0.009) between recovery time after 10 minutes and after 40 minutes of exposure (p = 0.007).

All fish exposed to 75 mgL^{-1} Benzoak for 10 minutes died, thus no further experiments were conducted with Benzoak.

Increased recovery time, after prolong exposure of Aqui-S, was first detectable after twenty minutes. Survival rate was only 33.3 % after exposure to Aqui-S for 20 minutes. All fish exposed for thirty minutes died. Thus, no experiment was conducted with exposure for 40 minutes. A significant difference was detected (Kruskal-Wallis: H (3) = 12.55, p = 0.006), recovery time after 30 minutes of exposure was significantly different from the recovery time after 15 minutes of exposure (p = 0.015).

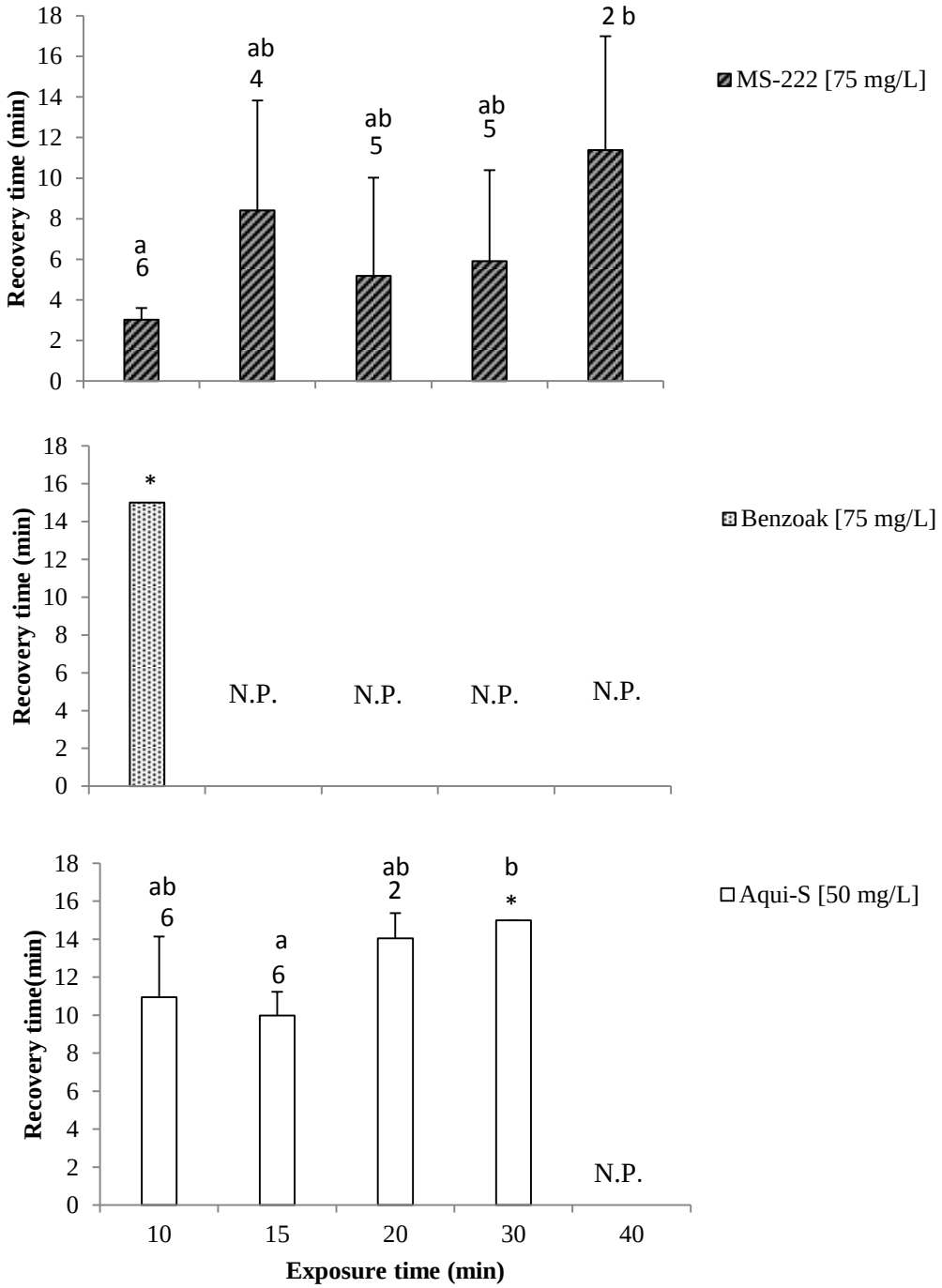

Figure 15 The mean recovery time (min + SD) (n = 6.) for bighead carp *Hypophthalmichthys nobilis* exposed to 75 $mgL^{-1}$of MS-222, 75 $mgL^{-1}$of Benzoak and 50 mgL^{-1} of Aqui-S for 10, 15, 20, 30 and 40 minutes. If recovery took more than 15 minutes, fish were noted as dead. Numbers indicate number of surviving fish. Exposures that killed all fish are indicated with *. Further experiments were not preformed (N.P.) in such incidents. Statistical significant differences between groups are indicated by different letters.

3.4.1.1 Weight and length of fish used in experiment 2-4

The total length and weight for fish used in experiments 2-4 (mean; 8.2 ± 2.2 cm, and 10.4 ± 1.7 g) are presented in the appendix A.3. No significant difference in length (ANOVA: $F(6) = 0.662$, $p = 0.888$) or weight (ANOVA: $F(6) = 0.345$, $p = 0.408$) was found among the different groups of fish used in experiment 2-4.

There was a significant difference in size between the fish used for experiment 1 and fish used in experiments 2-4, both for total length(ANOVA: $F(1) = 213.010$, $p < 0.001$) and weight(ANOVA: $F(1) = 163.59$, $p < 0.001$).

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3.5 Experiment 5: Controlled exposure to plant extract from mauwa - pilot study

The results of the effects of controlled exposure to plant extract from the plant mauwa are presented in **Table 5**. The concentration causing death to the fish, bighead carp, within 15 minutes of exposure was determined to be 65 ml of plant extract per two liters of water. Half of this concentration caused the fish to die 13.28 minutes after being moved to a restitution aquarium containing fresh water. Bleeding from the gills was observed in all fish that died (both during exposure and afterwards in the restitution aquarium). The blood was fresh, non-coagulated, and was not frothy. The imitated reaction of fish put into the exposure aquarium was frantic swimming and gasping for air at the surface. This settled down after some time, and the fish became subdued.

Table 5 Concentration and effect of plant extract from mauwa *Engelhardia spicata* on bighead carp *Hypophthalmichthys nobilis*.

Experiment	Concentration of plant extract	Observed effect
1	2,5 mlL^{-1}	Did not die but behavior was effected
2	7,5 mlL^{-1}	Died after 25 min in the restitution aquarium, bleeding from the gills
3	12,5 mlL^{-1}	Died during the night (in restitution aquarium), bleeding from the gills
4	15 mlL^{-1}	Died during the night (in restitution aquarium), bleeding from the gills
5	18 mlL^{-1}	Died after 17 min in the restitution aquarium, bleeding from the gills
6	22,5 mlL^{-1}	Died after 8.16 min in the restitution aquarium, bleeding from the gills
7	30 mlL^{-1}	Died after 2.22 min in the restitution aquarium, bleeding from the gills
8	32,5 mlL^{-1}	Died after 14.92 min in the exposure aquarium, bleeding from the gills
9	16,25 mlL^{-1}	Died after 13.28 min in the restitution aquarium, bleeding from the gills

3.6 Field exposure: Demonstration of how the plant mauwa is used by local fishermen - pilot study

Observations of effect on wild fish in Khimti river after exposure to plant extract from mauwa used by local fishermen is presented in **Table 6**. A total of twelve fish where caught. After being caught all the fish were put into a bucket with fresh water for further observation. Three of the fish showed no sign of life when they were caught. The remaining nine fish were less active than would be expected, but seemed fine otherwise. Within fifteen minutes all fish were more sluggish and inactive than when they were first caught. After 40 minutes three more fish had died, and after 50 minutes only three fish were still alive. Bleeding from the gills was observed in all fish that died (both during exposure and after, in restitution bucket).

Table 6 Observations of effect of plant extract from the plant mauwa used by local fishermen to catch fish.

Number of fish caught	Species	No sign of life when they were caught	Died during restitution	Recovered
11	Snow- trout (*schizothorax plagiostomus*)	3	6	2
1	Unknown			1

4 Discussion

4.1 Stress responses in bighead carp

The aim of this part of the experiment was to describe the primary and secondary stress response in bighead carp. Selected exposure method, as described by Einarsdóttir and Nilssen (1996), was used to produce a reproducible characteristic stress response. Fish were transferred to the dark exposure tanks more than 24 hours prior to the experiment in order to fully acclimatize the fish.

In the present study the plasma cortisol levels varied between 126 $nmolL^{-1}$ and 254 $nmolL^{-1}$. Control plasma levels in this study were 226 $nmolL^{-1}$. For common carp pre-stress levels of plasma cortisol have been reported as 20 $nmolL^{-1}$, while one hour after exposure to a handling stressor plasma cortisol levels were reported as 217 $nmolL^{-1}$ (Barton, 2002). Peak plasma cortisol levels in grass carp have been measured as 456 $nmolL^{-1}$, whereas resting levels were measured to be 61 $nmol \cdot L^{-1}$ (Gause et al., 2012). Thus the control fish in this study had cortisol levels comparable to post-stress levels in other studies with similar species, indicating that control fish were stressed.

None of the changes in plasma cortisol levels in the present study were significant: However cortisol levels dropped thirty minutes after exposure to the stressor. This was not as expected. Rather one would have expected the plasma cortisol level to peak after approximately thirty minutes based on what has been seen in other species, where plasma cortisol levels peak 0.5 - 1 hour after exposure to the stressor (Barton and Iwama, 1991). This adds further evidence that the fish were stressed prior to the experiment.

Plasma glucose levels in teleost fish have been found to increase significantly after exposure to stressors (Barton, 2000, Wedemeyer, 1976, Wells and Pankhurst, 1999). In this study none of the changes in plasma glucose levels were significant. Plasma glucose levels varied between 2.9 $mmolL^{-1}$ and 3.8 $mmolL^{-1}$. Glucose plasma level showed a slight increase 30 minutes after exposure to the stressor, and gradually dropped with time after exposure to the stressor. Glucose levels measured in control fish (3.4 $mmolL^{-1}$) were relatively high compared to the levels in post- stressor fish. The lack of a significant increase in plasma glucose levels,

and the relatively high control value, give further indications that the control fish were stressed.

Results from the analytical methods (RIA and glucose colorimetric assay) were considered accurate. Size did not vary significantly between groups in experiment, and did not significantly affect cortisol or glucose values. Plasma cortisol and glucose varied between individual fish sampled at the same time during the experiment, contributing to high SD reported in this study, but this is most likely to be due to natural biological variations commonly observed.

Thus, cortisol and glucose levels, and the lack of significant changes in cortisol and glucose levels in this study, indicate that control fish were stressed. The stress response could have been induced by the change in environment from fishponds to the experimental tanks. There is a possibility that fish might not have been fully acclimatized 24 hours after the change in environment. There is also a possibility that using MS-222 to induce anaesthesia in the fish before the blood samples, so as not to cause stress, may itself have caused the high cortisol levels. Although it is generally thought that anaesthetics reduce stress, some studies have found that MS-222 induces a stress response (Zahl et al., 2012, Ross and Ross, 2008).

These results emphasize the importance of correct handling, and knowledge of species specific physiological responses. The control fish were kept under calm condition; despite this a stress response was induced, which indicates that bighead carp is relatively sensitive and easily stressed.

4.2 Anaesthetic efficacy of chemicals

Description of anaesthetic efficacy of chemicals can be related to chemical properties, procedure, biotic factors, abiotic factors and observations. Therefore, when administering or comparing anaesthetic agents, both biological and environmental factors must be taken into account (Burka et al., 1997, Ross and Ross, 2008). In this study, optimal time for reaching surgical anaesthesia (s.a.) was set between three and five minutes, while optimal recovery time was set to be less than five minutes. The fish selected for the study of chemical efficacy were bighead fingerlings (10 cm, 8 g).

4.2.1 Induction time

Behavioral observations indicate that MS-222, at 75mgL^{-1} gives the most satisfactory induction time (3.4 minutes) in bighead carp fingerlings. This result corresponds with results for cod *Gadus morhua* (Mattson and Riple, 1989) and fathead minnow *Pimephales promelas* (Palić et al., 2006) which have been found to be efficiently sedated with 75 mgL^{-1} MS-222. This is also in similar to findings for rainbow trout (Wagner et al., 2003) and Atlantic salmon *Salmo salra* (Kiessling et al., 2009) where 60 mgL^{-1} was sufficient to induce s.a..

Behavioral observations indicate that Benzoak, at 75 mgL^{-1} gives the most satisfactory induction time (2.5 minutes) in bighead carp fingerlings. Similar results have been obtained for striped sea bass *Morone saxatilis* which were efficiently sedated with 55-80 mgL^{-1} (Gilderhus et al., 1991). In crucian carp *Carassius carassius* a slightly higher concentration, (100 mgL^{-1}), has been suggested as an optimal concentration for inducing s.a. (Heo and Shin, 2010). For Atlantic salmon (Iversen et al., 2003) and cod (Mattson and Riple, 1989) lower concentrations, 30 and 40 mgL^{-1} respectively, have been found to be sufficient.

Behavioral observations indicate that Aqui-S, at 50mgL^{-1} gives the most satisfactory induction time (of 4.5 minutes) in bighead carp fingerlings. The decision to opt for 50mgL^{-1} despite a slightly long induction time, was made because induction time for fish exposed to 100 mgL^{-1} was too short, and may have resulted in a long recovery time and a low safety margin. Results in this study are similar to results for koi carp *Cyprinus carpio haematopterus* where 40-80 mgL^{-1} was found to efficiently induce s.a. (Gladden et al., 2010). A higher concentration (80 mgL^{-1}) of Aqui-S has been determined necessary to induce s.a. efficiently in western rainbowfish *Melanotaenia australis* (Young, 2009), whereas in a study by Iversen et al. (2003) it was found that approximately 30 mgL^{-1} Aqui-S was sufficient to efficiently induce s.a. in Atlantic salmon.

The concentrations with optimal induction time in this study for Aqui-S, and for Benzoak were higher than those seen in studies performed with cold water fish, such as salmonids and cod (Iversen et al., 2003, Mattson and Riple, 1989). This may be due to the higher metabolic rate expressed in warm water (since fish are ectothermic), causing a faster metabolic clearance (Young, 2009). However, the same trend was not seen for MS-222.

4.2.2 Recovery time

Concentrations used for further determination of efficacy in this study were selected based on induction time. Thus for MS-222, Benzoak and Aqui-S the selected concentrations were 75, 75 and 50 mgL^{-1}, respectively.

Bighead carp fingerlings exposed to MS-222 recovered in less than five minutes. Similar results have been found for grass carp (Gause et al., 2012). Also for *Pomacentrus amboinensis,* a type of damselfish, recovery time from s.a. induced by MS-222 has been reported as rapid (Munday and Wilson, 1997). Ross and Ross (2008) also report rapid recover times for fish exposed to MS-222. However, there seem to be species differences, cod exposed to 40 mgL^{-1} MS-222, a large variation in recovery time between 3.9 and 10.8 min, has been reported (Mattson and Riple, 1989).

Recovery time for bighead carp fingerlings exposed to Benzoak was longer than five minutes, and accordingly not considered optimal. Recovery time for grass carp after sedation with Benzoak has also been reported as longer than optimal (Gause et al., 2012). Mattson and Riple (1989) also found that the most satisfactory concentrations of Benzoak for induction time had a longer than optimal recovery time for cod.

Bighead carp fingerlings exposed to Aqui-S had the longest recovery time (10.2 minutes) in the present study. This is in accordance with a study by Kiessling et al. (2009) with Aqui-S, Benzoak and MS-222, where Aqui-S was found to have the longest clearance rate in Atlantic salmon. Of the three drugs tested, Aqui-S is the most lipophilic, thus, possible deposition in adipose tissue may prolong the clearance rate of the drug (Kiessling et al., 2009). Ross and Ross (2008) also report longer than optimal recovery time for fish exposed to Aqui-S. In contrast, recovery time reported for western rainbow fish exposed to 80 mgL^{-1} was only three minutes (Young, 2009).

4.2.3 Prolonged exposure

The anaesthetic drug should preferably be effective at low doses, and the toxic dose should considerably exceed the effective dose, thus providing a wide safety margin. In aquaculture large numbers of fish are often immobilized in one anaesthetic bath at the same time. Anaesthetic agents must be safe enough to avoid medullary collapse in the event of handling

delays (Burka et al., 1997). The safety margin of the anaesthetics was in this study tested by prolonged exposure to the selected optimal concentrations of the respective chemicals.

Safety margin of MS-222 was relatively good for bighead carp fingerlings. Recovery time did not increase significantly until 40 minutes of exposure. However, it should be noted that deaths did occur after only 15 minutes of exposure. After 40 minutes of exposure, 33 % of the fish were still alive. Although this is an accordance with Ross and Ross (2008) who report a good safety margin for MS-222 in general, they also report that the safety margin is lower in warm water, thus a good safety margin in this study is rather unexpected. Burka et al. (1997) and Gilderhus and Marking (1987) also report a narrow safety margin for MS-222 further contradicting the results found in this study. However it should be noted that these results are based on studies with salmonids. Thus the safety margin of MS-222 seems to vary considerably between species.

There was a 100 % mortality rate after 10 minutes of exposure to 75 mgL^{-1} of Benzoak. This is in accordance with a study by Gilderhus (1990), where safety margin of Benzoak for spawning-phase chinook salmon *Oncorhynchus tshawytscha* and Atlantic salmon was reported as narrow. Ross and Ross (2008) also report a reduced safety margin for Benzoak in warm water.

The safety margin of Aqui-S was relatively wide for bighead carp. A detectable increase in recovery time was first seen after fish had been exposed for 20 minutes. However, none of the fish exposed to Aqui-S for 30 minutes recovered. This is in accordance with a findings for koi carp where it has been demonstrated that Aqui-S has a wide safety margin (Gladden et al., 2010).

4.3 Exposure of fish to mauwa plant extract – pilot study

In Nepal mauwa is used for traditional ethnical fishing. It may be an interesting plant for aquaculture and fish management since it has known biological effects which can potentially hold qualities that can further be developed for commercial use. Plants with piscicidal effects may be useful in aquaculture. Aqui-S is an example of a chemical originating from plant extracts which today has great value in aquaculture.

Despite the use of piscicidal plants for fishing being illegal, it is still practiced in rural areas of developing countries (Cannon et al., 2004). It may therefore be relevant to investigate possible ecological consequences for comparative use of the mauwa plant in Nepal.

This pilot study only gives indications of the physiological and ecological consequences of the use of plant extract from mauwa. Results from the aquarium test show that the plant mauwa is toxic. Fish exposed to 35.5 mlL^{-1} plant extract died within 15 minutes of exposure. Concentrations as low as 7.5 mlL^{-1} plant extract killed the fish within 24 hours after exposure was ended (exposure lasted fifteen minutes). Most of the fish died after being transferred back into fresh water. Mode of action is not clear, however, bleeding was observed in all fish that died due to the plant extract exposure. This suggests that the plant extract in some way damages the gills. This means that mauwa plant extract may also be toxic to other gill breathing aquatic organisms. It is also likely that the damage caused to the gills is non-reversible.

Since this is a plant which is still used by rural ethnical groups for fishing it can potentially have detrimental effects in the water system where it is used. Therefore an observational study of mauwa effect on wild fish in Khimti River was done in collaboration with local fishermen. In total, twelve fish were caught, nine of which died. The same effects were seen in these fish as the ones in the controlled exposure study. Most of the fish died after being transferred back into fresh water. Bleeding from the gills was also observed. Catching twelve fish to feed a large family does not seem like an unreasonable large amount. However, non-appearing sedated fish in the area would most likely die without being registered. This means the consequences of using plant extract from mauwa may have a significant ecological impact on the fish community.

4.4 Overall evaluation

High plasma cortisol levels indicate that also control fish were stressed in the present study. This may be due to the fish not being fully acclimatized after the change in environment. Therefore more than 24 hours of acclimatization is recommended for bighead carp. There is also a possibility that MS-222 may have induced the stress response.

Behavioral observations indicated that 75 mgL^{-1} of MS-222, 75 mgL^{-1} of Benzoak and 50 mgL^{-1} of Aqui-S gave the most satisfactory induction times for bighead carp fingerlings.

These concentrations were therefore used for further evaluation of efficacy of the anaesthetics. Recovery time for MS-222 was less than five minutes and accordingly considered optimal in this study. Both Benzoak and Aqui-S had longer than optimal recovery time. Safety margin of MS-222 and Aqui-S were determined as good. Benzoak on the other hand had a very low safety margin. In summary, the observations in this study show that of the selected anaesthetic agents and concentrations, 75 mgL^{-1} MS-222 gave the most satisfactory results for bighead carp fingerlings. Induction and recovery times were short, and there was a good safety margin.

Plant extract from mauwa is toxic to fish. The extract causes bleeding from the gills, which is most likely a non-reversible damage. Based upon this it is believed that the use of mauwa plant extracts for fishing in river systems may have great ecological consequences.

4.5 Conclusions

- Control fish were stressed, indicated by plasma cortisol and glucose levels, and stress profile for bighead carp was therefore not successfully determined.
- MS-222 at 75 mgL^{-1} had most satisfactory efficacy for inducing s.a. bighead carp fingerlings, both due to a short induction and recovery time and because of a good safety margin.
- Extract from the plant mauwa caused adverse effects in bighead carp fingerlings, and may also have negative impacts on ecology and free living fish populations.

4.6 Perspectives and recommendations

To determine a stress profile for bighead carp more studies must be conducted. In future studies the fish should be given longer acclimation time (more than 24 hours) to enable determination of reference levels for plasma glucose and cortisol. To ensure that true resting levels are determined, retrieving blood samples through dorsal aorta cannulation should also be considered.

It would also be of interest to evaluate whether MS-222 affect cortisol levels in bighead carp. This would potentially have implications for the future use of MS-222 in this species.

Further studies on efficacy of chemical anaesthetics in bighead carp should be conducted to optimize dosage for chemical anaesthetics. Effects of environmental variables on the efficacy of the anaesthetics should be explored. Also effects of the anaesthetic on the environment should be tested (changes in pH, changes in oxygen concentrations etc. due to the anaesthetic).

Similar tests to determine efficacy of anaesthetic drugs for larger fish, particularly bloodstock fish which are very valuable, should also be performed.

The pilot study performed in this study only gives indications of the physiological and ecological consequences of the use of plant extract from mauwa.

For further studies it would be recommended to quantify the toxicity, and determine the active ingredient in the plant extract.

It would also be interesting to explore the mode of action, this would give further indications of the ecological consequences of exposing whole water systems to this toxin (i.e. whether or not it is poisonous to all gill breathing aquatic organisms).

However, the most important factor is educating the rural populations still using this poison to fish with, in order to allow them to understand the long-term ecological effects.

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A Appendix

A.1 Standard curve for cortisol and glucose

A.1.1 Standard curve for cortisol radioimmunoassay

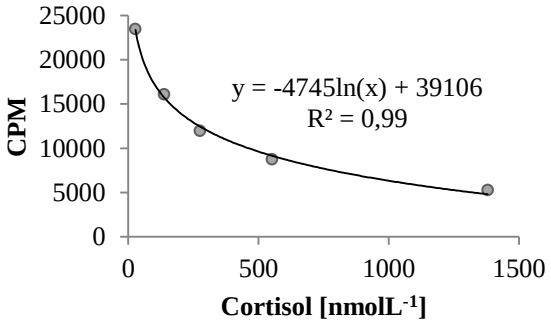


Figure A 1 Standard curve from cortisol radioimmunoassay based on measure of bound fraction of radioactively labeled cortisol (Ag*-Ab) (CPM: counts per minute).

A.1.2 Standard curves for glucose colorimetric assay

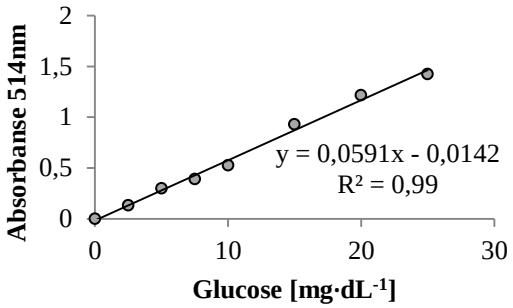


Figure A 2 Glucose standard curve (from plate one), used to determine glucose levels in plasma samples from bighead carp

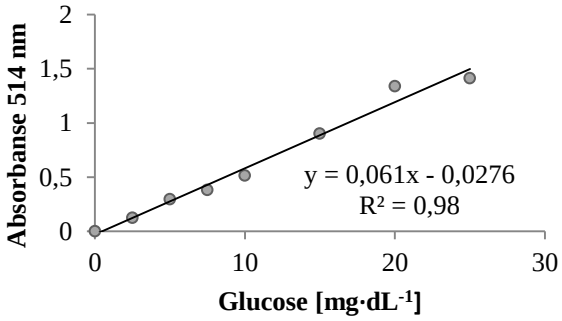


Figure A 3 Glucose standard curve (from plate two) used to determine glucose levels in plasma samples from bighead carp

A.2 ANOVA, Tukey-test and Kruskal-Wallis tables.

A.2.1 Experiment 1: Cortisol concentrations

Table A 1 Kruskal-Wallis test for cortisol concentration at different time interval after exposure to a stressor

	nmol/L
Chi-Square	6,020
df	5
Asymp. Sig.	,304

A.2.2 Experiment 1: Glucose concentrations

Table A 2 ANOVA test for glucose concentration at diffrent time interval after exposure to a stressor (data ln transformed to meet normaldistribution requierments)

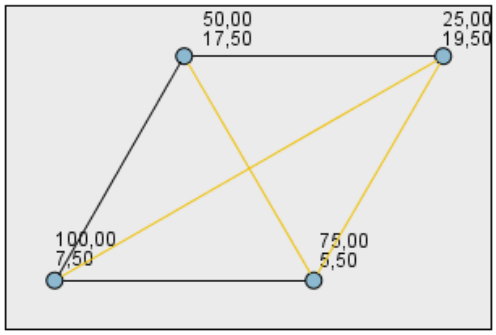
	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	,301	5	,060	,999	,433
Within Groups	2,050	34	,060		
Total	2,351	39			

A.2.3 Experiment 2: MS-222

Table A 3 Kruskal- Wallis test and multiple Pairwise Comparison of time until surgical anesthesia was reached after exposure to four different concentrations of MS-222

	Time
Chi-Square	19,142
df	3
Asymp. Sig.	,000

Pairwise Comparisons of mg/L



Each node shows the sample average rank of mg/L.

Sample1-Sample2	Test Statistic	Std. Error	Std. Test Statistic	Sig.	Adj.Sig.
75,00-100,00	-2,000	3,932	-,509	,611	1,000
75,00-50,00	12,000	3,932	3,052	,002	,014
75,00-25,00	14,000	3,932	3,560	,000	,002
100,00-50,00	10,000	3,932	2,543	,011	,066
100,00-25,00	12,000	3,932	3,052	,002	,014
50,00-25,00	2,000	3,932	,509	,611	1,000

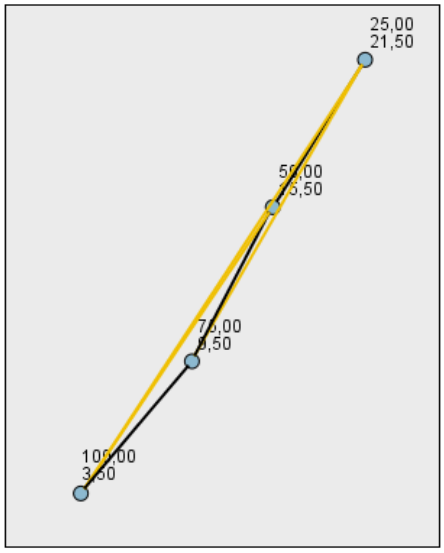
Each row tests the null hypothesis that the Sample 1 and Sample 2 distributions are the same.
Asymptotic significances (2-sided tests) are displayed. The significance level is ,05.

A.2.4 Experiment 2: Benzoak

Table A 4 Kruskal-Wallis test and multiple Pairwise Comparison of time until surgical anesthesia was reached after exposure to four different concentrations of Benzoak

	Time
Chi-Square	21,943
df	3
Asymp. Sig.	,000

Pairwise Comparisons of mg/L



Each node shows the sample average rank of mg/L.

Sample1-Sample2	Test Statistic	Std. Error	Std. Test Statistic	Sig.	Adj.Sig.
100,00-75,00	6,000	4,050	1,481	,139	,831
100,00-50,00	12,000	4,050	2,963	,003	,018
100,00-25,00	18,000	4,050	4,444	,000	,000
75,00-50,00	6,000	4,050	1,481	,139	,831
75,00-25,00	12,000	4,050	2,963	,003	,018
50,00-25,00	6,000	4,050	1,481	,139	,831

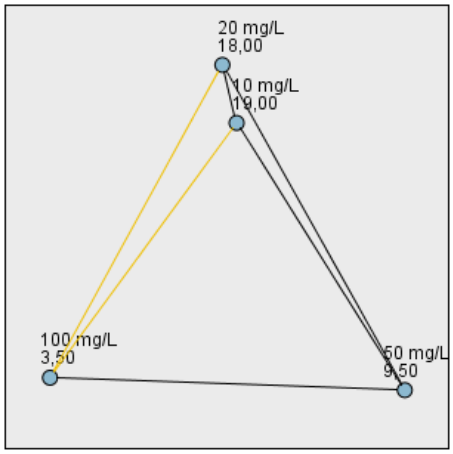
Each row tests the null hypothesis that the Sample 1 and Sample 2 distributions are the same.
Asymptotic significances (2-sided tests) are displayed. The significance level is ,05.

A.2.5 Experiment 2: AQUI-S

Table A 5 Kruskal-Wallis test and multiple Pairwise Comparison of time until surgical anesthesia was reached after exposure to four different concentrations of Aqui-S

	min
Chi-Square	21,583
df	3
Asymp. Sig.	,000

Pairwise Comparisons of mg/L



Each node shows the sample average rank of mg/L.

Sample1-Sample2	Test Statistic	Std. Error	Std. Test Statistic	Sig.	Adj.Sig.
100 mg/L-50 mg/L	6,000	3,880	1,546	,122	,732
100 mg/L-20 mg/L	14,500	3,880	3,737	,000	,001
100 mg/L-10 mg/L	15,500	3,880	3,994	,000	,000
50 mg/L-20 mg/L	8,500	3,880	2,190	,028	,171
50 mg/L-10 mg/L	9,500	3,880	2,448	,014	,086
20 mg/L-10 mg/L	1,000	3,880	,258	,797	1,000

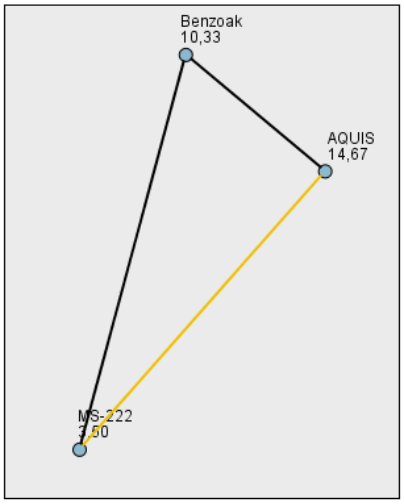
Each row tests the null hypothesis that the Sample 1 and Sample 2 distributions are the same.
Asymptotic significances (2-sided tests) are displayed. The significance level is ,05.

A.2.6 Experiment 3

Table A 6 Kruskal-Wallis test and multiple Pairwise Comparison of time until fish recovered after five minutes of exposure to MS-222, Benzoak and AQUI-S.

	Recoverytime
Chi-Square	13,414
df	2
Asymp. Sig.	,001

Pairwise Comparisons of Sedative



Each node shows the sample average rank of Sedative.

Sample1-Sample2	Test Statistic	Std. Error	Std. Test Statistic	Sig.	Adj.Sig.
MS-222-Benzoak	6,833	3,074	2,223	,026	,079
MS-222-AQUIS	11,167	3,074	3,632	,000	,001
Benzoak-AQUIS	4,333	3,074	1,410	,159	,476

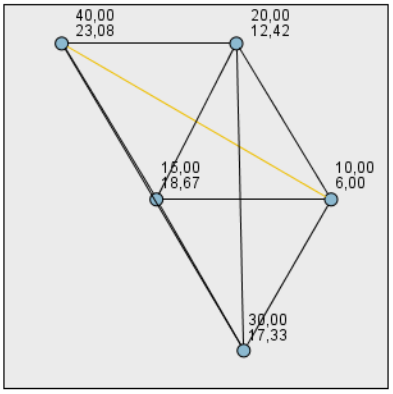
Each row tests the null hypothesis that the Sample 1 and Sample 2 distributions are the same.
Asymptotic significances (2-sided tests) are displayed. The significance level is ,05.

A.2.7 Experiment 4: MS-222

Table A 7 Kruskal-Wallis test and multiple Pairwise Comparison for recovery time after prolonged exposure to MS-222

	Recoverytime
Chi-Square	13,476
df	4
Asymp. Sig.	,009

Pairwise Comparisons of Exposuretime



Each node shows the sample average rank of Exposuretime.

Sample1-Sample2	Test Statistic	Std. Error	Std. Test Statistic	Sig.	Adj.Sig.
10,00-20,00	-6,417	5,033	-1,275	,202	1,000
10,00-30,00	-11,333	5,033	-2,252	,024	,243
10,00-15,00	-12,667	5,033	-2,517	,012	,118
10,00-40,00	-17,083	5,033	-3,394	,001	,007
20,00-30,00	-4,917	5,033	-,977	,329	1,000
20,00-15,00	6,250	5,033	1,242	,214	1,000
20,00-40,00	-10,667	5,033	-2,119	,034	,340
30,00-15,00	1,333	5,033	,265	,791	1,000
30,00-40,00	-5,750	5,033	-1,143	,253	1,000
15,00-40,00	-4,417	5,033	-,878	,380	1,000

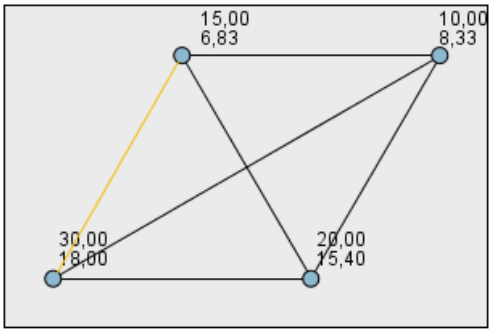
Each row tests the null hypothesis that the Sample 1 and Sample 2 distributions are the same.
Asymptotic significances (2-sided tests) are displayed. The significance level is ,05.

A.2.8 Experiment 4: AQUI-S

Table A 8 Kruskal-Wallis test and multiple Pairwise Comparison for recovery time after prolonged exposure to Aqui-S

	min
Chi-Square	12,552
df	3
Asymp. Sig.	,006

Pairwise Comparisons of Exposuretime



Each node shows the sample average rank of Exposuretime.

Sample1-Sample2	Test Statistic	Std. Error	Std. Test Statistic	Sig.	Adj.Sig.
15,00-10,00	1,500	3,697	,406	,685	1,000
15,00-20,00	-8,567	3,877	-2,209	,027	,163
15,00-30,00	-11,167	3,697	-3,021	,003	,015
10,00-20,00	-7,067	3,877	-1,823	,068	,410
10,00-30,00	-9,667	3,697	-2,615	,009	,054
20,00-30,00	-2,600	3,877	-,671	,502	1,000

Each row tests the null hypothesis that the Sample 1 and Sample 2 distributions are the same.
Asymptotic significances (2-sided tests) are displayed. The significance level is ,05.

A.3 Total length and weight of fish

Table A 9 Mean length and weight for fish used in experiment 1

	Total length (cm; mean ± S.D.)	**Weight (g; mean ± S.D.)**
Control	19,9 ± 3,3	63,7 ± 34,4
15 min	18,6 ± 1,3	46,6 ± 9,0
30 min	19,5 ± 2,2	54,3 ± 20,8
60 min	19,8 ± 2,2	60,9 ± 20,4
120 min	19,2 ± 2,0	54,3 ± 21,7
240 min	18,9 ± 2,0	48,3 ± 17,1

Table A 10 Mean length (cm) and weight (g) fish used in experiment 2-4. Fish from experiment 2 where only measured if they reached surgical anesthesia in

Exposure	**Total length (cm; mean ± S.D.)**	**Weight (g; mean ± S.D.**
Aqui-S 50 mg·L^{-1}	10. 1 ± 0.6	7.4 ± 1.2
Aqui-S 100 mg·L^{-1}	10.5 ± 0.4	8.2 ± 0.9
Benzoak 50 mg·L^{-1}	10.6 ± 0.5	8.3 ± 1.2
Benzoak 75 mg·L^{-1}	10.5 ± 1.5	8.9 ± 4.3
Benzoak 100 mg·L^{-1}	10.0 ± 0.9	7.6 ± 2.2
M-222 75 mg·L^{-1}	10.3 ± 1.2	8.1 ± 3.2
M-222 100 mg·L^{-1}	10.5 ± 0.4	8.9 ± 1.1

A.3.1 Experiment 1

Table A 11 ANOVA test for differences in weight between groups of fish used in experiment 1

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1586,891	5	317,378	,662	,655
Within Groups	16791,918	35	479,769		
Total	18378,809	40			

Table A 12 ANOVA test for differences in total length between groups of fish used in experiment 1

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	9,454	5	1,891	,366	,869
Within Groups	180,973	35	5,171		
Total	190,428	40			

Tabel A 13 ANCOVA test for covariance between cortisol and glucose level, and length and weight of fish used in experiment 1

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	2162,211[a]	7	308,887	1,530	,193
Intercept	1744,461	1	1744,461	8,638	,006
weight	644,776	1	644,776	3,193	,083
totallength	810,544	1	810,544	4,014	,054
Exposure	1365,032	5	273,006	1,352	,268
Error	6462,438	32	201,951		
Total	151216,534	40			
Corrected Total	8624,649	39			

a. R Squared = ,251 (Adjusted R Squared = ,087)

A.3.2 Experiment 2-4

Table A 14 ANOVA test for differences in weight between groups of fish used in experiment 2-4

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	11,422	6	1,904	,345	,908
Within Groups	193,068	35	5,516		
Total	204,491	41			

Table A 15 ANOVA test for differences in total length between groups of fish used in experiment 2-4

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1,793	6	,299	,379	,888
Within Groups	27,632	35	,789		
Total	29,425	41			

A.3.3 Comparison of fish used in experiment 1 and fish used in experiment 2-4

Table A 16 ANOVA test for differences in total length and weight between fish used in experiment 1and fish used in experiment 2-4

		Sum of Squares	df	Mean Square	F	Sig.
cm	Between Groups	1516,400	1	1516,400	213,010	,000
	Within Groups	583,750	82	7,119		
	Total	2100,150	83			
g	Between Groups	42881,243	1	42881,243	163,541	,000
	Within Groups	21500,764	82	262,204		
	Total	64382,007	83			