Aspects of Fish Welfare in Aquaculture Practices

Proefschrift

ter verkrijging van de graad van doctor
aan de Radboud Universiteit Nijmegen
op gezag van de rector magnificus prof. mr. S.C.J.J. Kortmann,
volgens besluit van het college van decanen
in het openbaar te verdedigen op vrijdag 20 december 2013
om 11.00 uur precies

door
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geboren op 1 augustus 1985
te Montpellier (Frankrijk)

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Aspects of Fish Welfare in Aquaculture Practices

Doctoral thesis, Radboud University Nijmegen

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Printed by Gildeprint Drukkerijen - Enschede

ISBN: 978-94-6108-539-9

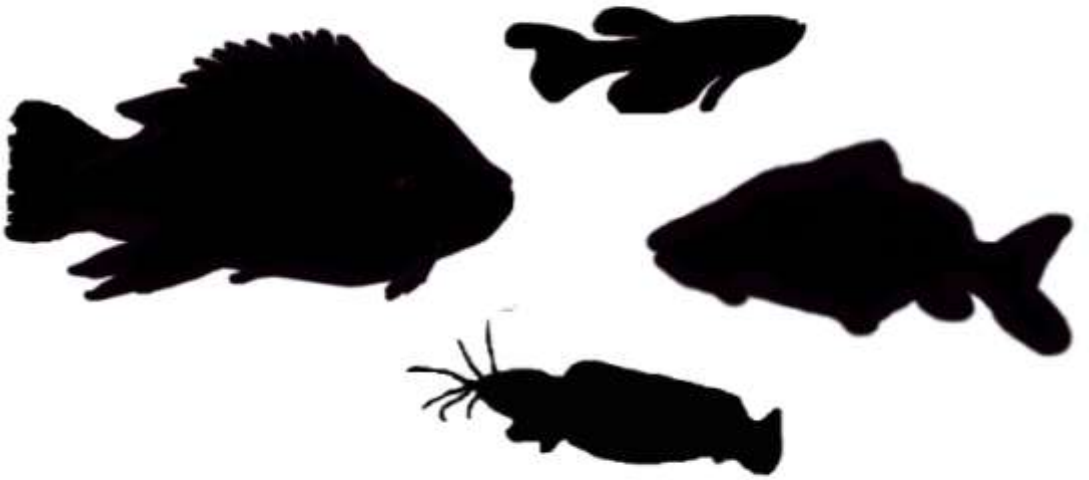
This research project has been carried out within the Knowledge Base Research / Policy Supporting Research / Statutory Research Task (Project BAS-project KB08 009001ASG) and within the frame of the beleidsondersteunend onderzoek (Project BO-07-011-028), for the Dutch Ministry of Economic Affairs

Cover design: Kiyoko Kuribara; cover picture: Constantin Paun

Table of contents

Chapter 1

General introduction



1. Aim of this thesis

The overall aim of this thesis is to increase knowledge on welfare of farmed fish through study of the effects of well-defined, relevant external stimuli on physiology and behavior. We investigated effects of acute and chronic discomfort, which served as models for the way current aquaculture practices may interfere with fish welfare. To apply acute discomfort we selected fin clipping and an electric shock, both applied to the tailfin. These treatments could evoke a pain sensation. For chronic discomfort we exposed fish for prolonged periods (weeks) to nitrogenous waste (ammonium, nitrite and nitrate) in the ambient water. Effects of discomfort on fish welfare were evaluated on the basis of physiological (*e.g.* endocrine stress responses, growth rate and osmoregulatory capacity) and behavioral parameters (*e.g.* scototaxis, *i.e.* a light/dark preference test and habituation to novelty). Our experiments were all carried out with fish kept in recirculating aquaculture systems (RAS). RAS offers the possibility to achieve a high production with minimal ecological impact (Martins et al, 2010). In the Netherlands essentially all fish aquaculture occurs in RAS. The species chosen reflect the fish commonly found in aquaculture practices (tilapia subspecies and common carp, *Cyprinus carpio*): African catfish, *Clarias gariepinus* is one of the major farmed species in the Netherlands; pikeperch is increasingly popular and a highly valued species and introduced to diversify aquaculture (Le-Francois et al, 2002). Zebrafish was used as the model of choice in the study on the effect of fin clipping on the habituation response, for which a protocol was recently published (Stewart et al, 2010; Wong et al, 2010). The versatility, ease of handling, inexpensiveness to grow and well annotated genome are reasons for its popularity as fish model in developmental biology, toxicology and genetics research (Ribas et al, 2013).

2. Fish in aquaculture

Every day, millions of fish are used by humans in various different ways, such as for recreational fishing, as pets, as models for scientific research, and most importantly from a quantitative point of view, as raw material for the production of food, non-food products and feed. The human demand for protein and lipid cannot be satisfied by fisheries practices alone. Natural stocks of several fish species have drastically declined due to a variety of causes (Naylor et al, 2000). The world-wide aquaculture production nowadays almost matches the total fisheries production, and shows yearly increases of 7% on average, whereas the fisheries production has leveled off the past decade (FAO, 2012).

There are several types of aquaculture; cage or pen-net cultures at sea, basins in estuaries, pond cultures and land-based recirculation cultures are the dominant ones (EFSA 2008a; 2008b; 2008c; 2008d). In Europe (FEAP 2012), the culture of Atlantic salmon (*Salmo salar*; 1.48 million tons), rainbow trout (*Oncorhynchus mykiss*; 359 kilotons), European seabass (*Dicentrarchus labrax*; 148 kilotons) and sea bream (*Sparus auratus*; 120 kilotons) rely mainly on cage culture at sea, whereas freshwater species such as common carp (*Cyprinus carpio*; 59 kilotons), tilapia species and African catfish (*Clarias gariepinus*) are produced mainly in ponds, in countries in Asia and Africa, but also in Eastern Europe. Asia accounted for 92% of world aquaculture finfish production by volume in 2010, whereas for Europe this was 5.2% (FAO, 2012).

As indicated above, in The Netherlands, production of farmed fish for consumption relies almost completely on culture in RAS. These are on-land systems that re-use 90-95% of the water by recirculating it through a series of treatments that remove the waste effectively to limit waste water (Bovendeur et al, 1987; Eding et al, 2006). The development of RAS enables fish farmers to be less dependent on natural water resources and reduce the impact on the environment (Piedrahita, 2003; Read and Fernandes, 2003; Martins et al, 2010).

In recent years, more and more Norwegian salmon farmers now switch from flow-through systems to RAS for the production of fingerling fish (very young fish are produced in on-land systems), where they stay until a range of morphological, physiological and behavioral changes that pre-adapt the smolts to marine life occur. Production of smolts in RAS is on the rise to limit the intake of freshwater, a requirement of the European Water Framework Directive (EC Directive 2000/60/EC, 2000).

RAS allows optimal control over the culture conditions and results in better specific growth rates compared to pond or cage culture. However, RAS is labor intensive and a technically demanding form of aquaculture. A downside of RAS is production and accumulation of various nitrogen waste products, such as ammonia, nitrite and nitrate, which may negatively affect the welfare of fish. The stocking densities in RAS are high and this may lead to a (nitrogenous) waste accumulation problem. The focus on production (high stocking densities) is a key issue in aquaculture. A similar situation holds for the various treatments that fish in captivity have to undergo: handling, sorting, crowding, stocking, tank dimensions and substrate qualities are among the factors that influence normal behavior and may strongly call upon the animal's adaptive capacity. Fish are exposed to a large variety of stimuli that, each or in combinations, will more or less substantially influence physiological and behavioral parameters, indicative for welfare of the animals.

3. Improving fish welfare in aquaculture

With a constant expansion and diversification of the aquaculture sector, the attention for the well-being of fish (fish welfare) has strongly increased. One could ask why? Welfare issues concern not only husbandry conditions during production, but also animal health and can be related to food quality and safety (EFSA, 2008e). An important barrier that may stand in the way of to optimize fish welfare are the economics of interventions at *e.g*. a fish farm. In the EU project Benefish (now completed), it has been shown that the interventions at the level of fish farms (better rearing procedures reducing mortalities and injuries) may not only improve fish welfare but also generate increased profits (Turnbull and Huntingford, 2012).

Due to increasing societal awareness, predominantly in Europe, Canada, Australia and New Zealand, the interest in welfare of fish in aquaculture is still growing (Branson, 2008; EFSA, 2009a; Van de Vis et al, 2012). Fish welfare is of interest for many different stakeholders: the industry, consumers, non-governmental organizations, retailers, scientists (including animal ethicists) and policy makers. It is known that animal welfare is a multi-sided concept and this raises the question how to define fish welfare, and how to measure it (Broom, 1998). Welfare can be defined as the quality of the life of an animal that is able to experience it (Torgersen et al, 2012). We will not discuss the concept of fish welfare in detail in this thesis, as it has been the subject in various review papers (Arlinghaus et al, 2007; Bracke and Hopster, 2006; Branson, 2008; Duncan, 2007; Galhardo and Oliveira, 2009; Hagen et al, 2011; Huntingford et al, 2006, Korte et al, 2007; Ohl and Van der Staay, 2012; Spruijt et al, 2001). Although for animal welfare various definitions exist, the issue is that poor welfare is associated with overtaxing the adaptive capacity of animals (allostatic overload; (McEwen and Wingfield, 2003). Allostatic overload may lead to chronic stress related physiology and behavior, pathology and increased mortality. Exposure to successive stressors or to an accumulation of stressors may overtax the adaptive capacity of an animal and lead to allostatic overload and poor welfare (Korte et al, 2007). In the model of allostasis fish welfare relates to stress load in a hyperbolic fashion. Low environmental challenges, *i.e.* hypostimulation, and environmental hyperstimulation may both produce poor welfare conditions (distress) (Korte et al, 2007). An environmental challenge varies as a result of intensity, duration, predictability and controllability of stressors. Exposure to a certain level of environmental challenges (eustress) will improve welfare (hyperbola above the neutral welfare line). When allostatic mechanisms in animal are over-stimulated or fail

to reset, allostatic overload arises; if not properly and timely counteracted, overload leads to poor performance, disease and death.

Methods to assess how welfare of fish can be influenced heavily rely on indirect measurements, such as physiological and behavioral assessments. Studies on the cognitive status (Braithwaite et al, 2013), the perception of pain (Sneddon, 2004) and fear in fish (Galhardo and Oliveira, 2009) are to date scarce and therefore more studies are needed in this field.

In their review Galhardo and Oliveira (2009) conclude that in fish, the regulation of stress involving psychological components is not well studied. Though our knowledge on how fish perceive events is increasing rapidly during the past decade, in the view of some scientists fish lack the capacity for mental assessment of stress situations (Rose 2002, 2007; Rose et al, 2012). Braithwaite and colleagues (2013) recently prepared an overview on current knowledge on the capacities of fish to perceive emotions. These authors show that the fish species studied have sufficient neural capacities to support complex cognitive abilities and, potentially, the experience of emotion. The number of fish species studied for behavioral and brain functions with respect to cognition and emotion is, however, limited. Acquired knowledge is species-specific, depending on life stage, experiences during early life, habitat, personality of the fish (shy *vs.* bold; Galhardo and Oliveira, 2009) (Braithwaite et al, 2013).

4. Acute discomfort: fin clip and electric shock as noxious, potentially painful stimuli

Pain is defined as an unpleasant sensory and emotional experience, associated with actual or potential tissue damage, or described in terms of such damages (IASP, 1979). In higher vertebrates (mostly mammals) two types of pain are generally distinguished: (1) acute and well-localized (transient) pain, mediated by myelinated A-δ fibers, and (2) chronic, poorly-localized dull and persistent pain, mediated by non-myelinated C-fibers (Lynn, 1994). In mammals and man, there is no doubt that acute pain is part of a basic biological warning mechanism, essential for survival of the individual and therefore of the species in question (Baars, 2001). However, especially when pain is very strong and prolonged (chronic), it may be detrimental and affect physiological and psychological welfare (Lamont et al, 2000). However, the degree of the experienced pain by mammals is difficult to determine, due to strong interindividual differences as to pain threshold and the many different modalities in which pain may occur.

Compared to pain research in mammals, the detection of (the degree of) pain in fish is an even bigger challenge. Fish are ectotherms (laymen say

cold-blooded), non-furry/non-fluffy, but slimy and expressionless animals that do not reveal any emotions, nor attract empathy at the first glance (Braithwaite, 2010). Furthermore, while working with aquatic organisms, one should take into account that the simple procedure of removing an organism from its environment to apply a stimulus is already a stressful procedure by itself and can thus influence the outcome of the experiment. The right choice of adequate controls for handling stress is therefore a prerequisite to determine which part of the response is due to the stimulus and which to the handling procedure *per se*. So, how to find out if they perceive pain?

Gregory (1999) proposed three steps to assess whether fish are capable of pain perception. The first step is showing that neural elements involved in pain perception in mammals also occur in fish. The second step is to give a stimulus that has been validated to evoke pain in mammals, and compare the fish response with the mammalian one. The third step is to test if this fish response can be blocked or at least attenuated by known mammalian analgesics.

In 2002, nociceptors were for the first time identified in the trigeminal nerve of rainbow trout, which resulted in pain research in fish starting to attract more attention (Sneddon, 2002). When such trout were injected into the lips with either bee venom or acetic acid, substances used in mammalian pain research, significantly delayed feeding resumption and increased opercular beat rate were the result (Sneddon et al, 2003a; 2003b). Nordgreen and colleagues (2007) further showed that in Atlantic salmon, the capacity to perceive painful stimuli and the adequate nociceptive fibers for the detection of potentially painful stimuli are present.

Furthermore, while working with aquatic organisms, one should take into account that the simple procedure of removing an organism from its environment to apply a stimulus is already a stressful procedure by itself and can thus influence the outcome of the experiment. The right choice of adequate controls for handling stress is therefore a prerequisite to determine which part of the response is due to the stimulus and which to the handling procedure *per se*.

4.1 Fin clipping

The tailfin clip was chosen as a noxious stimulus. Damage to the tail fin of fish is a common phenomenon and may result from natural injuries when fish fight or chase other fish (Chervova, 1997; Turnbull et al, 1998) and as a result of aquaculture practices, *e.g*. sorting, transportation; in laboratory settings fin clipping is used to mark fishes or genotyping; Sharpe et al, 1998).

Importantly, Chervova (1997) demonstrated that the caudal, dorsal and pectoral fins, and the skin around the eyes (as well as the more internal epithelium of olfactory sacs) are among the most sensitive nociceptive zones in fish. Damaged or cut fins will regenerate (Akimenko et al, 2003). Furthermore, it has the advantage that it does not cause serious side effect nor does it impair directly vital functions (Noble et al, 2012), and it can be applied conveniently in a standardized manner (Mogil, 2009). We reasoned therefore, that a tail fin clip is not a lasting and invalidating burden for the fish. We analyzed the tissue clipped by electron microscopy to assess whether and if so which nerve fibers were cut during the clip. The potentially noxious effect of a fin clip has not been investigated before.

4.2 Electrical shock

Electric shocks are used in human pain research (Rhudy and Meagher, 2000). Unlike a fin clip, an electric shock does not cause physical damages, if applied correctly. We applied electric shocks to the tail fin to compare their effects with those of fin clipping. The effects of electric shocks applied to the caudal tail were studied earlier in cod (*Gadus morhua marisalbi*) and steelhead trout (*Salmo mykiss*) (Chervova, 1997).

4.3 Readout parameters for acute discomfort

To find out whether clipping and electroshocks are noxious stimuli we studied a panel of primary, secondary and tertiary readout parameters for stress, including plasma cortisol, glucose, lactate, non-esterified fatty acids (primary), gill functional morphology (secondary), and behavior (tertiary). The rationale was that truly noxious ('painful') stimuli could evoke differential responses on top of the unavoidable stress responses related to the handling associated with the application of a clip or electroshock.

Several behavioral tests were recently adapted from mammalian models (rodents) and validated for zebrafish (Champagne et al, 2010). Among these, the light/dark preference (Maximino et al, 2010) and the novel tank tests (Stewart et al, 2010; Wong et al, 2010) are of interest for pain studies in fish. Changes in place preference, exploratory, risk-taking and abnormal behavior after a noxious, potentially painful stimulus were proven to be reliable parameters to monitor pain handling in mammals, so maybe also in fish. Recent adaptations of automated video-tracking software (such as Ethovision; Noldus, Wageningen, The Netherlands) have greatly improved and facilitated the analysis of fish behavior.

4.4 Model species used to study acute discomfort

Common carp and tilapia subspecies were chosen as they are important species cultured worldwide (FAO, 2004; 2005; van Duijn et al, 2010); importantly, these fish are among the best studied in fish stress research (Bongers et al, 1998; Burns et al, 1995; Flik et al, 1993; Ibrahim et al, 2013). The zebrafish is a popular model in scientific research (Ribas et al, 2013), for which a large panel of molecular tools and behavior paradigms are available, and many of them online (zfin.org). The light/dark preference chamber and the novel tank test (Maximino et al, 2010; Wong et al, 2010) are two examples.

5. Chronic discomfort: impacts of nitrogenous waste products

Nitrogenous waste products are an important aspect in control of water quality in RAS in relation to production and fish welfare. RAS are a labor intensive form of aquaculture with high investments and maintenance costs (De Ionno et al, 2006). The built of these systems allow for high fish densities and make the sector profitable (Martins et al, 2010). However, the high input of dietary nitrogen required in fast growing fish can lead to saturation and malfunctioning of the system and accumulation of nitrogenous waste in the form of ammonia, nitrite and nitrate. Fish, as all vertebrates, produce nitrogenous wastes through catabolism of amino acids (Wood, 1993); fish are either (mostly) ammonotelic (Smith 1929; Olson and From, 1971) or urotelic (Goldstein and Forster 1965; Cvancara, 1969), *i.e.* they produce ammonium/ammonia as end product of the ammonium cycle or urea, respectively. Urotely is less common than ammonotely. The former is well known from sharks that use urea as important compound to adjust plasma osmotic pressure (Smith, 1936); freshwater air-breathers increase their urea production when exposed to air (Saha and Ratha 1987), and so do fish living in extreme alkaline environments (Randall et al, 1989). Nitrogenous waste is always produced and may accumulate in aquaculture systems (obviously and in particular in recirculating aquaculture systems) as result of a variety of factors, including sub-optimal system performance (too low capacity of the bio-filter), high protein diets, incomplete digestion of food by the fish, and overfeeding to stimulate/hasten growth (Crab et al, 2007).

5.1 The nitrogen cycle: ammonia, nitrite and nitrate

Ammonia (NH_3) and its protonated ionic form ammonium (NH_4^+) may severely impede growth and may easily reach toxic levels when fish are kept at

high density (Boeuf et al, 1999). The toxicity is particularly ascribed to the un-ionized molecule which diffuses quickly (around 20 x 10^{-3} cm/sec) across cell membranes (Yuri et al, 1997; Colt, 2006). The ratio NH_3/NH_4^+ and therefore the ammonia toxicity is influenced by pH, ionic strength, pressure and temperature (Chen et al, 2012). Also the ionized ammonium may become toxic but at much higher concentrations compared to ammonia (Colt, 2006).

In present RAS, aerobic biofilters harbor denitrifying bacteria that convert ammonia via nitrite (NO_2^-) to nitrate (NO_3^-) in a two-step process (van Rijn, et al, 2006). Interesting developments in the dinitrification procedures are foreseen with the discovery of anaerobic ammonium oxidizers (microbes carrying out ammonium oxidation in the absence of oxygen, a process called anammox). Implementation of anammox reactors in RAS would allow for direct conversion of ammonium and nitrite into dinitrogen gas and water as end products, thus limiting the accumulation of nitrogenous wastes in the rearing water and therefore save significantly on the use of water (Kartal et al, 2011). Anaerobic ammonium-oxidizers have been demonstrated in marine RAS (Tal, 2006) and more recently also in freshwater RAS (van Kessel et al, 2010).

Uptake and excretion mechanisms of ammonia/ammonium clearly differ from those of nitrate and nitrite, although they which were originally suggested to be similar (Eddy and Williams, 1987; Williams et al, 1993). The mechanisms of branchial ammonia excretion remain under debate. In the 'traditional' model for branchial excretion, NH_3 diffusion is considered the predominant mechanism under normal conditions (Schram et al, 2010). At high ambient ammonia concentrations, when passive outward diffusion over the integument (gills) is impaired or even reversed, energized (active) NH_4^+ excretion pathways are activated in the gills and contribute to ammonia removal (Wright and Wood, 2009). Recently, Rhesus (Rh) glycoproteins have been identified in pufferfish (*Takifugu rubripes*) gills (Nakada et al, 2007). The presence of these specific ammonia transporters has led to a revision of the excretion mechanisms of ammonia in fish (Weihrauch et al, 2009; Wright and Wood, 2009). The present view is that a 'Na^+/NH_4^+ exchange complex that consists of a series of carrier proteins (Rhcg, V-type H^+-ATPase, Na^+/H^+ exchanger NHE-2 and/or NHE-3, Na^+ channel) in series secures movement of ammonia over the membranes of endothelial and branchial cells that form the barrier between blood and water (Wright and Wood, 2012; Shih, 2013).

Next to the gills, the intestinal tract must be considered as significant pathway for nitrogenous waste traffic. In the European flounder, *Platichthys flesus* water-born nitrite is taken up via the intestine (Grossel and Jensen, 2000). In seawater species, that must drink to counteract osmotic water loss,

but also in freshwater fish that will drink when stressed (Kobayashi et al, 1983), intestinal pathways for nitrogenous waste fluxes need consideration.

5.2 Toxicity of nitrogenous waste compounds

High ambient ammonia concentrations are neurotoxic for fish (Wilkie, 2002). Nitrite is toxic primarily by converting the oxygen carrier hemoglobin (Hb) into methemoglobin (MethHb), that does not transport oxygen (Kiese, 1974). Nitrate toxicity is thought to be similar to that of nitrite, but a lower apparent branchial permeability to nitrate compared to nitrite in fish (Stormer et al, 1996) contributes to the notion that nitrate is less toxic for fish than nitrite. In freshwater, nitrite enters the organism primarily via the chloride cells in the gills (Bath and Eddy, 1980). At high external concentrations, part of the branchial chloride uptake is shifted to NO_2^- uptake (Jensen, 2003). Therefore, fish with high branchial chloride turnover are more prone to nitrite toxicity (Williams and Eddy, 1986). This concerns mostly freshwater species, where active branchial chloride uptake is the main route of entry (Jensen, 2003). The presence of chloride ions in the water protects against nitrite toxicity, due to competition of Cl^- and NO_2^- at the carriers that mediate branchial chloride uptake (Crawford and Allen, 1977). This protective effect of chloride is varies significantly among species reflecting differences in chloride handling strategies (Lewis and Morris, 1986).

In RAS, nitrite accumulates in the rearing water when incomplete oxidation of ammonium by the bio-filter to nitrate occurs (Kroupova et al, 2005). Nitrite then accumulates in the blood plasma of the fish where it can reach concentrations over sixty times higher than those in the water (Fontenot and Isely, 1999). Thus the blood is the first and main target of nitrite toxicity. Nitrite penetrates the red blood cells and next hemoglobin is converted to methemoglobin. Methemoglobin concentrations in fish blood vary among species as well as among individuals in a population (Lewis and Morris, 1986). Concentrations 1.5 times above basal are considered potentially dangerous (Bowser et al, 1983). Nitrite toxicity in fish is not limited to methemoglobin formation; various key physiological processes such as ion regulation, respiration, heart action and kidney and gill functions depending on chloride handling (Kroupova et al, 2005) show correlation with elevated methemoglobinemia.

As alluded to above, nitrate is considered the least toxic of nitrogenous waste products. However, fish farmed in RAS may be chronically exposed to nitrate levels ranging from 7 to 70 mM, depending on RAS design and management (Van Rijn, 2010). Accumulation of nitrate in RAS is the direct

consequence of the biological conversion of ammonia to nitrate via the nitrite pathway. Nitrate toxicity occurs mainly at high (millimolar) concentrations and long exposure times; larger fish tend to be more tolerant to nitrate exposure, as are fish in seawater (Camargo et al, 2005). An important side effect of nitrate accumulation is reduction to nitrite where and when the systems offers anaerobic conditions (Colt, 2006).

5.3 Parameters of interest and experimental setup

Previous toxicological, acute-exposure studies on nitrogenous waste compounds mostly focused on 96 h LC_{50} values for ammonium, nitrite and nitrate. Such data are important, but less relevant for fish farmers, who are more interested in the concentrations at which growth and welfare of the animals become impaired.

Physiological parameters, such as markers of stress and ionic imbalance inform us on changes in RAS and the coping abilities of the fish. Feed intake and growth rate tell us directly about the welfare situation. Stressful conditions may immediately impact feeding and growth, with the energy used primarily to properly cope with the stressor instead of being used for growth. Stress responses come with endocrine responses. Stress evokes adrenalin and cortisol responses, hormones with great impact on essentially every aspect of physiology (Wendelaar Bonga, 1997). Among the targets for stress hormones gills take an important position as their functioning and organization and histology gives a predictable readout for the stressor imposed. Last but not least, the brain is not only a target for stress hormones, in fact the brain of a vertebrate coordinates the stress response and for that very reason stress can be anticipated to modulate or alter various behaviors.

We chronically exposed fish to different levels of ammonia, nitrite and nitrate and assessed effects of physiology and growth performance. We investigated realistic concentration ranges (*i.e.* concentrations seen in RAS). Our aim was to recognize subtle effects that may serve as early warning signals for the farmer, effects that may now go undetected but would allow action to restore proper conditions and, of course, give maximum guarantee of good welfare for the fish.

5.4 The choice of the species

The African catfish is a robust, fast growing species and popular in Dutch aquaculture (van Duijn et al, 2010); indeed, it is a rapidly emerging species also in African aquaculture (FAO, 2012). Its robustness does not imply

that no or little attention should be paid to, for instance, water quality; all too often combinations of stressors (must) underlie unexpected calamities at farms. The sturdy African catfish offers the researcher an excellent and resilient model to unravel contributions of individual stressors found in a complex setting of RAS (simulated in the laboratory) with its nitrogenous-waste stressors.

Pikeperch is being introduced in aquaculture in Northern and Central Europe next to common carp, rainbow trout and tilapia species to diversify aquaculture. It is a highly valued (and prized) rather stenohaline freshwater fish. In fundamental research this species is relatively new. Pikeperch is considered sensitive to stressors (Schulz et al, 2005). We reasoned that an analysis of its tolerance to nitrogenous waste products will benefit proper introduction of this species in intensive RAS farming, where stocking densities are higher than in open systems.

6. Outline of this thesis

After a general introduction on acute and chronic discomforts that a fish may experience in aquaculture practices, three chapters are presented with studies in which noxious stimuli were given to carp, tilapia and zebrafish, followed by four chapters in which studies on effects of chronic exposure to nitrogenous waste were evaluated in catfish and pikeperch (see Fig. 1).

In **chapter 2**, an electron microscope analysis of common carp (*Cyprinus carpio*) tailfin was performed to find ultrastructural evidence for the presence of A-δ and C-type axons, neurites transmitting pain signals in higher vertebrates/mammals. The tail fin of Nile tilapia (*Oreochromis niloticus*) was clipped and several hormonal, cellular and behavioral parameters were studied in a search for responses that might differ from those to the response due to mere handling and, therefore, could indicate perception of pain. In **chapter 3**, responses to a mild electric shock applied to the tailfin area were studied in Mozambique tilapia (*Oreochromis mossambicus*). Again, the responses were compared to acute stress responses evoked by the handling associated with the procedure. In **chapter 4**, the response to tailfin clipping was studied in zebrafish, (*Danio rerio*) by assessing parameters for anxiety and for habituation to novelty. The effects of elevated water content of ammonia (**chapter 5**), nitrate (**chapter 6**) and nitrite (**chapter 7**) levels in the water were studied in the African catfish (*Clarias gariepinus*). Effects of elevated water ammonia and nitrate concentrations were also tested in the pikeperch (*Sander lucioperca*) (**chapter 8**). In the general discussion, **chapter 9**, the results of the research

chapters are summarized and discussed for their significance in (RAS-based) aquaculture.

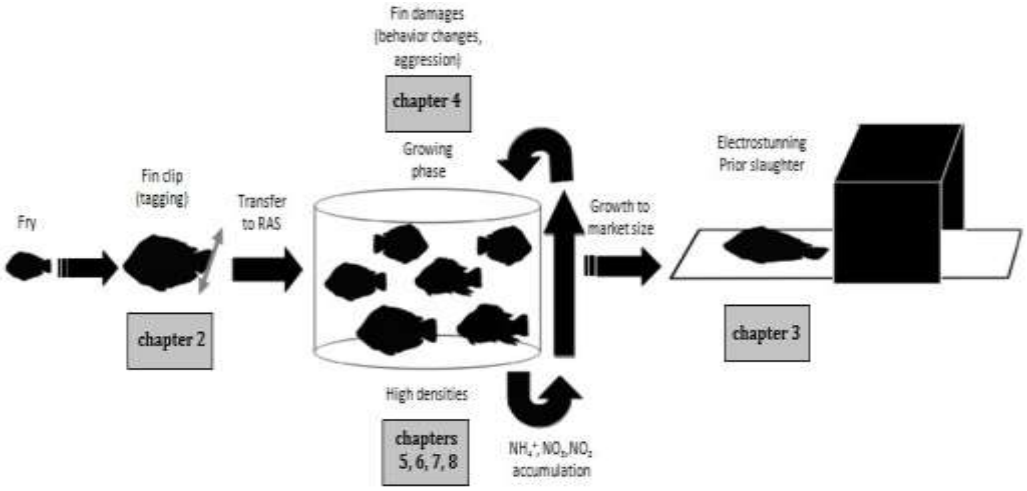


Fig. 1. The different phases in an aquaculture setup in which the different chapters of this thesis can be positioned. Fin clipping as model for damage by intraspecific aggression or when the fish come into contact with man (chapter 2). In their growing phase in RAS, fish are kept at relatively high densities, which may lead to both accumulation of nitrogenous wastes (chapters 5, 6, 7) and enhanced aggressive behavior that could target the fins (chapter 4). When the market size is reached, fish will be slaughtered. Prior to slaughter, stunning is advised. Electrical stunning (chapter 3) is an increasingly used in Dutch recirculation systems.

Acknowledgements

I would like to thank prof. dr. Eric Roubos for constructive comments on this general introduction.

Chapter 2

Tailfin clipping, a painful procedure: studies on Nile tilapia and common carp

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In: Physiology and Behavior (2010) 101:533-540

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Abstract

The fish welfare debate is intensifying. Consequently, more research is carried out to further our knowledge on fish welfare in aquaculture. We define here a series of key parameters to substantiate an acute response to a supposedly painful stimulus: a standardized tailfin clip.

Ultrastructural analysis of common carp (*Cyprinus carpio*) tailfin indicates the presence of A-δ and C-type axons, which are typical for transmitting nociceptive signals in (higher) vertebrates. In Nile tilapia (*Oreochromis niloticus*), responses to a tailfin clip were studied and the unavoidable acute stress associated with the handling required for this procedure. A series of key parameters for further studies was defined. The responses seen in 'classical' stress parameters (*e.g.*, changes in plasma cortisol, glucose and lactate levels) did not allow discrimination between the clipping procedure and the handling stress. However, 3 parameters indicated a differential, stronger response to the clip stimulus itself: first, swimming activity increased more and clipped fish spent more time in the light (in a tank where half the volume is covered by dark material); second, the gill's mucus cells released their content as observed 1 h after the clip, and this response is transient (no longer observed at 6 h post clipping). Third, branchial Na^+/K^+-ATPase activity assayed *in vitro* was not affected by the procedures, but a remarkable migration of Na^+/K^+-ATPase immunoreactive (chloride) cells into the lamellar epithelium was observed as of 6 h post clipping. We conclude that the differential response to clipping supports that this is a painful procedure that evokes a transient specific physiological status.

1. Introduction

In humans, awareness of pain, fear and stress depends on functions controlled and executed by the highly developed hippocampus, amygdala, and cerebral frontal lobes and neocortex (Apkarian et al, 2005). In fish, the telencephalon, which will evolve to these cerebral structures in higher vertebrates, is far less complex and anatomically and fundamentally different, which has led many to conclude that fish cannot experience pain, fear or stress (Bermond, 1997, Rose, 2002). One of the endeavors in research on fish welfare is the assessment of consciousness which is at the basis of pain and fear experience. There is ample evidence to conclude that fish experience stress and successfully mount behavioral and neuroendocrinological responses to cope with stress (Wendelaar Bonga, 1997).

Reviews by Braithwaite and Huntingford (2004) and Chandroo and colleagues (2004) present convincing evidence that fish, despite their less developed telencephalon, have learning abilities at a level that implies cognitive abilities. For some species (rainbow trout *Oncorhynchus mykiss*, Atlantic cod *Gadus morhua*, common goldfish *Carassius auratus,* and Atlantic salmon *Salmo salar*), the first evidence has been advanced that fish may have the capacity to perceive painful stimuli and have a nervous substrate to experience fear and to suffer (Sneddon, 2002; Nilsson et al, 2002; Nordgreen, 2009). However, it has to be emphasized that it is unlikely that fish, as well as other animals, except maybe higher primates, have the capacity to experience suffering as human do (Braithwaite and Huntingford, 2004). Nociception, the detection of potentially harmful stimuli, is at the very basis of experiencing pain, *i.e.*, interpreting the nociceptive stimulus. Pain perception thus involves both the nociceptive sensory machinery and the actual translation of harmful stimuli to the feeling of pain. Fish should possess then both a nociceptive system and some cognitive capacities to experience pain in a human sense. Indeed, a limited, yet firm, literature supports that fish detect harmful stimuli, respond to nociceptive stimuli and may conceptualize pain (Braithwaite and Huntingford, 2004; Chandroo et al, 2004; Sneddon, 2002; Sneddon, 2003; Sneddon et al, 2003a and 2003b).

Next to the feeling of pain, fear and stress are motivational affective states that are relevant to fish welfare. In their seminal reviews Braithwaite and Huntingford (2004), and Chandroo and coworkers (2004) conclude that these affective states may well be attributed to fish. Recently, Nilsson and coworkers (2002) demonstrated explicit memory in Atlantic cod and, therefore, it is

reasonable to hypothesize that fish indeed have capacities to have some form of consciousness and be aware of pain.

Studies that deal with the welfare of fish are limited to only a few out of an estimated total of 35,000 species; indeed, the knowledge on fish can only be called fragmentary. Beyond natural variation, human influences on fish, *e.g*., through prolonged farming and domestication, may impinge on welfare-related aspects such as aquaculture-related stress physiology (Pottinger and Pickering, 1997). Clearly, big gaps in the knowledge on fish welfare exist. Nevertheless, the current literature suggests that fish deserve a better moral consideration than they have received so far (Lund et al, 2007).

The international association for the study of pain (IASP) defined pain as an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage (IASP, 1979). Although pain has a subjective component that is difficult to convey without words, a non-verbal individual can still experience pain and benefit from pain-relieving treatment. In humans, methods to assess and quantify pain focus on cognitive abilities and subjective feelings. In studies on other mammals, emphasis is put on physiological parameters and behavioral activity, with little interest in the cognitive abilities and subjective feelings as is done for humans. However, few of these methods have been applied to demonstrate or quantify painful stimuli in fishes. A complicating factor in pain research is that the application of painful stimuli goes with an inherent stress response, for instance to handling (*e.g*., when blood is sampled) that interferes with the response to the fin clip. It is difficult to distinguish between stress responses and mild pain responses as these responses share a larger part of the stress physiology.

In this study, behavioral and stress-endocrine responses of the Nile tilapia (*Oreochromis niloticus*) to a presumed pain stimulus (tailfin clip) were investigated. In common carp (*Cyprinus carpio*), the clipped tissue was investigated at the ultrastructural level to identify nerve fibers classified in mammals and rainbow trout as pain fibers. Swimming activity was monitored and the fish's preference to reside in the lightened or darkened section of a compartmented aquarium. The stress parameters plasma cortisol, glucose and lactate, were measured. Parameters for osmoregulatory performance including Na^+/K^+-ATPase enzymic activity and chloride cell abundance and position in gills and plasma concentrations of Na^+, K^+ and Ca^{2+} were determined. In addition, mucus content of mucus cells in the gills was quantified.

This study was designed to discriminate the acute stress response inherent to the application of a fin clip as presumed pain stimulus from the fin clip proper through inclusion of the appropriate controls, and to select key parameters for future studies into this field of research.

Peripheral nerve fibers are categorized according to their diameter, conduction velocity and degree of myelinisation as A-α, A-β, A-γ, A-δ B- and C-fibers (Erlanger and Gasser, 1937). The A-fibers are myelinated for fast conduction of action potentials. The A-δ fibers are involved in the transmission of well-localized acute pain, while C-fibers lack a myelin sheet (are very simply isolated by glia) and therefore slowly conduct action potentials and involved in poorly localized unpleasant slow dull pain (Sneddon, 2002; Pottinger and Pickering, 1997; Lynn, 1994). Fibers conducting in the velocity range of A-δ and C-fibers were identified in the trigeminal nerve of the rainbow trout and characterized as nociceptive fibers by Sneddon (2002). A-δ fibers (25%) were predominant over C-fibers (4%); displaying a different pattern compared with other vertebrates, where C-fibers can comprise from 50% (cat, human) up to 65% (frog) of the total fiber type (Young, 1977). This difference in fibers composition is attributed to the water-to-land transition in vertebrate evolution (Sneddon, 2002).

A tailfin clip was chosen as pain stimulus; all the handling around the clipping procedure, but omitting the clip, served as control procedure to quantify the handling stress. Fins are vulnerable body parts that are easily damaged as a result of aggressive behavior between fishes or of aquaculture practices, such as sorting and transport.

2. Materials and methods

2.1 Ultrastructural analysis of common carp (Cyprinus carpio) tailfin

2.1.1 Nerve bundles

Tailfin clips of common carp were immersed in glutaraldehyde (2.5% v/v), $K_2Cr_2O_7$ (1% w/v) and OsO_4 (1% w/v) in 0.15 M cacodylic acid (pH 7.5) and embedded in Spurr's resin. Ultrathin sections (70–90 nm) were cut with an ultratome and mounted on square mesh nickel grids. On-grid sections were post-stained for 2 min with uranyl acetate and then lead citrate for 2 min and rinsed thrice with doubly distilled water. Nerve fiber types in cross sections were categorized based on diameter and the presence of myelin to distinguish A-α, A-β, A-δ and C-fibers (Sneddon, 2002; Lynn, 1994) (Table 1).

Fiber type	Bundle 1 (hypoder.)	Bundle 2 (hypoder.)	Bundle 3 (lepido.)	Bundle 4 (lepido.)	Bundle 5 (lepido.)	Average (SD)	Trigeminal nerve (Trout)
C and A-δ	46.7	38.7	33.3	26.8	47.8	**38.7 (8.9)**	**37**
A-β	40.0	48.4	56.9	57.1	41.3	**48.7 (8.2)**	**53**
A-α	13.3	12.9	9.8	16.1	10.9	**12.6 (2.4)**	**9**

Table 1. Neurite type frequency (in %) of 5 independent nerve sections in a tail of a common carp, following the classification as published for rainbow trout trigeminal nerve (average, Sneddon, 2002). No statistical differences between average frequencies were found among the 5 nerve cross sections analyzed (chi-square test, P = 0.9). Classification of the neurite types is based on diameter (Lynn, 1994). hypoder. = hypodermis, lepido. = lepidotrichia.

2.2 Responses of Nile tilapia (O. niloticus) to a tailfin clip

2.2.1 Fish

Female Nile tilapia, weighing around 200 g, were obtained from a local fish farm (Fishion Aquaculture BV, Mortel, The Netherlands) and after transport to the laboratory acclimatized for 2 weeks to the aquarium facilities of the Radboud University Nijmegen. The fish were kept in 140 L flow-through tanks with 9 fishes per tank; the fish received pellet feed at 2% of the total body weight daily (Trouvit, Trouw, The Netherlands). The water quality was monitored for nitrogenous waste products weekly (NO_2^- = 0.5 mg/L; NO_3^- = 12.5 mg/L; NH_4^+ = 0.5 mg/L; O_2 = 7.0 mg/L). Water pH (7.5 ± 0.2) and water temperature (25 ± 0.2 °C) were continuously monitored; the light regime was 12 h light: 12 h dark. The study was approved beforehand by the Animal Experimental Committee of Lelystad (Protocol: 2008139).

2.2.2 Fin clipping

Fish were caught with a net and restrained manually by one experimentator, while another clipped the caudoventral corner of the tailfin with a sharp, sterile pair of dissection scissors; next, the fish were returned to their original tank. In the control for handling stress treatment, fishes were handled the same way but not given the clip (instead gentle pressure was applied at the area the fin clip was provided to the other group).

2.2.3 Experimental set

Eight groups of 9 fish were used (Table 2). Two control groups were sampled 1 day prior the treatments of the 6 experimental groups. The results of the two control groups were pooled, since no differences were found between

these fish. Clipped and control for handling stress groups were sacrificed at 1, 6 and 24 h after the clip procedure. Fish were not fed 24 h before sampling.

2.2.4 Sampling

The fish were rapidly netted and deeply anaesthetized with 2-phenoxyethanol (1 mL/L; Sigma-Aldrich, St Louis, USA); this procedure took less than 2 min. Blood sampled by puncture of the caudal vessels with a heparinized syringe fitted with a 25 Gauge needle was immediately centrifuged at 4 °C and 13,000 rpm for 10 min to separate plasma and cells; plasma was snap-frozen and stored at −20 °C. Two gill arches were excised and stored in SEI buffer (150 mM sucrose, 10 mM EDTA, 50 mM imidazole; pH 7.4) for later determination of Na^+/K^+-ATPase enzymatic activity or fixed in Bouin's fixative (15 volumes saturated picric acid: 5 volumes formaldehyde: 1 volume glacial acetic acid) for mucus cell and chloride cell histology.

2.2.5 Dark-light preference and swimming activity

Tanks were covered with black plastic to make 50% of the volume dark and 50% illuminated. The preference to reside in the light or dark and general swimming activity of the fish was determined by snapshots through undisturbed camera-viewing of the tanks in the week before the experiment (control) and after administration of the fin clips, prior to sampling. The fish were scored for presence in the dark or light part of the tank. Data are expressed as ratio of fish present in (as a group) in the light *vs.* the dark. A score of 1.0 indicated that the fish were equally divided over the light and dark part of the tank. Control situation was assessed 1 h for 3 days prior the experiment started for every tank. Different time points of the days were chosen to have an overview of the daily position in the tanks. Snapshots were taken every 2 min during this period, as well as for the first hour of the experimental period, and every 15 min from the second hour till the end of the experiment.

2.2.6 Blood plasma

Plasma was analyzed for cortisol as described in detail elsewhere (Metz et al, 2003). Activities of Na^+, K^+, Ca^{2+}, pH, and concentrations of glucose and lactate in plasma were measured using Stat Profile pHOx plus analyzer (Nova Biomedical, Waltham, MA, USA).

2.2.7 Gill histology

Gill samples fixed in Bouin's for 24 h, were dehydrated in a series of alcohols and embedded in paraffin. The samples were cut at 7 µm and sections stained for the presence of mucus cells and chloride cells. Mucus was stained with Alcian blue. The mucus cell density was estimated by counting Alcian blue positive cells in designated representative cross-sections stretching along 400 lamellae of the sampled gill arch. Following stress stimuli, mucus cells expel their content resulting in a decreased frequency of Alcian blue positive cells. Mucus cell frequency was assessed for each fish twice by the same person. Mucus cells are found in this species on both the leading and trailing edge of the gill filament and were scored there to avoid any topological bias. Statistical analysis indicated that mucus cells are evenly distributed over the gill filament in this species (*t*-test for paired samples, $P > 0.05$; data not shown). Data from cell frequencies in the leading edge of the gill filaments are presented in this study.

The chloride cells in the gills were detected through staining of their abundant Na^+/K^+-ATPase by immunohistochemistry with a monoclonal antibody raised against a chicken Na^+/K^+-ATPase alpha-subunit (IgGα5, designed by Dr. Douglas Farmbrough from the Developmental Studies Hybridoma Bank, Department of Biological Sciences University of Iowa, USA). The Na^+/K^+-ATPase α-5 antibody has been used in a number of studies to localize Na^+/K^+-ATPase in fish gills (Dang et al, 2000; Metz et al, 2003). Chloride cells predominate in the trailing edge of the filament (where the water flow exits the gill) and the adjacent interlamellar space of the gill filamental epithelium (van der Heijden et al, 1997) and, therefore, sections of the trailing edge were observed for chloride cell distribution. Under stressful conditions chloride cells may migrate into the lamellar epithelium (Schram et al, 2010); we scored our samples for this migration. Enzymic activity of Na^+/K^+-ATPase activity as a measure of sodium pump capacity of the gills was determined by measuring the K^+-dependent and ouabain-sensitive ATP-hydrolytic activity in a gill homogenate (Metz et al, 2003). As the bulk of the Na^+/K^+-ATPase is restricted to the chloride cells of the gills, a homogenate results in proper reflection of the sodium pump capacity.

2.2.8 Statistics

Data are expressed as means and standard deviation (SD). Data were not normally distributed, and therefore, the non-parametric Kruskal–Wallis test was used throughout to assess statistical significance of differences.

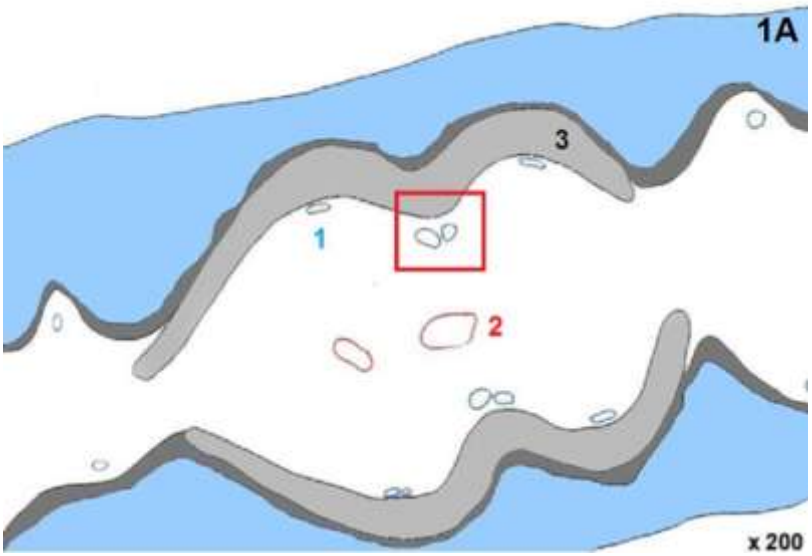
3. Results

3.1 Ultrastructural analysis of common carp (C. carpio) tailfin

In carp tailfin clips, nerve bundles were found, both within the lepidotrichia segment and in the soft tissue (hypodermis) between the fin rays. The nerves were symmetrically distributed (Figs. 1 and 2). Morphometric analyses revealed 4 categories of neurites, 3 types of myelinated A-fibers and 1 type of unmyelinated C-fibers (Fig. 2). Neurites in five nerves were analyzed for diameter to score them as C and A-δ, A-β and A-α type (Table 1). The neurite type distributions in the nerves were tested for homogeneity (chi-square test of homogeneity of proportions, $P > 0.05$).

3.2 Responses of Nile tilapia (O. niloticus) to a tailfin clip

Control fish preferred the darker side of the tank (Fig. 3). Following the tailfin clip, the fish showed increased swimming activity and more random movement through the tank. This response was visible from 1 h post treatment (significantly different that the control) and stronger at 6 h after the clip (significantly different than the control and stress group, Kruskal–Wallis test, $H(6, N = 589)$, $P < 0.01$) and had faded after 24 h. In the handling stress groups, the effect on swimming activity was mild at 1 h after handling and had faded as off 6 h following stress.



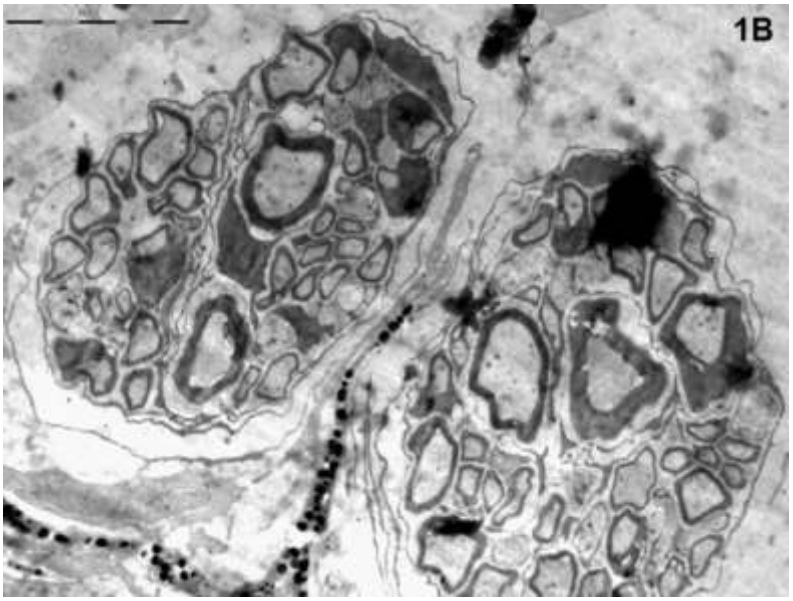


Fig. 1. A. nerves in tailfin of common carp (x 200), the red box is detailed in B, and shows a transverse section of the interior of the lepidotrichia segment of the tail ray showing 2 nerves (EM, Scale bar = 5 μm). 1: nerve bundle, 2: blood vessel, 3: lepidotrichial hemisegment.

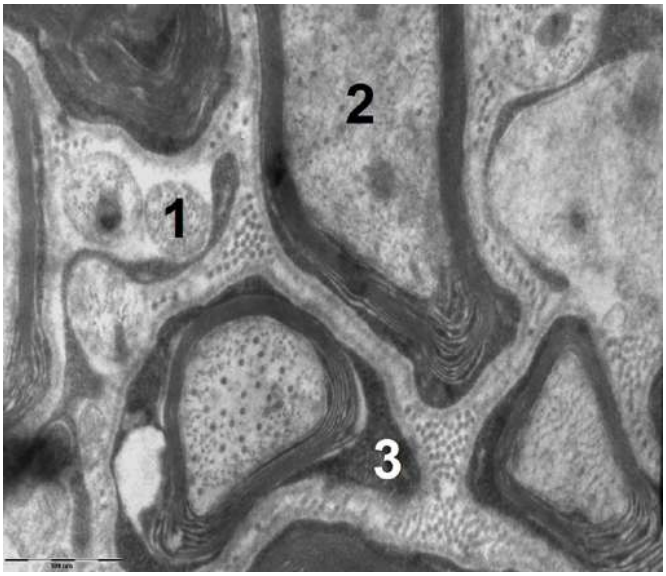


Fig. 2. Nerve fibers in tailfin of common carp (TEM, scale bar = 500 nm). Both C-fibers (1) and 3 categories of A-fibers (2) are present within the nerve. (3) Schwann cell producing the myelin sheets around A-fibers. Black spots in the neurite neuroplasm represent microtubules.

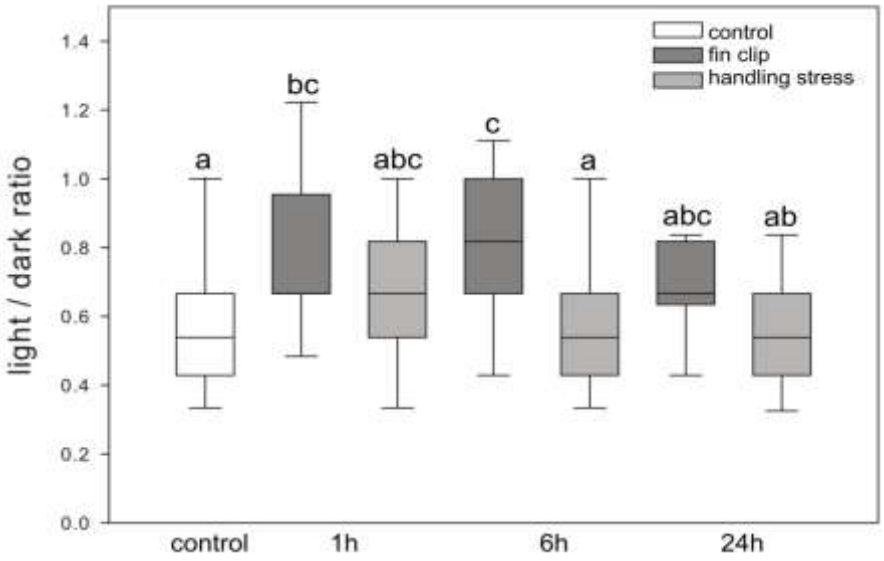


Fig. 3. Dark/light preference of Nile tilapia, in function of treatment. Compared to control, untreated fish, a fin clip induces a larger shift in preference than the handling stress alone. This effect lasts for at least 6 h. Different letters stand for significant differences at P = 0.05 (Post-hoc multiple comparisons after Kruskal–Wallis, H (6, N = 589), P < 0.01).

Group	Cortisol (nM)	Glucose (mM)	Lactate (mM)	pH	Na^+ (mM)	K^+ (mM)	Ca^{2+} (mM)	Osmolality (mOsmol/kg)	Na^+/K^+-ATPase (µmol P_i/h/mg protein)
H (Kruskal-Wallis)	H(6,71)=44.60 P < 0.01	H(6,71)=41.07 P < 0.01	H(6,70)=24.22 P < 0.01	H(6,71)=30.94 P < 0.01	H(6,71)=15.31 P = 0.018	H(6,71)=11.12 P = 0.085	H(6,71)=15.22 P = 0.019	H(6,70)=13.43 P = 0.037	H(6,71)=8.32 P = 0.22
Controls	24.6 (58.0) ^{a}	2.89 (0.75) ^{a}	2.56 (1.40) ^{a}	7.72 (0.11) ^{abc}	161.4 (3.0) ^{a}	3.76 (0.52)	1.14 (0.18) ^{ab}	321.8 (9.5)	7.92 (1.53)
Fin clip 1 h	57.6 (46.9) ^{abc}	4.63 (1.09) ^{b}	2.41 (1.30) ^{a}	7.81 (0.09) ^{ab}	157.9 (1.9) ^{ab}	3.52 (0.30)	1.09 (0.15) ^{ab}	314.3 (5.3)	10.01 (2.83)
Handling stress 1 h	46.4 (50.7) ^{abc}	4.88 (1.85) ^{b}	2.72 (2.42) ^{a}	7.83 (0.06) ^{b}	158.5 (3.4) ^{ab}	3.41 (0.32)	1.10 (0.14) ^{ab}	316.4 (9.9)	8.20 (2.31)
Fin clip 6 h	334.6 (292.2) ^{b}	3.68 (1.67) ^{ab}	1.41 (0.59) ^{ab}	7.68 (0.04) ^{c}	158.1 (5.2) ^{ab}	3.97 (0.48)	1.25 (0.23) ^{a}	314.9 (11.8)	7.55 (1.19)
Handling stress 6 h	256.4 (139.9) ^{b}	3.14 (0.34) ^{ab}	0.65 (0.13) ^{b}	7.72 (0.03) ^{ac}	158.7 (3.7) ^{ab}	3.76 (0.35)	1.22 (0.14) ^{ab}	311.3 (8.1)	7.42 (1.25)
Fin clip 24 h	15.7 (32.0) ^{ac}	2.34 (0.43) ^{a}	1.69 (0.64) ^{ab}	7.81 (0.04) ^{ab}	157.5 (1.7) ^{b}	3.62 (0.33)	0.99 (0.10) ^{b}	312.3 (6.3)	8.14 (1.29)
Handling stress 24 h	111.5 (82.1) ^{bc}	2.49 (0.43) ^{a}	1.39 (0.51) ^{ab}	7.81 (0.03) ^{ab}	157.9 (1.5) ^{ab}	3.47 (0.44)	1.05 (0.12) ^{ab}	312.1 (5.3)	7.44 (1.68)

Table 2. Plasma parameters and branchial Na^+/K^+-ATPase activity of Nile tilapia. Data are expressed as mean and standard deviations (SD). Different letters indicate significant differences at P = 0.05 (Post-hoc multiple comparisons after Kruskal–Wallis).

3.2.1 Stress and plasma analyses

Data on plasma concentrations of cortisol (Kruskal–Wallis test, $H (6, N = 71) = 44.60$, $P < 0.01$) and glucose (Kruskal–Wallis test, $H (6, N = 71) = 41.07$, $P < 0.01$) (Table 2) showed the predictable changes imposed by stress, but these parameters lack the resolution to discriminate between a clip and handling stress. Basal values in the untreated controls are in line with values reported for fish in stress-free conditions (Auperin et al, 1997).

3.2.2 Ionoregulation related parameters

The plasma levels of Na^+, K^+ and Ca^{2+} and the plasma pH are shown in Table 2 (Kruskal–Wallis tests: Na^+: $H (6, N = 71) = 15.31$, $P = 0.018$; K^+: $H (6, N = 71) = 11.12$, $P = 0.085$, Ca^{2+}: $H (6, N = 71) = 15.22$, $P = 0.019$; pH: $(H (6, N = 71) = 30.94$, $P < 0.01$).

The Na^+/K^+-ATPase enzymic activity transiently increased 1 h after the fin clip, although this effect was not statistically significant (Table 2) (Kruskal–Wallis test, $H (6, N = 71) = 8.32$, $P = 0.22$). No differences in Na^+/K^+-ATPase activity were found among the groups tested.

Both clipping and handling stress-induced migration of chloride cells towards lamellar regions. This migration was observed at 6 h post treatment and lasted at least for 24 h (Fig. 4). The cells had migrated to the tips of the lamellae.

3.2.3 Mucus cells

Mucus cells in the control group are observed between the lamella in the filamental epithelium, in the same region where chloride cells are found (Fig. 5A). In response to the tailfin clip, 1 h after the clip (Fig. 5B), the frequency of mucus-containing cells had drastically decreased (Kruskal–Wallis test: $H (6, N = 49) = 15.7$, $P = 0.016$). This we take to indicate stress-induced release of mucus. However, this response was not observed in any of the other groups and thus allows discrimination between handling stress and clipping. At 6 h and 24 h after the clip (Fig. 5C), mucus cells had restored their mucus content to control levels. In the groups at 1 h, 6 h and 24 h following handling stress, no difference in mucus cell frequency was found compared to the controls (Fig. 6).

Fig. 5 summarizes the quantification of mucus cell frequencies in controls and all experimental groups.

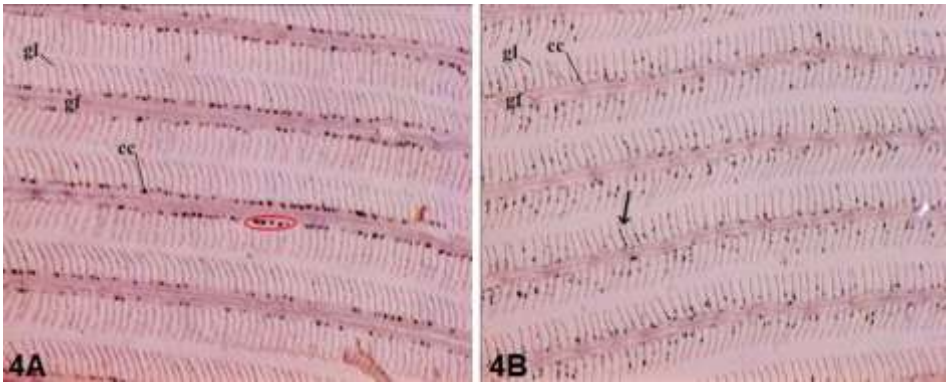


Fig. 4. Chloride cells (cc) of Nile tilapia, seen as dark dots with examples encircled, are situated in the filamental epithelium at the base of the lamellae (gl). Control fish A. In the 6 h and 24 h post treatment groups, chloride cells had migrated towards the apices of the lamella (arrow) B. This phenomenon was observed in both the clipped and handled fish. cc: chloride cell, gf: gill filament, gl: gill lamellae.

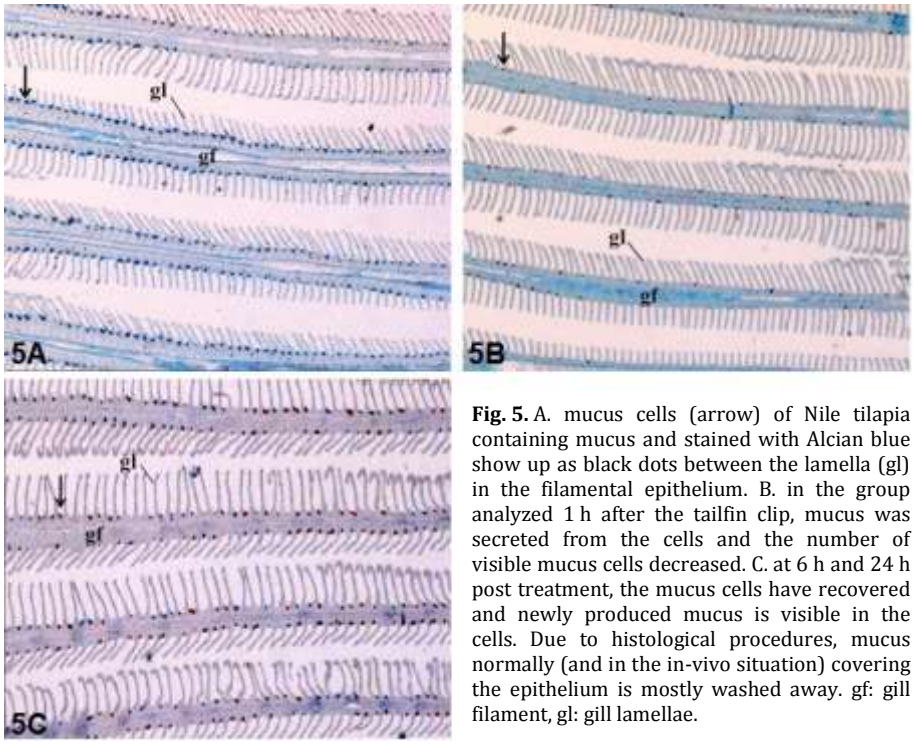


Fig. 5. A. mucus cells (arrow) of Nile tilapia containing mucus and stained with Alcian blue show up as black dots between the lamella (gl) in the filamental epithelium. B. in the group analyzed 1 h after the tailfin clip, mucus was secreted from the cells and the number of visible mucus cells decreased. C. at 6 h and 24 h post treatment, the mucus cells have recovered and newly produced mucus is visible in the cells. Due to histological procedures, mucus normally (and in the in-vivo situation) covering the epithelium is mostly washed away. gf: gill filament, gl: gill lamellae.

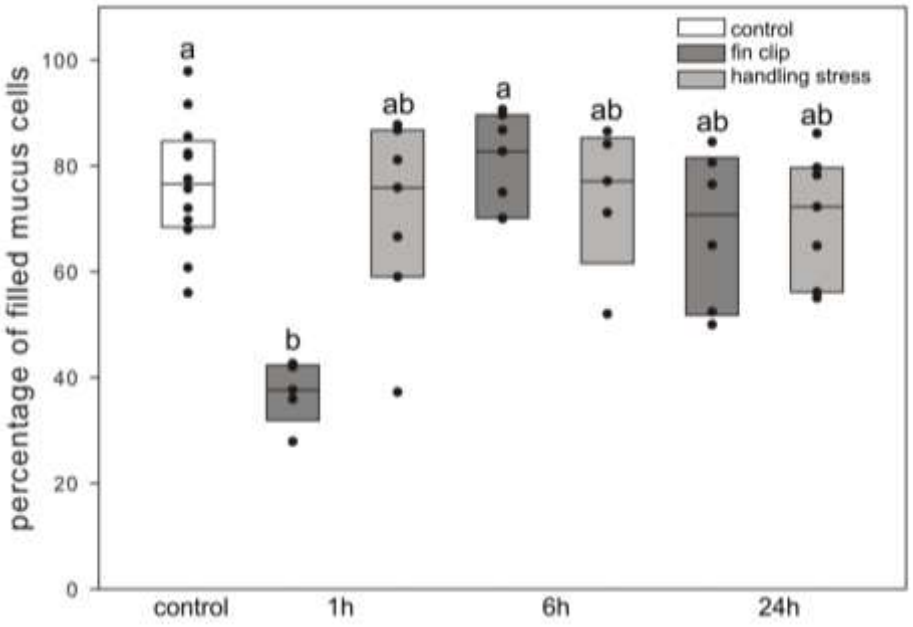


Fig. 6. Quantification of the mucus cells frequency in gills of Nile tilapia, in function of treatment. A significant decrease in mucus-filled mucus cells in the gill filaments in the 1 h after fin clip group. In the accompanying stress group, this decrease was not observed. Different letters stand for significant differences at P = 0.05 (Post-hoc multiple comparisons after Kruskal–Wallis).

4. Discussion

4.1 Ultrastructural analysis of common carp (C. carpio) tailfin

This study investigated acute physiological and behavioral responses of Nile tilapia to a presumed painful stimulus and the stress response inherent to the application of the painful stimulus (*i.e.*, the handling to clip the tailfin). In carp, the nerve in fin clips fulfilled all requirements to be designated as nerves that can carry noxious stimuli. Nervous tissues were observed in similar region of tail of the false mouth-breeder tilapia, *Tilapia melanopleura* (Becerra et al, 1983).

Nerves bundles were found between and within the fin rays. Four different types of neurites were identified in the nerves on the basis of their diameter (Sneddon, 2002; Lynn, 1994). C-fibers and A-δ fibers are involved in pain perception. In mammals, the unmyelinated C-fibers mediate slow dull pain signals and the myelinated A-δ fibers mediate acute pain (Sneddon, 2002; Erlanger and Gasser, 1937; Lynn, 1994). The presence of these 2 types of fibers in the clipped tissue, combined with the behavioral and physiological

parameters, support strongly that Nile tilapia discriminate nociceptive stimuli from handling stress, a conclusion in accordance with recent literature (Munro and Dodd, 1983; Braithwaite and Huntingford, 2004; Chandroo et al, 2004; Sneddon, 2003; Huntingford et al, 2006; Reilly et al, 2008a; 2008b).

The presence of nerves with remarkably similar neurites as seen in mammalian (and trout) nerves that carry noxious signals, makes the fin clip an easily applied stimulus to study acute pain responses in fish.

The transient character of the response to the handling stress *per se* and the clipping indicates that full recovery from this invasive procedure takes at least 6 h.

The relative abundances of C-fibers and A-δ fibers among the neurites we scored in cross-sectioned nerves are similar to those reported for the trigeminal nerve of rainbow trout (Sneddon, 2002). In trout, the low 4% C-fibers clearly contrasts with the percentage in terrestrial vertebrates where this type of neurite may represent 50% (Young, 1977) This low C-fibers percentage was also found in our study where it was estimated to ± 5% of the total amount of fibers.

The presence of C-type fibers in fish provides a further substrate for the discussion on pain perception in these animals. The 6 h duration of behavioral response in our tests suggests that signals comparable to those transducing lasting dull pain in mammals are carried by C-fibers (Sneddon, 2002; Lynn, 1994).

The presence of nerves in the carp tailfin with characteristics of pain nerves found in trout and mammals warrants similar analyses in other species of fish including, of course, Nile tilapia. We will analyze tissues at vulnerable sites such as the fins, opercula, mouth and lips and skin for neurites and through immunohistochemistry check whether these neurites penetrate the skin epithelium as seen in mammals (Oaklander, 2001).

4.2 Responses of Nile tilapia (O. niloticus) to a tailfin clip

Nile tilapia that receives a fin clip show more swimming activity and less preference for the darker part of the tank compared to controls. This response was found both 1 h and 6 h after the fin clip and indicates that the presumed harmful clip experience affects behavior and is remembered for several hours. Gill Na^+/K^+-ATPase activity, the enzymic correlate of the sodium pump, increased transiently in the fish that received the fin clip. This mild effect was only seen in fish 1 h after the fin clip. We speculate that the clip is a painful stimulus and resulted in a stronger adrenergic response, which evoked a temporary increased epithelial permeability to water and ions. An enhanced

sodium pump activity could counteract the imminent threat of ion leakage. This assumption is corroborated by the constant plasma ion levels observed.

A similar transient response was seen in the branchial mucus cells that secreted their content 1 h after a clip, an effect not seen after the handling stress only, and this discriminates again the clipping procedure from the handling and suggests that clipping could impose pain. The mucus secretion would reflect than a stronger adrenergic response induced by the fin clip. There is a neurological substrate for this reasoning, as we found nerves in the clips that fulfill all criteria for nerves that can transmit noxious, potentially painful, stimuli.

The fish that received the fin clip increased their swimming activity for at least 6 h and this was not observed in handled groups. In an earlier study, it was shown that rainbow trout enhanced their ventilation behavior as well as delayed time to resume feeding for several hours, following a noxious stimulus, as we observed here. Clearly, behavioral studies are instrumental to study pain perception in fish (Sneddon, 2003; Sneddon et al, 2003a).

The plasma cortisol level increased in response to handling and clipping, but did not differ between the two conditions. Basal plasma cortisol levels in our fish were in the range considered normal (27.6–55.2 nM; Wendelaar Bonga, 1997). The handling and clipping increased cortisol levels up to 334.6 (292.2) nM and 256.4 (139.9) nM 6 h post treatment for the fin clipped and handling controls, respectively. Increases up to 165.6 nM (60 ng/mL) are generally referred to as a mild response, while rapid increases above 276 nM (100 ng/mL) are generally considered to reflect a severe stress response (Wendelaar Bonga, 1997). When fish experience chronic stress, plasma cortisol level should remain elevated compared to controls (Wendelaar Bonga, 1997), but in our fish cortisol levels returned to control values by 24 h, which indicates that the fish recovered from the procedures. A significant inter-individual variation, as indicated by large standard deviations, was observed at 6 h following the clipping or handling. Four fish had cortisol levels above 276 nM, interpreted as severe stress, two individuals had values that go with mild stress and three had cortisol levels comparable to controls. This suggests either a strong individual subjective element, or individual variation related to differences to neuroendocrine responses. So called proactive fish may show a flight–fight response by high activation of the brain–sympathetic–chromaffin cell axis, while reactive fish may show a freeze-hide response that is characterized by an activation of the hypothalamo–pituitary–interrenal axis (Henry and Stephens, 1977).

In the same species as used here, acute intense light was reported to induce cortisol to rise from less than 100 nM to over 500 nM; after 8 h cortisol levels had returned to basal (Biswas et al, 2004). Copper exposure may induce even higher cortisol levels (over 600 nM; Monteiro et al, 2005). Such results indicate that fin clipping represented a relatively mild stress when evaluated by cortisol response. The individual variation in basal levels and in cortisol responses confound these parameters as suitable indicator of pain; only if multiple samples of the same individual are collected one could possibly assess differences in sensitivity towards painful stimuli or differential response to handling and clipping. Clearly, the behavioral response has more potential to make such discrimination.

Plasma glucose and lactate levels followed the changes observed in cortisol levels, with mildly increased glucose levels compared to the controls, but no differences between the pain and stress groups. Monteiro and colleagues (2005) reported glucose levels between 1.32 and 3.03 mM in control and copper-exposed Nile tilapia, values in line with those measured in the present study for control and stressed fish. Clearly this parameter is suited to indicate stress but lacks the resolution to discriminate between handling and clipping. Lactate levels had slightly decreased 6 and 24 h post treatment, and no difference between the stress and pain groups was observed (Kruskal–Wallis tests: $H(6, N = 70) = 24.22$, $P < 0.01$). The lower levels of lactate do not seem to correlate with enhanced swimming activity induced by the handling stress or clipping procedure, for which we have no explanation.

Concentrations of Na^+, K^+ and Ca^{2+} as well as plasma osmolality were essentially unchanged after the fin clip and handling. This supports the relative mildness of the stressor applied and indicates no major loss of control over permeability to water and ions, as is often seen in severely stressed fish, due to catecholamine-induced epithelial lifting and dysfunction of the gills (Wendelaar Bonga, 1997).

The chloride cells harbor the majority of the in Na^+/K^+-ATPase activity in the gills. In response to the pain and stress treatment, increased migration of the cells from the filaments towards the lamella was observed. This phenomenon occurred in the 6 and 24 h post-treatment groups, whereas at 1 h post treatment, migration was not yet visible. The time kinetics of this response makes it a parameter of choice in many settings, a notion that needs and deserves further attention in our welfare research.

We did not assay catecholamine levels, so we can only assume that the fin clip evoked an adrenergic response that may increase the branchial permeability to water and ions (Wendelaar Bonga, 1997). The rather constant plasma ion levels do not support this prediction. A rapid transient rise in

Na^+/K^+-ATPase activity as observed in the most severely stressed fish (those receiving the fin clip) could contribute to counteract an imminent loss of ions.

The increase in activity and the migration of the chloride cells are a combined adaptive osmoregulatory response to the fin clip and the likely endocrine changes occurring in the fish. The migration of chloride cells is secondary to that in time and suggests an alternative adaptive strategy. The phenomenon of migrating chloride cells from the filaments to the lamellae is a well described adaptation strategy of euryhaline fish in the transition of salt to brackish water (Hirai et al, 1999). In our fish it seems unlikely though that new cells contribute significantly to the migration, rather a redistribution of cells seems to occur. More research is needed to investigate the combined response of the activity of the enzyme and the migration of the chloride cells in response to a fin clip.

The group clipped 1 h previously an increased mucous secretion was observed compared to the controls. In the group clipped 6 h previously, the cells had recovered and were re-filled with mucus, suggesting the observed effect is an acute reaction to the fin clip to increase the protective mucus layer on the gills. The accompanying stress response had no effect on the mucus cells in the gills.

Mucus is produced in the goblet cells produce mucine granules. When these cells come into contact with the water they burst at the cell surface and subsequently the mucus is released (Verdugo, 1991). Mucus has a very high water content captured by glycosaminoglycans and glycoproteins (Fletcher et al, 1976). In addition, mucus contains substances, such as lysozyme, IgM's, calmodulin and pheromones (reviewed in Shephard, 1994). Mucus serves an array of functions in fish (and all other animals). On the gills, it forms an extra unstirred layer and influences ion and water movements and gas exchange and imposes an immune barrier for pathogens. Further mucus provides protection against chemical and physical disturbances (Shephard, 1994).

The multidisciplinary gills and the protective function of mucus highlights the importance of further studies into the differential responses of the mucus cells in the gill filaments to the fin clip and the stress response. Several aspects of mucus biology in relation to the pain response can be studied. Finding the trigger for the differential mucus release seems an intriguing task, analyzing the composition of mucus and the possibility of different types of mucus with subsequent different release triggers and receptors can be investigated. The excretion profile of mucus after a shorter time period then 1 h after a fin clip and the mucus on the tail section that received the fin clip deserve attention.

5. General conclusions

This experiment aimed to confirm involvement of pre-selected parameters in the response to a presumed pain stimulus in the form of a fin clip and to select key parameters for future studies into this field of research. In addition, the study aimed to confirm differential responses to the fin clip compared to the accompanied stress response. A wealth of new insights was obtained with great promise for the near future of our welfare research in fishes.

The response that was found for several parameters and the presence of the nerve bundles show that the fin clip stimulus was rightly predicted to be painful. The differential response to the fin clip and the handling stress shows that the fish experience different degrees of discomfort.

Several promising parameters have now been tested and selected for future research. However, pain may also be studied in future experiments by measuring substance-P, endorphins, EEG-measurements or some combination thereof.

The results confirm a differential response of the fish to the fin clip and the stress treatment for the behavioral response, enzymic osmoregulatory activity and the mucus cell response and these will be the focus for future experiments.

Acknowledgements

We thank Tom Spanings (Radboud University Nijmegen) for the fish husbandry and invaluable assistance during the experiments.

This research project has been carried out within the Knowledge Base Research / Policy Supporting Research / Statutory Research Task for the Dutch Ministry of Agriculture, Nature and Food Quality. Project BAS-project KB08 009001ASG.

Chapter 3

Physiological and behavioral responses to an electrical stimulus in Mozambique tilapia (*Oreochromis mossambicus*)

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In: Fish Physiology and Biochemistry (2012) 38:1019-1028

Abstract

Consumer awareness of the need to improve fish welfare is increasing. Electrostunning is a clean and potentially efficient procedure more and more used to provoke loss of consciousness prior to killing or slaughtering (reviewed by van de Vis et al, 2003). Little is known how (powerful) electrical stimuli, which do not stun immediately, are perceived by fish.

We investigated responses of hand-held Mozambique tilapia (*Oreochromis mossambicus*) to a standardized electric shock applied to the tailfin. The handling with the resulting unavoidable acute stress response was carefully controlled for. Fish responses were analyzed up to 24 h following the shock. Electric shock resulted in slightly higher levels in plasma cortisol, lactate, ionic levels, and osmolality, than handling alone. Plasma glucose had significantly increased 6 h after shock compared to handling, indicative of enhanced adrenergic activity. Mucus release from the gills, branchial Na^+/K^+ ATPase activity, and chloride cell migration and proliferation, parameters that will change with strong adrenergic activation, were not affected.

Decreased swimming activity and delay in resumption of chafing behavior indicated a stronger and differential response towards the electric shock. Responses to handling lasted shorter compared to those to an electric shock. The differential and stronger responses to the electric shock suggest that fish perceived the shock potentially as painful.

1. Introduction

The international association for the study of pain (IASP) defines pain as an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage (IASP, 1979). The questions of pain, pain awareness, fear and stress in fish are still subject of controversies. In humans, these processes depend on functions controlled and executed by the highly developed hippocampus, amygdala, and cerebral frontal lobes of the neocortex (Apkarian et al, 2005). The absence of identical/comparable structures in teleostean fish has led some researchers to conclude that fish cannot experience pain, fear or stress (Bermond 1997; Rose, 2002). Recently, homologies between the telencephalic medial pallium of the teleosts and the amygdala of mammals as well as between the teleostean lateral pallium and the mammalian hippocampus have been identified (Portavella et al, 2002). This suggests that parts of the fish telencephalon could function to interpret processes related to pain, pain awareness and fear, as do their homologues in mammals. Differences in development and organization of fish brain, in particular the eversion of the telencephalon *vs.* inversion in mammals, has contributed significantly to a late recognition of a neural substrate for fish cognitive abilities, and assigning consciousness to fish which is at the basis of pain and fear experiences in mammals.

Reviews by Braithwaite and Huntingford (2004) and Chandroo and coworkers (2004) present evidence that fish, despite their less developed telencephalon, have learning abilities at a level that implies cognitive abilities. For selected species (rainbow trout, *Oncorhynchus mykiss*; Atlantic cod, *Gadus morhua*; goldfish, *Carassius auratus;* Atlantic salmon, *Salmo salar*) evidence has been advanced that fish do have the capacity to perceive painful stimuli and the adequate nociceptive fibers for the detection of potentially painful stimuli (Sneddon, 2002; Nilsson et al, 2002; Nordgreen, 2009); we have recently shown that tailfin clipping may be a painful experience in Nile tilapia, *Oreochromis niloticus* and common carp, *Cyprinus carpio* (Roques et al, 2010). However, it should be emphasized that it is unlikely that fish, as well as animals in general, except maybe higher primates, have the capacity to experience suffering as humans do (Braithwaite and Huntingford, 2004). Nociception, the detection of potentially harmful stimuli, is at the very basis of experiencing pain, *i.e.* interpreting a nociceptive stimulus. Two types of nerve fibers are involved in the process of nociception: the myelinated A-fibers are involved in the transmission of well-localized acute pain, while unmyelinated C-fibers (simply isolated by glia) are involved in poorly-localized unpleasant slow dull pain (Sneddon, 2002; Pottinger et al, 1997; Lynn, 1994). Sneddon (2002) identified these two types of fibers in the head the rainbow trout. More recently, Roques

and coworkers identified these fibers in the tail of common carp, where the stimulus of the current study was given (Roques et al, 2010).

A pain experience by definition involves both the nociceptive sensory machinery and the actual translation of harmful stimuli into a feeling of pain. Fish should possess then both a nociceptive system and cognitive capacities to experience pain in analogy to humans. Indeed, a limited, yet firm, literature supports that fish do detect harmful stimuli, respond to nociceptive stimuli but also may conceptualize pain (Braithwaite and Huntingford, 2004; Chandroo et al, 2004; Sneddon, 2002; Sneddon, 2003; Sneddon et al, 2003a; 2003b; Roques et al, 2010). Nilsson and coworkers (2002) demonstrated explicit memory in Atlantic cod. Other examples of learning abilities include individual positioning in a social network, prey-predator relationship, avoidance of dangerous sites associated with negative experience and decision-making based on outcomes of fights with conspecifics (Reviewed by Galhardo and Oliveira, 2009). Therefore, it is reasonable to hypothesize that fish have a neural substrate for some form of consciousness and may also experience pain. As fish learn to avoid painful conditions there must be a memory for such adverse events.

The aim of the present study was to assess the behavioral, physiological and endocrine responses of Mozambique tilapia (*Oreochromis mossambicus*) to a presumed and standardized pain stimulus (electrical shock applied to the tail).

Swimming activity (number of crossings from dark to light sections of an aquarium) was monitored under the hypothesis that a stressor alters light/dark preference (Maximino et al, 2010). The delay of resuming the stereotypical chafing behavior was monitored following an electrical shock given to the tail fin; the handling associated with the shock treatment was controlled for. When chafing, fish shoot downwards to the bottom, lay themselves on their flank, and chafe over the substrate, for up to 10 times before rising again and resuming their previous position (Galhardo et al, 2008). Stress-related plasma parameters together with parameters for osmoregulatory performance and branchial release of mucus were analyzed. This study was designed to discriminate the response to the application of an electric shock to the tailfin (a presumed painful stimulus), from handling stress.

Stress is a well-known confounder in pain research as the application of painful stimuli often goes with handling and induces a stress response that may obscure the response proper to, in this case, the electric shock. It may be difficult to distinguish between a stress response and a mild pain response as these responses are part of the fish's stress physiology. Therefore, we included for every group that received the electric shock a control for handling stress.

2. Materials and Methods

2.1 Fish

Mozambique tilapia (*Oreochromis mossambicus*), weighing around 120 g, were obtained from laboratory stock. Two weeks before the start of the experiment, fish were randomly divided into 7 groups, housed in 140-L aquaria with 10 fish each; the fish received pellet feed at 2% of the total body weight daily (Trouvit, Trouw, The Netherlands). The water quality was monitored for nitrogenous waste products daily (NO_2^- < 0.5 mg/L; NO_3^- < 12.5 mg/L; NH_4^+ < 0.5 mg/L; O_2 > 7.0 mg/L). Water pH (range: 7.3 - 7.7) and water temperature (25 ± 0.2°C) were continuously monitored; the light regime was 12h light: 12h dark. The study was approved by the Animal Experimental Committee at Lelystad, the Netherlands (Protocol: 2009143.c).

2.2 Electrical shock

Individual fish were caught by net and restrained in a V-shaped box covered with a wet towel to immobilize it. The electrodes were placed at a caudoventral corner of the tailfin (Fig. 1). Chervova (1997) concluded that caudal fins are among the most sensitive zones for damage, due to aggressive behavior, in White Sea cod (*Gadus morhua marisalbi*) and steelhead salmon (*Salmo mykiss*). Fin damage is frequently observed in the wild as well as in aquaculture practices, with sorting and transport activities as major causes. Furthermore, A-δ and C-fibers, involved in nociception were demonstrated in this fin area of common carp, *Cyprinus carpio* (Roques et al, 2010). Electricity (15 Volts dc, 64 ± 34 mA) was applied for 1 sec to the tailfin, and subsequently the fish were immediately returned to their tank. Control for handling stress fish were handled the same way except that the electric shock was omitted (only the gentle pressure of the electrode application to the fin was given).

Seven groups of 10 fish were used (Table 1), including 1 (untreated) control group that was sampled for plasma analyses the day before the six experimental groups. Fish receiving an electric shock and controls were sacrificed 1, 6 and 24 h after the shock or handling stress was given. Fish were not fed as of 24 h before sampling.

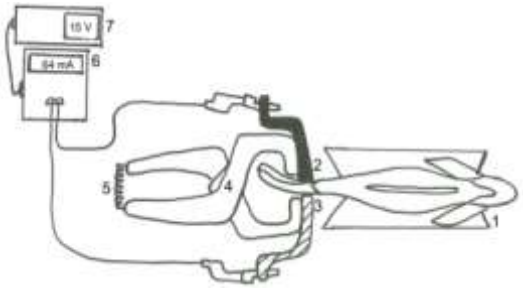

Fig. 1. Scheme of the electrical system used to provide the standardized electroshock. (1) V-shaped box covered with a wet towel to avoid desiccation. Fish were gently and manually restrained, (2) anode, (3) cathode, (4) pliers, (5) spring, adjusted to ensure standard stimulus, (6) stimulator with digital indication of the current delivered, (7) electronic integrator with fixed value of voltage delivered (15 V per pulse).

2.3 Sampling

The fish were quickly (within 20 sec) netted and deeply anaesthetized with 2-phenoxyethanol (0.1% v/v in water; Sigma-Aldrich, St Louis, USA); the fish lost equilibrium within 30 sec and got deeply anaesthetized within 2 min. Blood samples obtained by puncture of the caudal vessels with a heparinized syringe fitted with a 25 Gauge needle were immediately centrifuged at 4°C and 10,000 g for 10 min to separate plasma and cells; plasma was snap-frozen and stored at -20°C.

Two gill arches were excised and stored in SEI buffer (150 mM sucrose, 10 mM EDTA, 50 mM imidazole; pH:7.4) for later determination of Na^+/K^+-ATPase enzymic activity or fixed in Bouin's (15 volumes saturated picric acid : 5 volumes formaldehyde : 1 volume glacial acetic acid) for mucus cell and chloride cell (immuno-)histochemistry.

2.4 Behavior

Tanks were covered with black non-transparent plastic to make 50% of the volume of the tank dark and 50% illuminated. Behavior was recorded with a Samsung SHR-2040 4-Channel DVR Security System recorder linked with Sanyo's bullet video cameras. Activity was monitored continuously from 1 h prior stimulus to 6 h post stimulus, for 1.5 h prior the lights were switched off (9 to 10.5 h post stimulus) and for 1 h on the next morning (23 h post-stimulus). The number of moves from one compartment to the other was registered. Transition from one to the other compartment was scored when the whole head of the animal crossed the border between the two compartments. Controls were analyzed similarly over a period of 5 days prior the stimulus application. Results are presented as averages of four periods of 15 min per h.

We further investigated the delay to resume chafing behavior which is prominent in tilapia. This may occur as a single act or in bouts of 10 or more within only a few seconds (Wyman and Walters-Wyman, 1985).

2.5 Blood plasma

Plasma was analyzed for cortisol as described in detail before (Metz et al, 2005). Plasma glucose and lactate were measured with commercially available enzymatic test kits (Instruchemie, Delfzijl, The Netherlands), with protocols adapted to a 96-well microtiter plate. For glucose, 10 µL sample or standard (5.55 mM glucose) was mixed with 200 µL reagent and incubated for 10 min at 25°C. Absorbance was read within 60 min at 495 nm. For lactate, 10 µL sample or standard (4.44 mM lactate) or blank (8% perchloric acid) was mixed with 290 µL of lactate reagent and incubated for 20 min at 37 °C. Absorbance was read at 355 nm. Plasma osmolality (sample volumes: 50 µL) was measured with a cryoscopic osmometer (Osmomat 030, Gonotec, Germany). Deionized water (0 mOsmol/kg) and a standard solution (300 mOsmol/kg) were used as reference.

2.6 Gill histology

Gill samples fixed in Bouin's were dehydrated in a series of alcohols and embedded in paraffin. The samples were cut at 7 µm and sections stained for the presence of mucus cells and chloride cells. Mucus was stained with Alcian blue. The mucus cell density was estimated by counting Alcian blue positive cells in designated representative cross-sections stretching along 300 lamellae of the sampled gill arch (leading edge). Following noxious stimuli, mucus cells may expel their content resulting in a decreased frequency of Alcian blue positive cells. Mucus cell frequency was assessed for each fish twice by the same person. Mucus cells are found in this species on both the leading and trailing edge of the gill filament and were scored on both locations to avoid topological bias. Statistical analysis indicated that mucus cells are evenly distributed over the gill filament in this species ($P < 0.05$; data not shown). Data from cell frequencies in the leading edge of the gill filaments are presented.

The chloride cells in the gills were detected through staining of their abundant Na^+/K^+-ATPase by immunohistochemistry with a monoclonal antibody raised against chicken Na^+/K^+-ATPase alpha-subunit (IgGα5, a generous gift of Dr. D. Farmbrough, Developmental Studies Hybridoma Bank, Department of Biological Sciences University of Iowa, USA). The Na^+/K^+-ATPase α-5 antibody has been used in a number of studies to localize Na^+/K^+-ATPase in fish gills including in tilapia species (Dang et al, 2000; Metz et al, 2003). In tilapia, chloride cells predominate on the trailing edge of the filament (where

the water flow exits the gill) and in the adjacent interlamellar space of the filamental epithelium (Van der Heijden et al, 1997). Sections of the trailing edge were scored for chloride cell incidence. Under stressful conditions chloride cells may migrate from filamental to lamellar epithelium (Roques et al, 2010; Schram et al, 2010); we scored our samples for this migration. Enzymic activity of Na^+/K^+-ATPase activity as a measure of sodium pump capacity of the gills was determined by measuring the K^+-dependent and ouabain-sensitive ATP-hydrolytic activity in a gill homogenate (Metz et al, 2003). As the bulk of the Na^+/K^+-ATPase is restricted to the chloride cells of the gills, a homogenate results in proper reflection of the sodium pump capacity of the chloride cell compartment.

2.7 Statistics

Physiological data are expressed as means plus or minus standard deviation (SD) (Table 1). When data were normally distributed (Kolgomorov-Smirnov test) and equality of variances verified (Levene's test), differences between groups were analyzed using one-way ANOVA followed by Bonferroni's post-hoc test. When the conditions of validity were not met, the non-parametric Kruskal-Wallis ANOVA followed by the multiple comparison Wilcoxon rank-sum test was used to assess statistical significance of differences. Even mucus distribution over the gill filament was tests with a *t*-test for paired samples. Statistical differences for behavior data were assessed by the non-parametric Mann-Whitney *U*-test.

3. Results

3.1 Swimming activity

In control situations, the fish were very active in the early morning (7:00 until 11:00). Then activity declined gradually from 11:00 until the middle of the afternoon (16:00). There is an apparent revival in activity in the last 1.5 h prior to lights off (*i.e.* from 18:00-19:30). For both the handled-only group and the shocked group a significant decrease in activity during the first 2 h post-stimulus. Three hours post-stimulus the activity is still significantly lower for the group that received the electrical stimulus. Recovery seems to occur after 4 h in this group, while it is achieved after 3 h for the handling-only group (Fig. 2A and 2B).

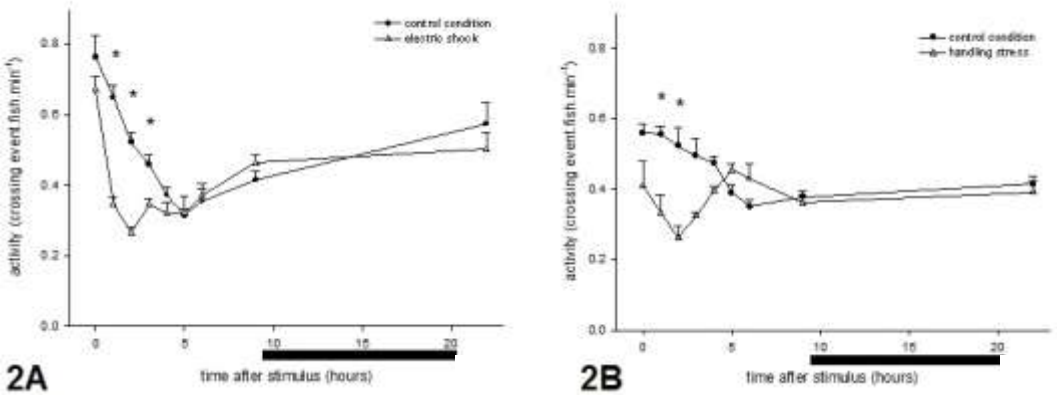

Fig 2. Quantification of the general swimming (crossing) activity in Mozambique tilapia following several treatments; 2A: electric shock *vs.* its control; 2B: handling stress *vs.* its control situation; analysis for a period of 24 h. Data are presented in number of crossing events per fish and per min per periods of 1 h, (10 fish per tank). Controls were analyzed similarly over a period of 5 days prior to the stimulus application. Results are presented as averages of four periods of 15 min per h, and S.E.M. Tanks are divided into two distinct zones (covered *vs*. uncovered). Fish were considered to cross when their entire head was in the other compartment.

3.2 Chafing behavior

In controls chafing was seen for all fish from 8:00 until 13:00, regardless of the sex or social status of the individuals. From 13:00 to 16:00 the incidence of this behavior declined, and it was mainly performed by dominant individuals. Performance incidence of this behavior gradual increased from 16:00 until lights were off (19:30). Both shocked and handled groups totally stopped displaying chafing behavior after the stimulus or the handling. The delay to resume chafing was 1h 55 min for the handled-only group, and 2h 10 min (15 min later) for the group receiving the electroshock.

3.3 Stress and plasma analyses

Data on plasma concentrations of cortisol ($H (6, N=68) = 39.92$, $P < 0.01$) and glucose ($H (6, N=68) = 37.80$, $P < 0.01$) (Table 1) showed the predicted changes resulting from stress. No significant differences between a shock and handling-only stress at 1 h after manipulation were observed. After 6 h, cortisol and glucose levels of the shocked group remained elevated compared to controls; only for glucose there was a significant difference between shocked and handled-only fish. Lactate levels ($H (6, N=61) = 25.02$, $P < 0.01$) remained constant in both groups 1 h after treatment before decreasing, significantly for the shocked group, at 6 h. Levels in both groups are back to control after 24 h.

Group	Cortisol (nM)	Glucose (mM)	Lactate (mM)	Na^+ (mM)	Cl^- (mM)	Osmolality (mOsmol/kg)	Na^+/K^+-ATPase activity (µmol P_i/h per mg protein)
H (Kruskal-Wallis):	H(6, 68)=39.92	H(6, 68)=37.80	H(6, 61)=25.02	H(6,45)=13.65)	(Bonferroni)	H(6, 68)=35.80	H(6, 58)=15.51
Significance	P < 0.01	P < 0.01	P < 0.01	P = 0.034	P = 0.91	P < 0.01	P = 0.017
Control	33.9 (29.5) ^{a}	2.08 (0.63) ^{a}	2.92 (1.01) ^{ab}	156.6 (6.5) ^{ab}	192.7 (22.8)	0.338 (0.007) ^{a}	1.73 (0.48)
Electroshock 1 h	220.1 (112.1) ^{b}	4.03 (1.33) ^{b}	3.15 (1.02) ^{a}	153.3 (3.3) ^{ab}	180.0 (21.3)	0.321 (0.006) ^{bc}	2.00 (1.25)
Handling stress 1 h	210.2 (127.4) ^{b}	3.54 (1.66) ^{ab}	2.71 (0.77) ^{ab}	156.1 (6.3) ^{a}	173.8 (21.2)	0.325 (0.010) ^{ab}	2.13 (1.08)
Electroshock 6 h	171.5 (88.4) ^{b}	4.28 (1.03) ^{b}	1.48 (0.85) ^{b}	147.3 (4.2) ^{b}	168.6 (31.5)	0.310 (0.005) ^{c}	3.01 (0.93)
Handling stress 6 h	106.1 (78.4) ^{ab}	2.12 (0.56) ^{a}	1.64 (0.68) ^{ab}	151.2 (6.1) ^{ab}	172.4 (25.5)	0.318 (0.006) ^{bc}	3.13 (1.16)
Electroshock 24 h	51.4 (51.4) ^{ab}	2.24 (1.14) ^{a}	1.65 (1.03) ^{ab}	153.2 (6.1) ^{ab}	191.6 (23.3)	0.320 (0.004) ^{bc}	2.84 (1.56)
Handling stress 24 h	22.5 (23.8) ^{a}	1.89 (0.43) ^{a}	3.05 (0.63) ^{a}	150.6 (4.9) ^{ab}	172.7 (24.8)	0.322 (0.006) ^{ab}	2.87 (0.74)

Table 1. Plasma parameters, branchial Na^+/K^+-ATPase activity of Mozambique tilapia. Data are expressed as mean and standard deviations (SD). Different letters indicate significant differences at P = 0.05 (Post-hoc multiple comparisons after Kruskal-Wallis, when the data were not normally distributed, one-way ANOVA followed by Bonferroni post-hoc test when the data were normally distributed).

3.4 Ionoregulation related parameters

The plasma levels of Na^+ and Cl^- are shown in table 1. No significant differences in both plasma ionic concentrations were found (Na^+: H (6, N=45) = 13.65, P = 0.034 ; Cl^-, P = 0.91). No differences in Na^+/K^+-ATPase activity were found among the groups tested (H (6, N=58) = 15.51, P = 0.017). Plasma osmolality (H (6, N=68) = 35.80, P < 0.01) significantly decreased from 1 h post stimulus in the shocked group, recovery was not observed after 24 h. In the handled group, a significant decrease was observed only after 6 h, and full recovery was seen after 24 h.

3.5 Mucus cells and chloride cells migration

Mucus cells in the control group are observed between the lamella in the filamental epithelium, in the same region where chloride cells are found (Roques et al, 2010). In all the groups, regardless of the treatment and time point, no difference in mucus cell frequency was found compared to the controls. No migration of the chloride cells was observed during the experiment.

4. Discussion

4.1 Swimming activity

The fish that received the electric shock significantly decreased their general swimming activity for at least 3 h. Fish only handled showed decreased activity (compared to controls) for 2 h; fish that received an electroshock showed decreased activity for up to 4 h, when compared to their controls. This difference between the two treatments indicates that the combination of handling and the electric shock has a stronger effect than the handling procedure alone. The response differed from that observed in Nile tilapia, *Oreochromis niloticus*, subjected to fin clip. In the latter study the activity was enhanced after the fin clip for at least 6 h (Roques et al, 2010). This may be related to the different type of stimuli used. For the fin clip, the harmful stimulus was accompanied by tissue damage. The clip was speculated to result in a strong adrenergic response, that will affect both behavior and branchial mucus cell release shortly following the stimulus in Nile tilapia (Roques et al, 2010).

The caudal fin is an easy target and therefore often subjected to attacks, both in the wild and in husbandry conditions, resulting in conditions comparable to the fin clip procedure. In such case, the animal may have the tendency to flee from the place it was hurt (Wendelaar Bonga, 1997). This

would explain the enhanced activity after receiving the clip, a stimulus with physical damage. The electric shock is a novel experience for the fish and did not provoke physical damage under the conditions applied. The apparent quietness in the first hours following the electroshock in Mozambique tilapia, *Oreochromis mossambicus*, likely reflects the different nature of the stimulus compared to the fin clip, but may also be a species-specific response. The faster recovery in the handled group compared to the shocked group is an indicator that the stimulus was perceived as noxious, potentially painful. Nevertheless, the recovery seems faster in the current study (compared with the fin clip), suggesting that the electrical stimulus was potentially perceived as less noxious than the fin clip.

4.2 Chafing behavior

Mozambique tilapia that were subjected to either an electric stimulus or only handling completely stopped to display chafing behavior for almost up to 2 h. Fish that received the electric shock seemed to recover slower (by 15 min) than the handled-only group.

Chafing has been widely observed among numerous families of teleost fish, including Cichlidae and Mozambique tilapia (Oppenheimer and Barlow, 1968; Barlow and Green, 1970; Wyman and Walters-Wyman, 1985). It is considered as a maintenance behavior, with the primary goal to remove parasites or particles from the body surface of the fish (Galhardo et al, 2008; Wyman and Walters-Wyman, 1985). In case of fish raised in captivity, with poor environmental conditions it was speculated that chafing may reflect a redirected behavior when the natural environment is unavailable, or in response to an adverse context (Galhardo et al, 2008; Wyman and Walters-Wyman, 1985).

Galhardo and colleagues (2008) observed that chafing is more important in Mozambique tilapia when substrate is not provided in comparison which substrate-enriched tanks. Furthermore, she speculated that this behavior might serve as a coping mechanism, revealing conflict, frustration or disturbance due to the presence in an unfavorable environment. In the present study, we can stipulate that fish in the control condition can be considered in a state of frustration as she described above, due to the relatively poor enrichment of the environment (standard laboratory conditions: glass aquaria, half covered, without substrate). When the electric shock is applied to the fish, they may be emotionally affected and therefore stop to display such type of stereotypical behavior as a result of a disturbance. Individuals receiving the noxious stimulus (electric shock) can be seen as more affected in comparison with the handled-only fish, since they start to display this behavior later. The

performance of such behavior may apparently be overruled by a noxious, potentially painful stimulus.

The changes in both chafing and general swimming (crossing) activity indicate that there is a differential response between the shocked group and the handled-only group, the latter one recovering faster. These behavioral pattern changes are clear indications that the electric shock is perceived as potentially painful. This underlines the importance to monitor behavioral parameters in welfare and pain-related studies in teleosts (Sneddon, 2003; Sneddon et al, 2003; Roques et al, 2010).

4.3 Stress and plasma analyses

The plasma cortisol level increased in response to handling only and the electric shock, but did not differ between the 2 conditions. Basal plasma cortisol levels in our fish were in the range considered normal for non-stressed fish, *i.e.* 33.9 (29.5) nM (Wendelaar Bonga, 1997). The handling and shocked groups increased significantly cortisol levels 1 h post treatment both for the electric-shock and handling controls. and remained significantly elevated at 6 h post treatment compared to the control. Increases up to 165.6 nM (60 ng/mL) are generally referred to as a mild response, while rapid increases above 276 nM (100 ng/mL) are generally considered to reflect a severe stress response (Wendelaar Bonga, 1997). When fish experience chronic stress, plasma cortisol level should remain elevated compared to controls (Wendelaar Bonga, 1997), but in our fish cortisol levels returned to control values by 24 h, which indicates that the fish recovered from the procedures. In the same species as used here, 2 h of net confinement were reported to induce cortisol to rise from 5-8 nM to 440 nM (Nolan et al, 1999).

Plasma cortisol in Nile tilapia receiving tailfin clip or submitted to handling stress only rose significantly after 6 h (334.6 nM (292.2) and 256.4 nM (139.9), respectively) (Roques et al, 2010). No differences were observed between the 2 treatments, as in current study. Such results indicate that the electroshock given to the fin represented a relatively mild stress when evaluated by cortisol response in comparison with other stimuli. This plasma parameter showed a different pattern in comparison with the fin clip study, stressing once more the difference between the 2 types of stimulus, targeting the same body part. Furthermore, as no differences were observed between the shocked groups and their related handling stress groups, plasma cortisol seems not suitable to assess the actual effect of such mild noxious stimulus in term of potential pain indicator.

Plasma glucose and lactate levels followed the changes observed in cortisol levels, with significant increased glucose levels compared to the

controls after 1 h for both groups. Plasma glucose of the shocked group remain significantly higher compared to the control and handled groups at 6 h. Lactate levels had slightly decreased after 6 h, and no difference between the two groups was observed. It appears that both treatments affect the fish; cortisol has a stimulatory effect on glycaemia that lasted longer in the case of the electric shock. This long-lasting effect was not observed for the fin clip (Roques et al, 2010). For this parameter, an electroshock seems to have a stronger effect. This could be due to the nature of the stimulus, the clip inducing acute strong adrenergic response due to the tissue damage, while the electroshocks induce a longer lasting endocrine and behavioral response, probably due to its unusual (unpredictable and novel) nature. A decrease in plasma lactate can be interpreted as use of lactate as metabolic substrate for gluconeogenesis to cope with the adverse situation. The endocrine mediators involved in this process have not been investigated in this study. Polakof and Soengas (2008) demonstrated in rainbow trout injected either intraperitoneally (IP) or intracerebroventricularly (ICV) with l-(+)-lactate that lactate metabolism was apparently involved in glucose homeostasis through changes in plasma glucose levels and glucose production in liver. They suggested that lactate was probably being converted into glucose by the liver, resulting in higher plasma levels of glucose, and, as a result, an increase of glucose availability.

Plasma concentrations of Na^+ and Cl^-, the two main determining components of plasma osmolality, did not significantly change after the shock and handling. There was a tendency for the level of these two ions (independently) to decrease after both treatments over the time. Indeed, plasma osmolality (determined mostly by the levels of these two ions) did decrease significantly for both groups, with an apparent stronger effect for the shocked group. These observations support the relative mildness of the stimuli applied and indicate a mild loss of control over permeability to water and ions, as is often seen in stressed fish, due to catecholamine-induced epithelial lifting and dysfunction of the gills (Wendelaar Bonga, 1997). This mild loss of ions is counteracted at the level of the gills by a slight increase in Na^+/K^+-ATPase activity, observed for both stimuli, after 6 h.

Unlike in a previous experiment (Roques et al, 2010) where an increased mucus secretion was observed 1 h post stimulus, mucus secretion was not enhanced in the present experiment. This is additional evidence for a differential response of the fish towards two different stimuli: the fin clip induced a stronger acute adrenergic response associated with the tissue damage than the response to an electric shock.

5. General conclusions

A fin clip and an electric shock elicit differential responses, qualitatively and quantitatively.

While the fin clip elicited a strong acute adrenergic response, especially at the level of the gills (mucus secretion, chloride cells migration), accompanied by an enhanced swimming activity and a preference for the dark compartment, this was not observed in the current study. The stereotypical chafing behavior provides a reliable marker for discomfort. Physiological parameters were mildly affected mainly from 6 h post stimulus (glucose, lactate, osmolality), with only glucose levels significantly different compared to handled controls.

Behavior showed an opposite pattern, the animals being less active following the stimulus, with a slower recovery in shocked fish compared to handled-only fish. We ascribe these differences to the different nature of the stimuli; the tissue-damaging fin clip induces a strong and acute adrenergic response of short duration, the electric shock, a novel stimulus, elicited a longer-lasting reaction.

Our results show that exposure of Mozambique tilapia to a mild electric shock impairs its welfare. This is relevant as the European Food Safety Authority (EFSA) recognizes that farmed fish at slaughter run the risk to be exposed to currents too low to provoke immediate loss of consciousness. Our study supports the EFSA recommendations on electronstunning conditions for fish.

Acknowledgements

We would like to thank Henk Gunnink (Livestock Research, Wageningen UR) for providing the video recording equipment.

This research project has been carried out within the Knowledge Base Research / Policy Supporting Research / Statutory Research Task for the Dutch Ministry of Agriculture, Nature and Food Quality. Project BAS-project KB08 009001ASG.

Chapter 4

Effects of a tailfin clip on habituation to novelty in zebrafish (*Danio rerio*): a pilot

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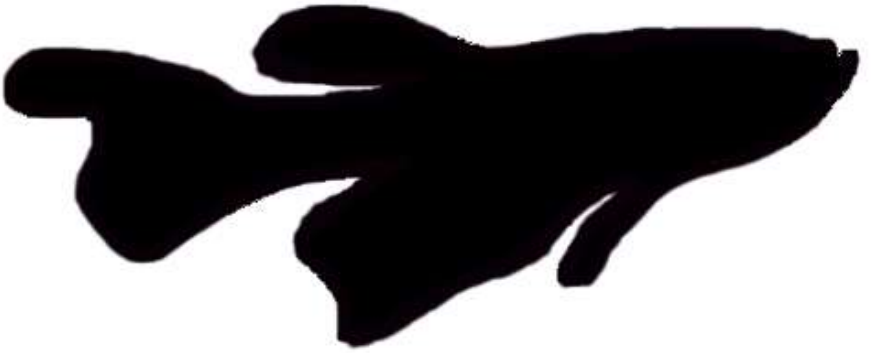
Submitted to Animal Welfare



Abstract

The habituation response of long-fin zebrafish (*Danio rerio*) receiving a standardized noxious and potentially painful stimulus (tailfin clip) was studied over a period of 8 days following the fin clip in the novel tank paradigm. The control group was transferred daily for 6 min to the novel tank, and showed signs of habituation to the procedure from the 5th day. Two experimental groups were transferred similarly and received in addition the tailfin clip either on day 1 (FC D1) or 5 (FC D5), respectively. Traditional parameters affected by anxiety, *viz.* time spent in the upper zone of the tank, zone transition frequency, 'freezing' behavior were not affected by this clipping procedure. Total distance swum was significantly lower on day 5 in the group receiving the stimulus on that day, while no differences were found when the clip was given on day 1. Freezing was seen occasionally in all groups on day 1, only three individuals from the FC D5 group showed this behavior on day 5. We conclude that tailfin clip does not affect habituation to novelty when applied on day 1, but when the fin clip is applied on day 5, no competing emotions exist; pain-related behavior is expressed, independently from novelty.

1. Introduction

The questions of pain, pain awareness, fear and stress in fish are subject of controversy; the brain structures that control these emotional processes in mammals, the hippocampus, amygdala, and cerebral frontal cortex as such are absent in teleostean fish. This has led some researchers to conclude that fish cannot experience pain, fear or stress (Bermond, 1997; Rose, 2002, 2007). More recently, the medial and lateral pallium in the teleostean telencephalon were established to be functionally similar to mammalian amygdala and hippocampus, respectively (Portavella et al, 2002). The lateral pallium is linked with processing both spatial memory and temporal relationships whereas the medial pallium is associated with fear-related and aversive responses. Some authors refer to this structure as a place that specifically processes 'emotional memory' (Broglio et al, 2005).

Evidence of learning that implies cognitive abilities have been reported for several fish species (Braithwaite and Huntingford, 2004; Chandroo et al, 2004). Fish learn to avoid painful conditions and thus there must be a memory for such adverse events. Therefore, it is reasonable to hypothesize that fish have some form of consciousness that can include the capability to experience a form of pain (Braithwaite and Huntingford, 2004).

The aim of the present study was to assess the behavioral responses of zebrafish (*Danio rerio*) to a presumed painful stimulus, a standardized tailfin clip. We used the commercially available mutant 'Tupfel long-fin' zebrafish, which has longer fins than wild type zebrafish, but no other phenotypic differences (Géraudie et al, 1995). In a previous experiment, we identified in the tailfin of common carp (*Cyprinus carpio*) A-δ and C-fibres, that are involved in nociception in mammals (Roques et al, 2010). In this same study, Nile tilapia (*Oreochromis niloticus*) responded to a tailfin clip by an increased swimming activity (Roques et al, 2010). Here we propose to study behavior of zebrafish receiving this stimulus in a novelty test.

Several behavioral tests were recently adapted from mammalian models (rodents) and validated for zebrafish (Champagne et al, 2010). Changes in place preference, exploratory, risk-taking and abnormal behavior after a noxious and potentially painful stimulus are reliable parameters to monitor responses to potential pain stimuli.

In this study, we used hitherto naïve zebrafish in a novel tank paradigm (Cachat et al, 2010; Wong et al, 2010), and investigated habituation to a novel environment, and the effect of a fin clip thereupon. Habituation is the most basic form of learning (Bolivar, 2009). Habituation is important in filtering the most important information received from the environment; once habituated to

a novel situation, the animal can focus on other more important features, such as exploration. If habituation is impaired, the animal may not be able to distinguish neutral stimuli from immediate threat, which can result in overstimulation of the stress axis and impaired welfare. Wong and colleagues (2010) demonstrated habituation of zebrafish to a novel environment through quantification of fearful behaviors (erratic movements, freezing) and enhancement of exploratory behavior. The initial response to novelty of zebrafish would be diving to the bottom of the tank, freezing and erratic movements and low exploration, characterized by few moves to the upper part of the novel tank. In normal conditions, those features would be less frequent as time passes and the fish would gradually and increasingly explore the novel environment, typically by augmenting the number of entries and time spent in the upper zone (Levin et al, 2007).

We monitored both swimming activity and spatial preference over a period of 8 days, under the hypothesis that stress/anxiety (fin clip) alters habituation (Wong et al 2010). We designed the experiment procedure to discriminate between the response to a tailfin clip (a presumed painful stimulus) and the unavoidable handling stress that goes with the clip. Stress is a well-known confounder in pain research.

Two groups received the fin clip at distinct time points; one at the beginning of the experiment (day 1; group FC D1) and the other at day 5 (FC D5), when habituation to novelty should have become manifest (Wong et al, 2010). We chose to apply the fin clip on those two time points on the expectation that the behavior observed on the first day would be the result of a motivational conflict between the reactions to novelty and to the fin clip. On the 5^{th} day, the fish are expected to be habituated to the novel tank, and therefore the behavior observed should be caused by the clip itself.

2. Material and methods

2.1 Fish

Adult zebrafish, weighing 0.32 (± 0.04) g, from the local breeding stock of the laboratory aquarium facilities of the Radboud University Nijmegen were used for this experiment. Fish received flakes feed at 2% of the total body weight daily. The water quality was monitored weekly for nitrogenous waste products (NO_2^-, NO_3^- and NH_4^+). Water pH and temperature were continuously monitored; oxygen was provided to saturation; the light regime was 12 h light : 12 h dark. The study was approved by the Animal Experimental Committee of Nijmegen (Protocol: 2012-043).

2.2 Experimental setup

Prior to the experiment, fish (8 per group divided in 2 aquaria of 4 fish each) were transferred to a climate room. The fish were placed into 2-L acrylic transparent aquaria and acclimatized to the new system for 4 days. One fish died during this acclimatization period (control group tank; reason not known) and was not replaced.

2.3 Novel tank exposition

Behavioral testing was performed using the novel tank test, representing a 1.5-L trapezoidal tank (15.2cm height×7.1cm width×27.9cm top×22.5cm bottom length, as described by Wong et al, 2010. Fig. 1). During the 8 days of experiment, fish were daily transferred from their home tanks to the novel tank setup where behavior was recorded for a period of 6 min using a Samsung DVR coupled with Sanyo bullet video-cameras. After those 6 min of exposure, fish were returned to their home tank. Fish from the control group were individually caught, restrained in the net for 15 sec and transferred without further manipulation to the novel tank (Fig. 2A and 2C). Experimental fish were individually caught, restrained in the net and received either on day 1 (FC D1) or on day 5 (FC D5) a fin clip, prior to being transferred to the novel tank (see the section below and Fig 2B.). the whole fin clip procedure lasted 15 sec per animal. Those fish were caught, restrained in the net for 15 sec and transferred without further manipulation the other days to the novel tank (Fig. 2A and 2C).

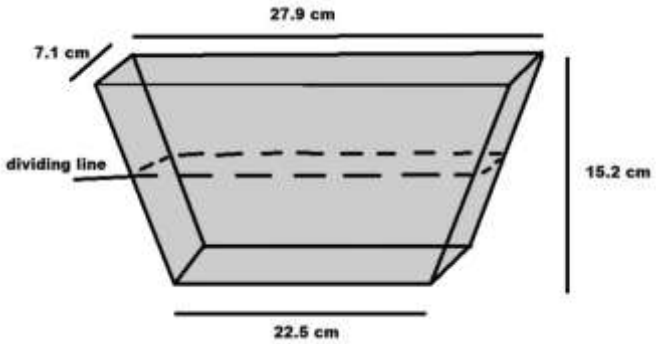


Fig. 1. Tank used to test for habituation to novelty. A 1.5-L trapezoidal tank (15.2cm height×7.1cm width×27.9cm top×22.5cm bottom length filled up to 3 centimeters below the top of the tank with water from the fish holding system.

The novel tank was filled with the same water from the fish setup, and thoroughly rinsed with demi-water between each fish trial. Novel tanks rested on a level, stable surface and were surrounded by white, non-reflecting walls. Behavioral testing took place between 11:30 and 13:00. The behavior of each fish was recorded for 6 min and later analyzed with the software Ethovision 8.0 (Noldus, Wageningen, The Netherlands). Swimming patterns of fish on days 1 and 5 are shown in appendices 1 and 2.

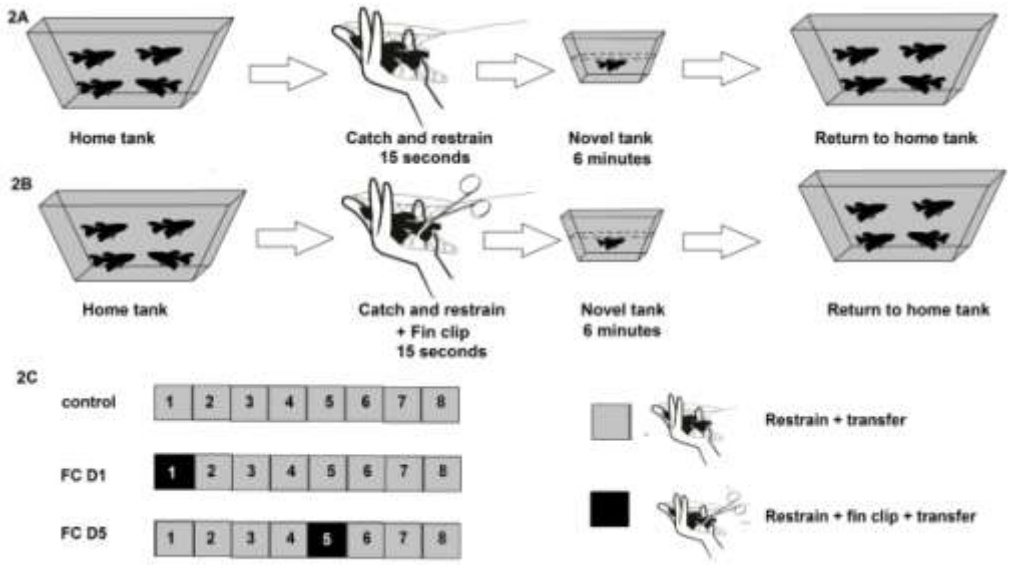


Fig. 2. Experimental procedure. 2A: Experimental manipulation of the control group (days 1 – 8), the FC D1 group (days 2 – 8) and FC D5 group (days 1 – 4; 6 – 8). Fish were individually caught, restrained in the net for 15 sec and transferred without further manipulation to the novel tank 2B: Experimental manipulation of the FC D1 group (on day 1) and FC D5 group (on day 5); experimental fish were individually caught, restrained in the net and received the fin clip prior to being transferred to the novel tank. 2C: Summary of the manipulation over the days.

2.4 Fin clip electron microscopy

Fin clips were immersed in glutaraldehyde (2.5% v/v), $K_2Cr_2O_7$ (1% w/v) and OsO_4 (1% w/v) in 0.15 M cacodylic acid (pH 7.5) and embedded in Spurr's resin. Ultrathin sections (70 – 90 nm) were cut with an ultratome and mounted on square mesh nickel grids. On-grid sections were post-stained for 2 min with uranyl acetate and then lead citrate for 2 min and rinsed thrice with doubly distilled water. Nerve fiber types in cross sections were categorized based on

diameter and the presence of myelin to distinguish A-δ and C-fibres (Lynn, 1994; Sneddon, 2002).

2.5 Video analyses

The novel tanks were virtually divided into a lower and upper part. The following parameters were scored: time spent in the upper part of the tank, number of transitions (entries) to the upper part, number and duration of freezing bouts, total distance swam and mobility. The immobile threshold was determined at 3% and the highly-mobile threshold at 75%, with a 10 samples averaging interval (Cachat el al, 2011). Freezing was defined as total absence of movement except opercular movements and eye movements for 2 sec or longer (Wong et al, 2010). Freezing was scored manually on days 1 and 5 when the fish showed immobile behavior (mobility below 3% of total body length). Changes in these parameters reflect high stress and anxiety (Wong et al, 2010; Cachat et al, 2011).

2.6 Statistics

Data are expressed as means ± standard deviation (SD). When data were normally distributed (Shapiro-Wilk test) and variances homogenously distributed (Levene test), a one-way ANOVA was performed to assess statistical significance of differences between treatments, followed by Tukey-test as post-hoc test. When data were not normally distributed, the non-parametric Kruskal–Wallis test was used to assess statistical significance of differences between treatments. On independent samples Student *t*-test was performed to confirm habituation after 4 days of procedure, by comparing the following parameters: total distance travelled, time spent in the upper/lower sections, frequency in the upper section, immobility duration, freezing bouts and freezing duration between the period days 1 – 4 and the period days 5 – 8, for the control group (Table 1).

3. Results

3.1 Electron microscopy

Nerve bundles were identified in the tailfin region of long-fin zebrafish (Fig. 3). The presence of A-δ and C-fibres was confirmed based on diameter and presence/absence of myelin sheet.

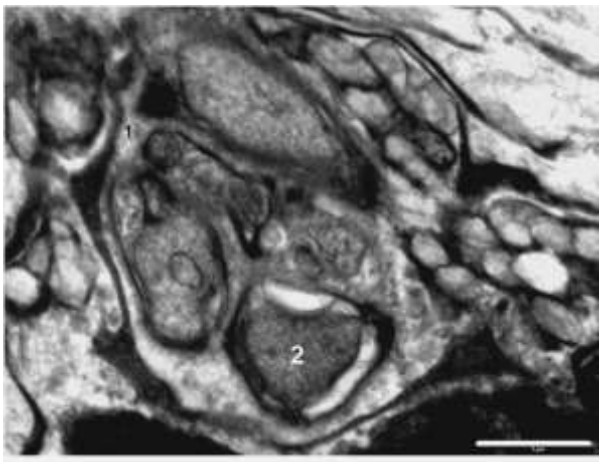


Fig. 3. TEM of zebrafish *Danio rerio* caudal fin sections showing nerve bundles (scale bar is 1 μm). Both unmyelinated C-fibres (1) and myelinated A-fibres (2) are present within the nerve.

3.2 Habituation to the novel tank procedure.

During the second period of the experiment (days 5 – 8), the control fish significantly swam a greater distance, frequented more the upper zone of the novel tank and reduced freezing-duration compared to the first period of the experiment (days 1 – 4). The number of freezing bouts and duration of freezing decreased over time (Table 1).

Parameter	Average (SD) days 1 – 4	Average (SD) days 5 – 8	P value
Total distance (mm)	1413 (556)	1837 (550)	P < 0.01 *
Time in lower region (s)	282.9 (64)	262.5 (67)	P = 0.24
Time in upper region (s)	77.1 (64.3)	97.5 (67.1)	P = 0.24
Frequency in upper region	18.5 (12.7)	26.1 (14.4)	P < 0.05 *
Immobile duration (s)	55.4 (96.2)	23.5 (35.7)	P = 0.22
Freezing bouts	1.1 (2.3)	0.2 (0.4)	P > 0.05
Freezing duration (s)	18.9 (43)	2.1 (5.9)	P < 0.05 *

Table 1. Averages (SD) of the total distance travelled, Time spend in the lower and upper regions, frequency in the upper region, immobile duration, freezing bouts and freezing durations for the periods between days 1 – 4 (N=28) and days 5 – 8 (N=28), for the control group (7 fish). Statistical differences between the two periods are highlighted by an asterisk.

3.3 Behavior: day 1

Total distance travelled is presented in Fig. 4. No statistical difference was observed on day 1 between the three groups concerning total distance travelled (Fig. 4, Kruskall-Wallis; H (2, N = 22) = 4.35, P = 0.80), zone preference (Fig. 5) or mobility (Fig. 6A). Fish from all treatment groups occasionally froze

on day 1, without significant differences between groups both regarding freezing bouts and freezing duration (Kruskal-Wallis, H (2, N = 22) = 0.54, P = 0.76). Within treatment groups fish showed great variety in freezing frequency as well as duration (inter-individual differences) (Fig. 7A).

3.4 Behavior: day 5

On day 5, the group receiving the fin clip on this day (FC D5) swam significantly less than the control and the group receiving the fin clip on the 1st day (FC D1); 1039 (521) cm *vs.* 1961 (669) cm and 2150 (843) cm, respectively (Fig. 4; One-way ANOVA followed by Tukey test, P = 0.01).

In addition to the reduced swimming distance, there is a trend on day 5 towards reduced activity and freezing. Only the group receiving the fin clip on this day showed occasional immobility (< 3% body length per 10 samples average) events (Fig. 6B); no statistically significant difference was found (Kruskal-Wallis, H (2, N = 23) = 5.77; P = 0.06). Only 3 individuals from the group receiving the clip on this day (FC D5) occasionally froze (Fig. 7B). No freezing occurred for the two other treatments (control and FC D1) and no statistically significant difference was found (Kruskal-Wallis, H (2, N = 23) = 6.15, P = 0.05). No difference in time spent in the upper zone between the 3 treatments was found (Fig. 5).

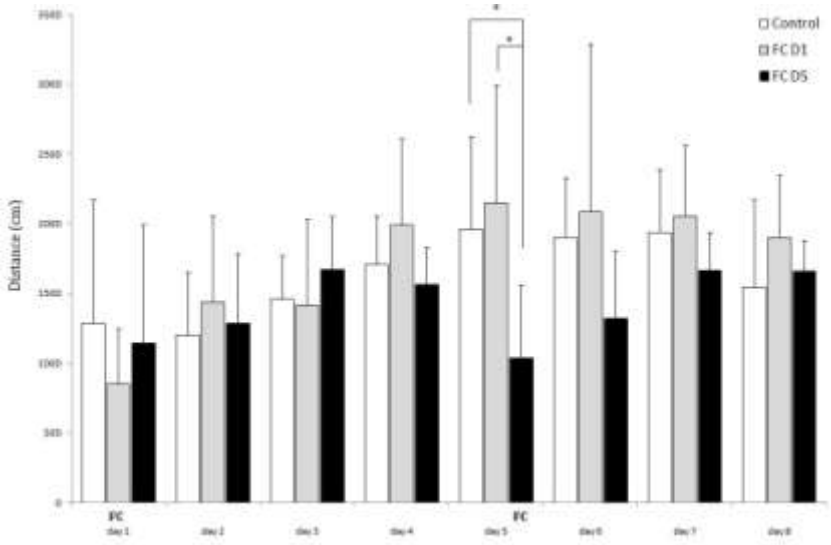


Fig. 4. Averages (SD) of the total distance travelled during the daily 6-min trial (cm). FC: fin clip given to the designed group: day 1 for the FC D1 group and day 5 for the FC D5 group. Asterisks stand for significant differences (one-way ANOVA followed by Tukey test, P = 0.01).

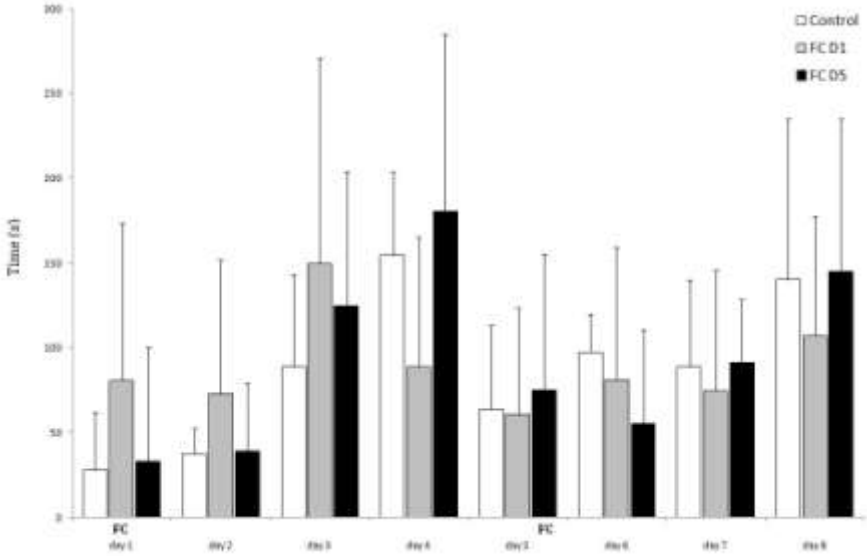


Fig. 5. Average (SD) time spent in the lower section of the novel tank during the daily 6-min trial (cm). FC: fin clip given to the designed group: day1 for the FC D1 group and day 5 for the FC D5 group

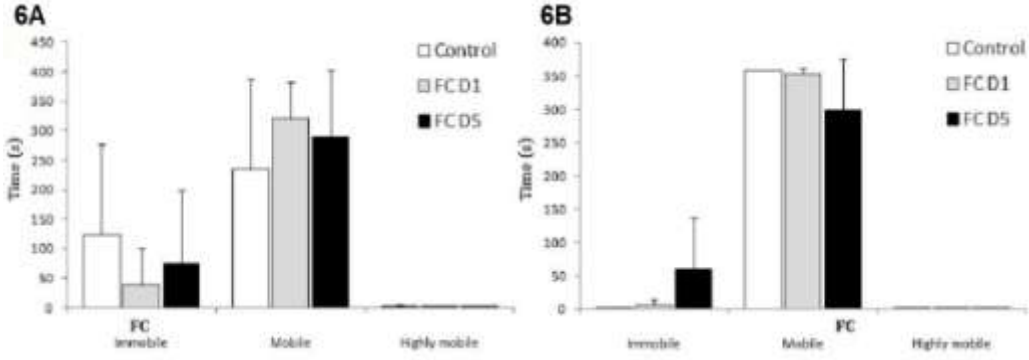


Fig. 6. Average mobility (s) during the 6-min trial on day 1 (6A) and day 5 (6B). Immobile threshold was set at 3% total body length and highly mobile threshold at 75% total body length, with a 10 samples average. FC: fin clip given to the designed group: day1 for the FC D1 group and day 5 for the FC D5 group.

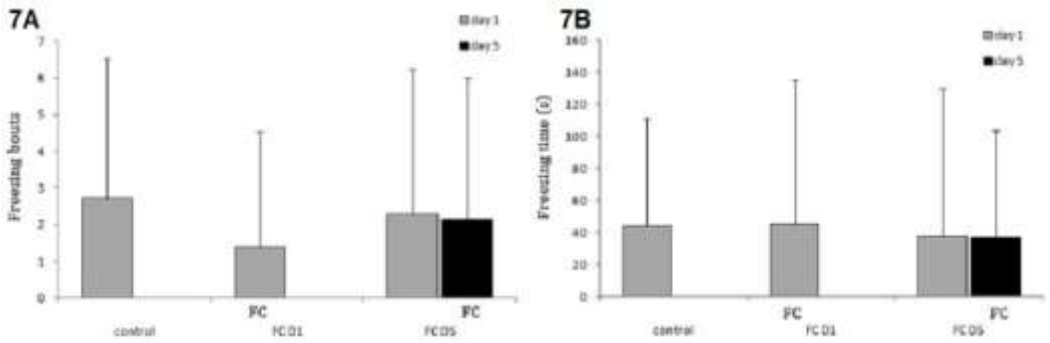


Fig. 7. Freezing bouts (7A) and freezing duration (7B) during the 6 min trials on day 1 (grey) and day 5 (black). FC: fin clip given to the designed group: day1 for the FC D1 group and day 5 for the FC D5 group.

4. Discussion

4.1 Parameters affected and comparison with classical studies

The tailfin clip did not affect 'classical' parameters in the novel tank test such as freezing and compartment preference. No effect regarding spatial preference and latency to first explore the upper zone or freezing behavior was found, regardless of the day when the fin clip was given. On the 1st day, we observed no differences between the treatment groups, regardless whether they received the fin clip (FC D1 group) or not. The apparent no-effect of the fin clip on the 1st day could be explained by the existence of a motivational conflict: the novelty test seems to affect more the behavior of the fish than the fin clip. Freezing behavior was observed in all the treatment groups, mobility was not different between groups; fish were mostly mobile and only some and occasionally fish were immobile. On the 5th day of procedure, the fish receiving the tailfin clip were less active than the 2 other groups: they swam significantly less, and the freezing behavior occurred in three individuals from this group only, while none of the fish in the two other groups did. This overall reduction of activity can be attributed to the painful stimulus itself; there is no motivational conflict on that day; zebrafish are habituated to the novel tank procedure after 4 days (Wong et al, 2010). When the fish received the potentially painful stimulus on the 5th day (when we anticipated that habituation had occurred), the fish indeed were disturbed again by the clip. The behavioral changes of the group receiving the fin clip on the 5th day, where no motivational conflict exist, are clear indications that a tailfin clip has an effect on habituation and therefore might be considered first of all noxious, and potentially painful alike.

4.2 Choice of the pain stimulus, behavioral test and model species

In a previous study on Nile tilapia (*Oreochromis niloticus*) we have shown that a tailfin clip may be considered a potentially painful stimulus for fish (Roques et al, 2010). In this study on zebrafish we demonstrated that behavior of the fish was clearly affected by the fin clip, altering swimming activity up to 6 h post stimulus. In a follow-up study, where we gave an electric shock to the tail of Mozambique tilapia (*Oreochromis mossambicus*), we observed a significant decrease of the general activity compared to the handled-only group (Roques et al, 2012), which was taken to indicate a species-specific response to a noxious stimulus. In the current study, we aimed to investigate in more detail the effect of a tailfin clip on fish behavior related to anxiety, using the novel tank paradigm and the model species zebrafish, *Danio rerio*. The novel tank paradigm has been intensively studied in this species, regarding habituation to this procedure and effects of anxiolytic drugs (Becan et al, 2009; Stewart et al, 2010; 2012).

An array of anxiolytic drugs has been shown to suppress the initial response (diving to the bottom, freezing, erratic movements and decreased exploration; Levin et al, 2007), with more rapid and enhanced exploration of the novel environment (Stewart et al, 2010). Habituation to this procedure, also shown by this reduction of initial avoidance response and enhanced exploratory behavior has been shown to occur after 4 days consecutive exposure to this novel tank test (Wong et al, 2010). The comparison of several parameters such as total distance travelled, frequency in the upper zone and freezing duration for the control group, between periods days 1 – 4 and days 5 – 8 confirms the habituation to the novel tank procedure demonstrated by Wong and colleagues (2010). In the second period (days 5 – 8), the fish had habituated to the procedure and showed more exploratory behavior. Therefore, we decided to apply a tailfin clip on the 1st day (where the behavioral response is affected by both the novelty and the fin clip procedure) and the 5th day (when the fish are habituated to the novel tank) of the experimental period and compare our results with existing literature. The cutting of the fin *per se* did not affect the swimming capacity as swimming was not different between the different groups on day 1. But the motivation to swim seems affected as we concluded from the response to the fin clip given on day 5.

4.3 Limitations of this study and perspectives

This study demonstrated an effect of a tailfin clip on habituation of zebrafish to novelty. We observed high individual variation within treatment

groups; not all fish behaved similarly, resulting in high variation. Inter-individual behavior variance is normal for every animal species, including fish (Pike et al, 2008; Wolf and Weissing, 2012). The effects seen in our studies warrant more studies with larger groups of fish to substantiate individual variation.

The fin clip procedure requires a manual restraint of the animals out of the water, albeit for a very short period of time. This procedure is not ideal as air exposure in particular is known to be a severe stressor for fish (Arends et al, 1999). It is know that stress may lead to analgesia. This potential confounder was overcome/controlled by handling the fish from the other groups for a similar (short) period of time. Ideally, the painful stimulus should be applied under water, which is not feasible with zebrafish tailfin clipping.

Zebrafish are social animals and a certain hierarchy in each home tank was established by the fish, with four individuals per tank. The next step of this line of work would be to investigate the effect of a tailfin clip on a group, either by clipping the whole group or few individuals of a cohort and monitor group effects, with possible involvement of alarm substances and effects on behavior, as demonstrated before (Ashley et al, 2009).

Also, analysis of the 3-dimensional behavior (Cachat, 2013) may add resolution to the analysis of subtle responses to stress and pain.

Acknowledgements

The authors thank Tom Spanings for excellent animal care, Dr. Jonathan Cachat and prof. Allan Kalueff from Tulane University for their help and advices, and Andrew Spink from Noldus for technical help with Ethovision.

Chapter 5

The impact of elevated water ammonia concentration on physiology, growth and feed intake of African catfish (*Clarias gariepinus*)

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In: Aquaculture (2010) 306:108-115

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Abstract

The threshold concentration for NH_3 in rearing water of African catfish (*Clarias gariepinus*) was assessed. African catfish with an initial mean (SD) weight of 141.0 (24) g were exposed to 5 different T_{amm} [sum of NH_3 and NH_4^+] concentrations: 0.37 (Control), 1.06, 2.12, 5.16 and 19.7 mM, which concurs with NH_3 concentrations of 4 (Control), 14, 38, 176 and 1084 µM. Plasma concentrations of NH_4^+, cortisol, glucose and lactate, plasma osmolality, gill morphology, branchial Na^+/K^+-ATPase activity, feed intake and specific growth rate were monitored. No effect of water NH_3 on plasma NH_4^+ concentrations was detected. Feed intake and specific growth rate were severely affected at exposure to water NH_3 concentrations above 90 µM (calculated EC_{10} values: 89 and 122 µM). No major disturbances in physiological blood parameters were observed at these NH_3 concentrations, but gill morphology (a remarkably sensitive stress indicator) deteriorated significantly. Based on the lower limit of the 95% confidence interval for EC_{10}, we advise for African catfish not to exceed a water NH_3 concentration of 24 µM (0.34 mg NH_3-N/L). This finding is relevant for design and management of African catfish production systems.

1. Introduction

Fish produce nitrogenous wastes through catabolism of amino acids (Wood, 1993). The majority of fresh water and marine teleost fish are ammonioteles and excrete most of their nitrogenous wastes as ammonia across the gills to the water (Wilkie, 2002). The mechanisms involved in branchial ammonia excretion remain controversial. In the most recently proposed model for branchial ammonia excretion, simple NH_3 diffusion down the partial pressure gradient is the predominant mechanism under normal conditions. At high water ammonia concentrations, when NH_3 diffusion is impaired or even reversed, several active NH_4^+ excretion pathways, involving Rhesus (Rh) glycoproteins as membrane transporters, facilitate ammonia efflux (Wright and Wood, 2009).

High water ammonia leads to rapid accumulation of ammonia in plasma and tissues (Wright et al, 2007), where it is mainly present as NH_4^+ at physiological pH (Wilkie, 2002). High internal NH_4^+ causes neurotoxicity (Cooper and Plum, 1987 in Wilkie, 2002).

High water ammonia, caused by high feed loads and high fish densities, is an important limiting factor for intensive aquaculture (Boeuf et al, 1999). Water ammonia should therefore be kept below species-specific threshold levels.

The African catfish (*Clarias gariepinus*) is empirically known to be highly tolerant to ammonia toxicity (Ip et al, 2004a). Several defense strategies allow this fish to cope with increased internal ammonia, for instance during prolonged air exposure or during periods of draught, when the fish survive in mud pools. The defense strategies include active excretion of NH_4^+, reduced ammonia production by reduction of proteolysis and/or reduced amino acid catabolism and a high ammonia tolerance of tissues and cells. Moreover, it appears that this catfish reduces membrane and skin permeability to NH_3 in response to high water ammonia concentrations (Ip et al, 2004a).

The NH_3 threshold concentrations for physiological disturbances, feed intake and growth are unknown for African catfish. As a result it is unclear whether intensive farming of this fish species at high water NH_3 concentrations results in physiological disturbances, reduced feed intake and reduced growth, and thus may impinge on the welfare of the fish. In the present study, African catfish was exposed to increased water ammonia for 34 days to establish NH_3 threshold concentrations.

2. Materials and methods

2.1 Experimental conditions

African catfish (*Clarias gariepinus*) were obtained from Fleuren-Nooijen BV, Someren, The Netherlands. Fish (n = 168) were randomly divided over 12 30-L rectangular glass tanks and allowed to acclimatize to the experimental conditions for 7 days. At the start of the 34 days experiment, the overall initial mean (SD) individual weight was 141.0 (24) g. The resulting mean stocking density was 65.8 kg/m^3, well below fish densities found at commercial farms for this size class (100 to 300 kg/m^3, Van de Nieuwegiessen et al, 2009). The treatment of the fish was in accordance with Dutch law concerning animal welfare, as tested by the ethical committee for animal experimentation of Wageningen UR Livestock Research (number 2009045.a).

We aimed at a threefold ammonia concentration increase for five consecutive treatments and a concentration range around the highest total ammonia concentrations observed at commercial farms (4.2 to 5.0 mM) without exceeding the acute toxic total ammonia concentration (96 h LC_{50}) of 380 mM (Britz, 1988 in Ip et al, 2004b). Five (1 to 5) different total ammonia [T_{amm} = sum of NH_3 and NH_4^+] concentrations in the rearing water were used: 0.37 (Control), 1.06, 2.12, 5.16 and 19.7 mM. These T_{Amm} concentrations concurred with NH_3 concentrations of 4 (Control), 14, 38, 176 and 1084 µM (Table 1). Treatments were executed in duplicate and assigned randomly to the tanks. Treatments are hereafter referred to as 4, 14, 38, 176 and 1084 µM NH_3.

During the acclimatization and experimental period, all tanks were supplied with local tap water via a header tank at a flow of 185 L per day for each tank. During the experimental period, experimental ammonia concentrations were realized by infusion of ammonium chloride (NH_4Cl) stock solutions (Table 1). Stock solutions were pumped into the tanks by a peristaltic pump (Watson Marlow 505 S; Rotterdam, The Netherlands) at a flow of 4.75 L per day per tank. Each tank was equipped with an air stone to mix the stock solution with the tank water. Flows were monitored and adjusted as required to reach the experimental ammonia concentrations. Sodium bicarbonate ($NaHCO_3$) was added to the stock solutions to adjust the pH. In addition, sodium chloride (NaCl) was added to the stock solutions to compensate for the differences in chloride concentrations arising from NH_4Cl addition. Total predicted sodium concentrations in the tanks from $NaHCO_3$ and NaCl combined were equal among treatments (Table 1). Fresh stock solutions were prepared daily. The salinity of the tank water resulting from the infusion of stock

solutions did not exceed 5 g/L. According to Clay (1977) African catfish tolerate salinities up to 10 g/L.

Treatment	NH_4Cl (g/10 L)	$NaHCO_3$ (g/10 L)	NaCl (g/10 L)	Total Cl^- dose (g/10 L)	Total Na^+ dose (g/10 L)	Predicted tank $[Na^+]$ (g/L)	Predicted tank $[Cl^-]$ (g/L)
1	0	0	1555	933	622	1.6	2.4
2	15	9	1549	939	622	1.6	2.4
3	45	47	1523	943	622	1.6	2.4
4	135	180	1432	948	621	1.6	2.5
5	404	530	1192	982	620	1.6	2.5

Table 1. Compositions of the daily prepared treatment specific stock solutions and the calculated[a] TAN, sodium and chloride concentrations in the tanks for all treatments.
[a] Based on equal flow rates per tank of 4.75 L/day for the stock solutions and 185 L/day for the tap water flow.

Water quality (Table 2) was monitored by daily (between 13:00 and 14:00) measurements of total ammonia (T_{Amm}) concentrations (photometrically, Hach Lange DR2800), water temperature, pH, dissolved oxygen concentrations (Hach Lange HQ 40 multimeter) and conductivity (WTW Cond 315i) in all individual experimental tanks. NH_3 concentrations were calculated from the temperature, pH and salinity dependent molar fraction of NH_3 and the measured T_{Amm} concentrations (Creswell, 1993). Ammonia concentrations were gradually increased to the designated concentrations during the first 4 days of the experimental period. Mean water temperature was 27.0°C throughout the experimental period.

Treatment	NH_3		T_{Amm}		DO	Water temperature	Conductivity	pH range
	(µM)	(mg N/L)	(mM)	(mg N/L)	(mg/L)	(°C)	(mS/cm)	
1	4	0.06	0.37	5.2	4.8	27.0	7.18	7.17–7.72
2	14	0.19	1.06	14.8	4.5	27.0	7.07	7.07–7.64
3	38	0.53	2.12	29.7	4.9	27.1	7.44	7.30–7.83
4	176	2.47	5.16	72.2	5.1	27.0	7.68	7.26–8.18
5	1084	15.2	19.7	275.1	5.6	27.0	8.78	7.46–8.66

Table 2. Mean values per treatment for NH_3, total ammonia (T_{Amm}) and dissolved oxygen (DO) concentrations, water temperature, conductivity and the pH range in the treatments during the experimental period.

2.2 Plasma sampling

One day before ammonia exposure started (day 0), the fish from two tanks were sampled. After 34 days exposure to ammonia, the fish from the 10

remaining tanks were sampled (two tanks for each of the five treatments, 12 fish per tank). Fish were rapidly caught with a net and quickly anaesthetized in 0.1% (v/v) 2-phenoxyethanol (Sigma, St. Louis, USA). Within 2 min, blood had been taken by puncture of the caudal vein using a lithium heparinized Vacuette blood collection system (Greiner Bio-One GmbH, Kremsmünster, Austria). The blood was centrifuged for 10 min (14,000 g, 4 °C) and the plasma obtained was stored at −20 °C.

2.3 Plasma NH_4^+

Plasma NH_4^+ was determined using a commercially available test kit (Instruchemie, Delfzijl, The Netherlands), with a protocol adapted for a 96-well microplate application.

2.4 Plasma cortisol

Cortisol was measured by radioimmunoassay (Metz et al, 2005) with commercially available antiserum (Campro Scientific, Veenendaal, The Netherlands). Samples of 10 µL of 1:5 (v/v) water diluted plasma were incubated overnight at 4 °C with 100 µL first antibody (IgG-F-1; 1:400), 2000 cpm ^{125}I-cortisol (Amersham, Buckinghamshire, UK) and 100 µL secondary antibody (GARGG; 1:160). All constituents were dissolved in cortisol RIA buffer [0.063 M Na_2HPO_4, 0.013 M Na_2EDTA, 0.02% (w/v) NaN_3, 0.1% (w/v) 8-anilino-1-naphthalene sulfonic acid (Sigma) and 0.1% (w/v) bovine gamma globulin (Sigma)]. Immune complexes were precipitated by addition of 1 mL ice-cold 5% (w/v) polyethylene glycol and 2% (w/v) bovine serum albumin (Sigma) and subsequent centrifugation (20 min, 2000 g, 4 °C). Pellets were counted in a gamma counter (1272 Clinigamma, LKB Wallac, Turku, Finland).

2.5 Plasma glucose and lactate

Plasma glucose and lactate were measured with commercially available enzymatic test kits (Instruchemie, Delfzijl, The Netherlands), with protocols adapted to a 96-wells microplate. For glucose, 10 µL sample or standard (5.55 mM glucose) was mixed with 200 µL reagent and incubated for 10 min at 25 °C. Absorbance was read within 60 min at 495 nm. For lactate, 10 µL sample or standard (4.44 mM lactate) or blank (8% perchloric acid) was mixed with

290 µL of lactate reagent and incubated for 20 min at 37 °C. Absorbance was read at 355 nm.

2.6 Osmolality

Plasma osmolality (sample volumes: 50 µL) was measured with a cryoscopic osmometer (Osmomat 030, Gonotec, Germany). Deionized water (0 mOsmol/kg) and a standard solution (300 mOsmol/kg) were used as reference.

2.7 Gill morphology

One gill arch was removed immediately after blood sampling and placed overnight in Bouin's fixative (75 mL saturated picric acid, 25 mL saturated formaldehyde, 5 mL acetic acid). Gill sections were made to include the trailing edge of the filament where the chloride cells reside. Gill sections were prepared according to Dang and colleagues (2000). After dewaxing, blocking of endogenous peroxidase with 2% (v/v) H_2O_2 and blocking of non-specific sites with 10% (v/v) normal goat serum, slides were incubated overnight with a monoclonal antibody against chicken Na^+/K^+-ATPase (final dilution of 1:500; IgGα5, Developmental Studies Hybridoma Bank, Department of Biological Sciences, University of Iowa, USA). Goat anti-mouse (Nordic Immunology, Tilburg, The Netherlands) was used as a second antibody (1:150). The slides were subsequently incubated with mouse peroxidase anti-peroxidase (1:150) (M-PAP, Nordic Immunology). In the peroxidase reaction 0.025% (w/v) 3,3′-diaminobenzidine (DAB) was used as chromogen in the presence of 0.0005% (v/v) H_2O_2.

2.8 Branchial Na^+/K^+-ATPase activity

The specific, Na^+- and K^+-dependent, ouabain-sensitive ATPase activity was measured in homogenates of gills preserved in SEI buffer (150 mM sucrose, 10 mM EDTA, 50 mM imidazole: pH 7.4) as described in detail by Metz and colleagues (2003). Aliquots (5 µL in triplicate) of homogenate (protein content of 1 mg/mL) were incubated in assay medium for 15 min at 37 °C. The specific activity was calculated by subtracting the K^+-independent, ouabain-insensitive ATPase activity from total ATPase activity. ATP hydrolysis was assessed by the amount of inorganic phosphate formed per min per mg of protein. Sample

protein content was estimated with a commercial protein kit (BioRad, Hercules, CA, USA), and bovine serum albumin as standard.

2.9 Specific growth rate, feed intake and feed conversion rate

On day 34, the fish in each tank were counted and individually weighed (Mettler PM 34 Delta range) to the nearest 1 g, to calculate the specific growth rate (SGR) as follows:

$$SGR = (\ln(W_t) - \ln(W_1)) \times \frac{100}{t}$$

Where SGR = specific growth rate (%/d), W_t = mean weight at day 34 (g), W_1 = mean weight at day 1 (g) and t = number of days.

Feed (Catfish type Me-3; Skretting, Boxmeer, The Netherlands) with 49% crude protein and 11% crude lipids was administered twice daily at 9:00 and 17:00 until visually observed satiation. Feed loads per tank were recorded. Uneaten pellets were collected from each tank 1 h after the two daily feeding sessions. Feed loss per tank was calculated as the total number of uneaten feed pellets multiplied by 0.0966 g per pellet, determined by weighing 100 feed pellets. Daily feed intake per tank was defined as the difference between daily feed load and feed loss. Total feed intake per tank resulted from the sum of the daily feed intake.

Total feed intake and biomass increase per tank were used to calculate feed conversion rate (FCR) as follows:

$$FCR = \frac{TFI}{(n_t \times W_t - n_1 \times W_1)}$$

Where FCR = feed conversion rate (g/g), TFI = total feed intake (g), W_t = mean weight at day 34 (g), W_1 = mean weight at day 1 (g), n_t = number of fish at day 34 and n_1 = number of fish at day 1.

2.10 Statistics

2.10.1 Physiological parameters

Physiological parameters are expressed as mean (SD) of the individual measurements per treatment. For each treatment, 24 fishes were sampled; in some instances not all samples collected were analyzed, either because samples were accidentally lost or because predicted low within treatment variation in readout allowed assessment of significance with lower sample numbers. Where necessary, data were log-transformed to obtain homogeneity of variance of residuals across treatment levels. Mean values for physiological parameters were tested for differences among the treatments using linear mixed models (REML) with treatments as fixed effects and tank as a random effect. Only in case significant treatment effects were detected, a least significance difference (LSD) post-hoc analysis was used to estimate the level of significance between mean values. For both REML and LSD analysis the fiducial limit was set at 5%.

2.10.2 Feed intake and growth

Total feed intake, specific growth rate (SGR) and feed conversion rate (FCR) were expressed as mean per treatment (N = 2). Mean values per treatment were tested for significant differences among the treatments by one-way ANOVA. Only in case significant treatment effects were detected, a least significance difference (LSD) post-hoc analysis was used to estimate the level of significance between mean values. For both ANOVA and LSD analysis the fiducial limit was set at 5%.

2.10.3 Concentration–effect curves

NH_3 concentration-effect curves were fitted for specific growth rate (SGR) and total feed intake (TFI) using a log-logistic model (Seefeldt et al, 1995). As a blank could not be included, the effects are expressed as absolute values. Curve-fitting was carried out with the Marquadt and Levenberg algorithm (Moré, 1978) as provided in the PRISM 4.00 software package (Graphpad Software, Inc.). The 10% effect concentrations (EC_{10}) and their 95% confidence limits were calculated (Miller and Miller, 2000).

3. Results

3.1 Plasma NH_4^+

Plasma NH_4^+ concentrations did not differ among treatments (REML, P = 0.10). Mean plasma NH_4^+ concentrations ranged between 159.2 and 217.8 μM in catfish exposed to water NH_4^+ levels ranging from 4 to 1084 μM.

3.2 Plasma cortisol

Plasma cortisol concentrations did not differ among treatments (REML, P = 0.25). The mean (SD) plasma cortisol concentration in the control (4 μM NH_3) was 44.5 (35.1) nM. In the other experimental groups, mean (SD) concentrations ranged between 33.6 (26.4) (1084 μM NH_3) and 72.9 (52.7) nM (38 μM NH_3). The initial concentration (t = 0) was 30.0 (18.2) nM.

3.3 Plasma glucose and lactate

A significant treatment effect was observed for plasma glucose concentrations (REML, P = 0.002). In the control (4 μM NH_3) the glucose concentration was slightly but significantly higher at 3.51 mM than the concentrations seen in fish kept in 14, 38 and 176 μM NH_3; the highest concentration (4.8 mM) was observed in the 1084 μM NH_3 treatment. Plasma lactate concentrations were similar in all groups (REML, P = 0.25). The mean plasma lactate concentration in the control was 3.84 mM. The concentrations in the 14, 38 and 176 μM NH_3 treatments were within the same range.

3.4 Plasma osmolality

A significant treatment effect was observed for plasma osmolality (REML, P = 0.002). The mean plasma osmolality in the control (4 μM NH_3) was 267.1 mOsmol/kg The osmolality in the 14, 38 and 176 μM NH_3 treatments were within the same range and no significant differences were detected among these. Plasma osmolality in the 1084 μM NH_3 treatment group rose to 347 mOsmol/kg, a significantly higher value than found in any other treatment.

Treatment	Water NH_3 (µM)	Plasma cortisol (nM)	n	Plasma glucose (mM)	n	Plasma lactate (mM)	n	Plasma NH_4^+ (µM)	n	Plasma osmolality (mOsmol/kg)	n	Na^+/K^+-ATPase activity (µM $Pi.h^{-1}$. mg $prot^{-1}$)	n
t = 0		30.0 (18.2)	24	3.4 (1.0)	24	2.74 (0.78)	24	167.9 (27.8)	23	251.2 (10.5)	24	2.5 (1.2)	10
1	4	44.5 (35.1)	24	3.5 (0.6)^{b}	24	3.84 (1.58)	24	159.2 (39.9)	16	267.3 (8.1)^{a}	24	3.4 (1.5)^{a}	12
2	14	43.3 (20.9)	24	3.0 (0.5)^{a}	24	4.17 (1.58)	24	200.1 (19.3)	15	278.7 (24.3)^{a}	24	3.9 (1.9)^{a}	12
3	38	72.9 (52.7)	24	3.1 (0.8)^{ab}	24	3.88 (1.16)	24	205.7 (31.0)	16	276.8 (8.3)^{a}	24	3.6 (0.9)^{a}	6
4	176	62.1 (38.6)	24	3.0 (0.8)^{a}	23	4.02 (0.97)	24	213.4 (13.5)	22	278.3 (13.8)^{a}	22	5.5 (2.3)^{a}	12
5	1084	33.6 (26.4)	23	4.8 (0.9)^{c}	23	6.23 (1.53)	23	217.8 (17.8)	21	347.0 (16.9)^{b}	23	10.2 (3.6)^{b}	9
P-value		0.21		0.002		0.25		0.1		0.002		< 0.001	

Table 3. Mean (SD) values at the start (t = 0) and per treatment for the end (t = 34 d) of the experiment for plasma cortisol, plasma glucose, plasma lactate and plasma NH_4^+ concentrations, plasma osmolality and branchial Na^+/K^+-ATPase activity. Mean values with different superscripts are significantly different (REML, P values as shown). SD = standard deviation of means values per treatment, n as indicated in the table. t = 0 values were not considered in the statistical analysis.

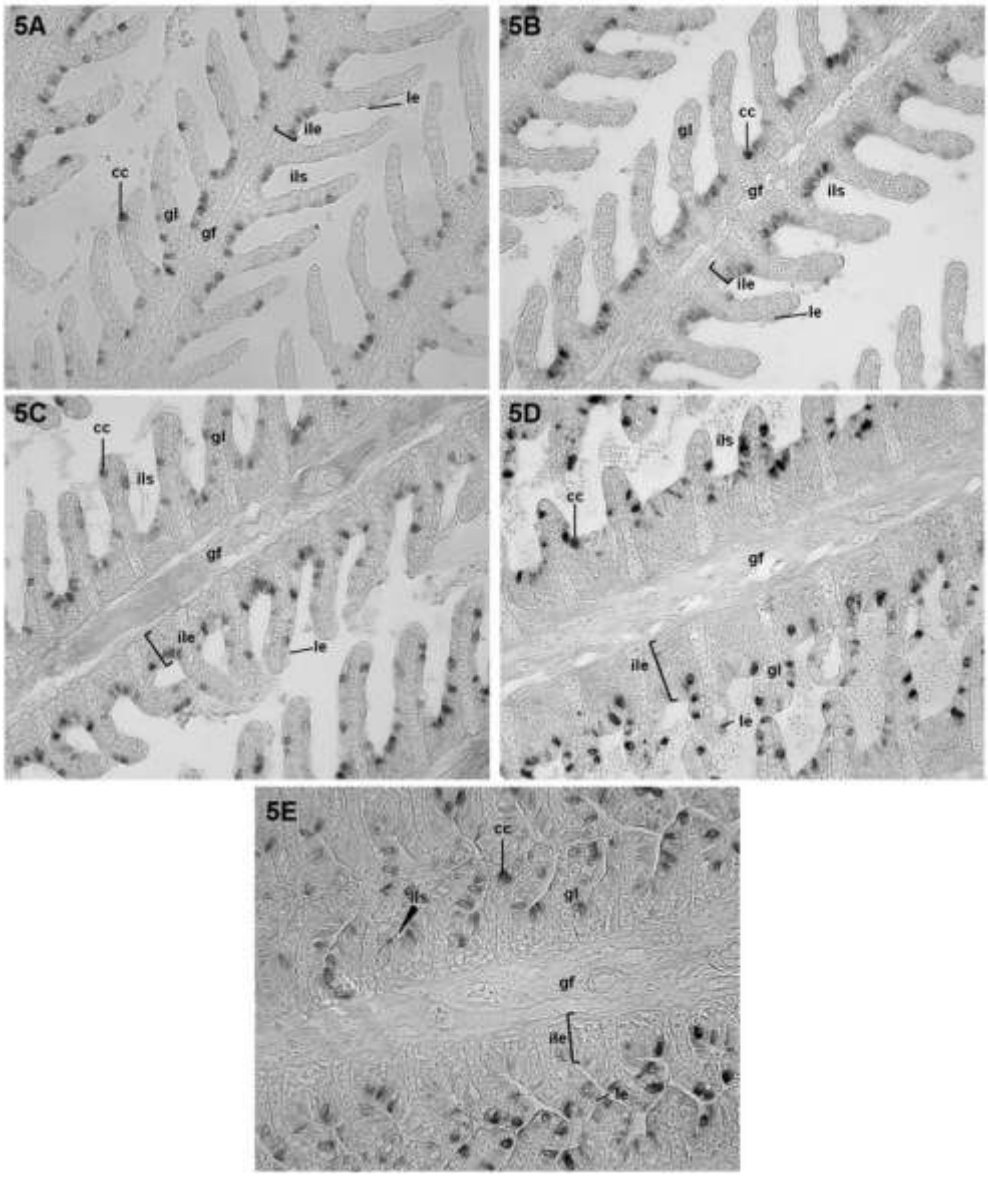

Fig. 1. Histology of gill epithelium immunohistochemically stained for Na^+/K^+-ATPase-rich cells (chloride cells) of the 4 μM (A) 14 μM (B) 38 μM (C) 176 μM (D) and 1084 μM (E) NH_3 treatment groups (400× magnification). Thickening of inter-lamellar and lamellar epithelium (inter-lamellar space reduction) increases gradually with increasing water ammonia level (C). The 1084 μM treatment (E) reveals distal and basal hyperplasia with lamellar fusion, epithelial hypertrophy accompanied with enhanced mucus secretion. Legend: ile = inter-lamellar epithelium; le = lamellar epithelium; ils = inter-lamellar space; cc = chloride cell; gf = gill filament; gl = gill lamellae.

3.5 Gill morphology

Gill morphology deteriorated with increasing water ammonia concentration (Fig. 1A–E): the inter-lamellar and lamellar epithelium thickened and the inter-lamellar space got reduced. The effect was directly visible in the 38 μM NH_3 treatment (Fig. 1C) and most profound in the 1084 μM treatment (Fig. 1E). In the latter group, distal and basal hyperplasia with lamellar fusion, epithelial hypertrophy and enhanced mucus secretion were observed.

Chloride cells in the control (4 μM NH_3, Fig. 1A) were mainly present in the inter-lamellar area and to a lesser extent in the lamellae. With increasing NH_3 exposure, the number of chloride cells increased, as did the number of chloride cells that had migrated to the lamellae (Fig. 1B–E).

3.6 Branchial Na^+/K^+-ATPase activity

A significant treatment effect was observed for branchial Na^+/K^+-ATPase activity (REML, $P < 0.001$). In the control (4 μM NH_3) the mean branchial Na^+/K^+-ATPase activity was 3.4 (1.5) μM P_i/h per mg protein. The activity in the 14, 38 and 176 μM NH_3 treatments were within the same range. Na^+/K^+-ATPase activity in the 1084 μM had significantly increased (more than 2-fold) compared to the other treatments at 10.2 (3.6) μM P_i/h per mg protein.

3.7 Feed intake, specific growth rate, feed conversion rate and mortality

No mortality was observed during these experiments. Total feed intake differed among the treatments (one-way ANOVA, $P < 0.01$, Table 4). The total feed intake was highest in the 14 and 38 μM NH_3 treatments. Total feed intake was lower in the 176 μM NH_3 treatment compared to the control and 14 and 38 μM NH_3 treatments. Total feed intake was strongly reduced in the 1084 μM NH_3 treatment compared to all other treatments. The differences in total feed intake among treatments developed over time (Fig. 2).

Specific growth rate (SGR) differed among treatments (ANOVA, $P < 0.01$, Table 4). The highest SGR was observed in the 4, 14 and 38 μM NH_3 treatments. The SGR in the 176 μM NH_3 treatment was lower than observed for the 14 and 38 μM NH_3 treatments, but equal to the SGR observed in the control (4 μM NH_3). The SGR in the 1084 μM NH_3 treatment was lower than in all other treatments.

Feed conversion rates (FCR) differed among treatments (one-way ANOVA, P = 0.03), with an approximately 30% higher mean FCR for the 1084 μM NH_3 treatment than for the other treatment (Table 4).

Treatment	Ammonia (μM)	Final weight (g)	Total feed intake (g)	SGR (%/BW/d)	FCR
1	4	412 (1.2)^{ab}	2339 (2)^{a}	3.25 (0.01)^{ab}	0.72 (0.004)^{a}
2	14	468 (10.4)^{a}	2705 (104)^{b}	3.64 (0.07)^{a}	0.69 (0.005)^{a}
3	38	466 (44.8)^{a}	2668 (170)^{b}	3.61 (0.29)^{a}	0.69 (0.004)^{a}
4	176	370 (17.1)^{b}	2017 (181)^{c}	2.92 (0.14)^{b}	0.73 (0.011)^{a}
5	1084	224 (9.6)^{c}	986 (32)^{d}	1.40 (0.13)^{c}	1.00 (0.15)^{b}
P-value		< 0.01	< 0.01	< 0.01	0.03

Table 4. Mean (SD) values per treatment (N = 2) for final weight, specific growth rate (SGR), total feed intake and feed conversion rate (FCR). Mean values with different superscripts are significantly different (one-way ANOVA, P values as shown).

3.8 EC_{10} for total feed intake and SGR

The concentration–effect curves for total feed intake and SGR in relation to the water NH_3 concentration (Fig. 3A and B), demonstrate that for total feed intake the EC_{10} for NH_3 is 89 μM (1.24 mg NH_3-N/L), with a 95% confidence interval from 24 to 321 μM. For SGR, the EC_{10} for NH_3 is 122 μM (1.70 mg NH_3-N/L), with a 95% confidence interval from 44 to 330 μM.

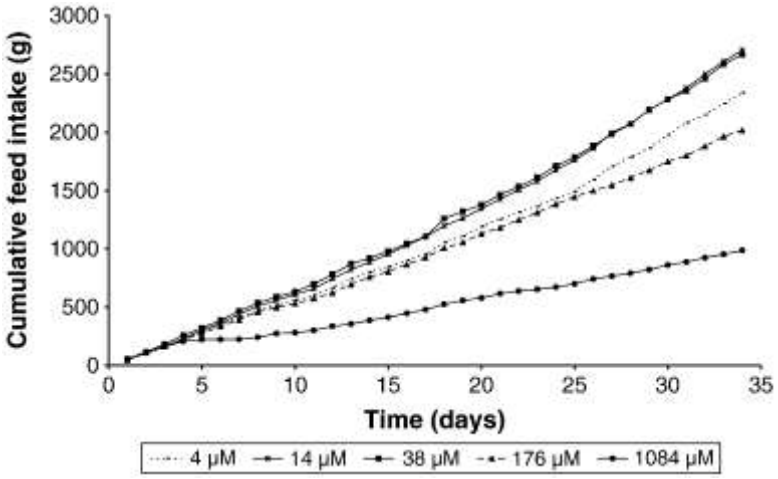

Fig. 2. NH_3 exposure and mean (N = 2) cumulative feed intake.

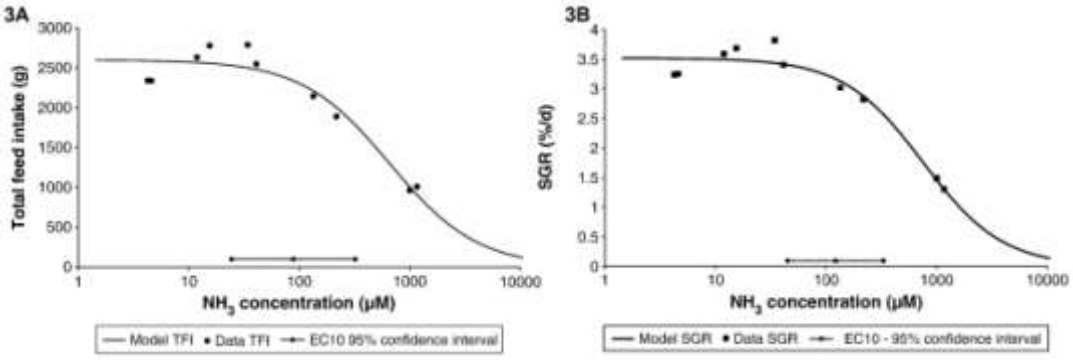

Fig. 3. Concentration–effect curves for total feed intake (TFI) (a) and specific growth rate (b) in relation to the water NH_3 concentration. TFI = 2559 / (1 + $10^{(2.819 - \log[NH3])}$)* – 1.095 (r^2 = 0.93) and SGR = 3.519 / (1 + $10^{(2.877 - \log[NH3])}$)* – 1.205 (r^2 = 0.95), where [NH_3] is the NH_3 concentration (μM).

4. Discussion

African catfish, *Clarias gariepinus*, successfully control plasma NH_4^+ concentrations within physiological concentrations over a wide range of water ammonia concentrations that would be lethal to many other fishes. However, the high ammonia concentrations did affect the fish, as revealed by other parameters: plasma glucose, plasma osmolality, branchial Na^+/K^+-ATPase activity, gill morphology, specific growth rate (SGR), total feed intake (TFI) and feed conversion rate (FCR) were affected, albeit that this species is very tolerant to ammonia compared to other fish.

4.1 Plasma NH_4^+

In African catfish plasma, T_{Amm} is predominantly present (84–98%) as NH_4^+ (Ip et al, 2004b). The capability to maintain a low plasma NH_4^+ concentration as seen in this study during exposure to millimolar water NH_3 has been previously demonstrated for African catfish (Ip et al, 2004b): plasma NH_4^+ concentrations after 5 days exposure to 0.69 mM ambient NH_3 came to 2.12 mM, a value approximately 10 times higher than reported here after 34 days exposure of the same species to up to 1084 μM NH_3. This suggests that in the African catfish exposure to high water NH_3 initially results in a plasma NH_4^+ peak due to an NH_3 influx, followed by the on-set of NH_3 defense mechanisms over time and a subsequent decline of plasma NH_4^+ concentrations to basal levels. Time-kinetic studies are needed to exactly define this pattern for

African catfish, but our results do support a successful acclimation to rather extreme NH_3 concentrations.

African catfish actively excretes NH_4^+ against an inward concentration gradient as the major defense mechanism against ammonia toxicity (Ip et al, 2004a; Ip et al, 2004b). The low plasma NH_4^+ levels observed in the present study can possibly be attributed to this mechanism. Indeed, as sodium is a counter ion in this NH_4^+ export, the 5 g/L salinity in our experiments may have facilitated NH_4^+ excretion. We do not exclude contribution of other defense mechanisms (see Introduction), but we have no data to directly support such mechanisms.

The maintenance of low plasma NH_4^+ at a chronically high water ammonia concentration is exceptional among farmed fish species. Plasma ammonia concentrations in Atlantic salmon (*Salmo salar*) and European seabass (*Dicentrarchus labrax*) were found to increase linearly up to 1 mM with chronic exposure to high water ammonia with no signs of rebound or acclimation (Knoph and Thorud, 1996; Lemarié et al, 2004).

4.2 Plasma cortisol, glucose and lactate

Plasma cortisol concentrations were (typically) rather variable among individual fish and no significant differences were found among treatments (Table 3). In fish, acute stress results in rapid 10 to 100 fold increase of the plasma cortisol concentration, followed by a return to basal concentrations within hours. Basal concentrations are generally low but variation among life stages, sexes, individuals within a population, and species exist (Wendelaar Bonga, 1997). In case of chronic stress, such as high water ammonia, plasma cortisol concentrations may remain elevated above basal concentrations, although at lower concentrations than the concentrations associated with acute stress (Wendelaar Bonga, 1997). In the present study, mean plasma cortisol concentrations ranged from 30.0 to 72.9 nM, well below a basal concentration of 122.8 nM (45.5 ng/mL) reported earlier for the same species (Martins et al, 2006a; Martins et al, 2006b). We conclude that the current experimental design did not induce chronic stress in the fish, except maybe for the highest NH_3 group. The lower plasma cortisol values of the 1084 µM NH_3 treatment group could be interpreted as an exhaustion of the pituitary–inter-renal-axis as a prolonged hyperactivity of this system (Hontela et al, 1992). This is supported by the observation of significantly elevated plasma glucose in the 1084 µM NH_3 treatment; the effects are still mild, corroborated by no more than a tendency for plasma lactate concentrations to go up in this treatment. High plasma glucose and lactate concentrations are both common to stressed fish

(Wendelaar Bonga, 1997). The elevated plasma glucose concentration in 1084 μM NH_3 treatment can possibly be explained by the energy demand required to fuel active NH_4^+ excretion. The elevated plasma lactate concentrations in this treatment suggest the use of lactate as substrate for gluconeogenesis (Wendelaar Bonga, 1997). This adaptation of the energy metabolism may be related to the energy demand of active NH_4^+ excretion while energy (food) intake is reduced (Table 4).

4.3 Plasma osmolality

Plasma osmolality was very similar among the 4 to 176 μM NH_3 treatments and ranged from 267.3 to 278.3 mOsmol/kg. Compared to these treatments the plasma osmolality was significantly elevated in the 1084 μM NH_3 treatment to 347 mOsmol/kg (Table 3). Teleostean fishes are strong regulators, tightly regulating their plasma osmolality in a species-dependent range of salinities (Varsamos et al, 2005). The water osmolality among treatments in this study was essentially constant (see Section 2.1 and Table 1) and no significant differences in conductivity of the water were observed among treatments (Table 2). The differences in plasma osmolality must therefore be attributed to the experimental treatments. Unfortunately, we ran out of plasma (the samples compromised the economical pHOx-equipment we normally use for plasma mineral analysis) and therefore we cannot support plasma osmolality data with sodium and chloride values. However, the elevated plasma osmolality in the 1084 μM NH_3 treatment group could very well be a consequence of facilitated influx of NaCl to the blood in exchange for NH_4^+ excretion as the higher (compared to normal fresh water) ambient Na^+ (1.6 g/L) and Cl^- (2.5 g/L) levels in the tanks would favor this. On the other hand, reduced ion exchange with the environment may decrease plasma volume and increase of plasma osmolality (Wendelaar Bonga, 1997), and this could also explain this observation.

4.4 Gill morphology/Na^+/K^+-ATPase activity

The branchial epithelium is where gas exchange, ion regulation, acid–base balance and nitrogenous-waste excretion occur (Evans et al, 2005). The direct contact with the medium and delicate structure make gills vulnerable for water pollutants and a sensitive site to develop anomalies (Erkmen and Kolankaya, 2000), such as epithelial hypertrophy, epithelial lifting, necrosis and hyperplasia with lamellar fusion (Evans, 1987; Erkmen and Kolankaya, 2000).

The gradual changes in gill morphology observed with increasing water ammonia concentrations culminated in drastic lesions in the 1084 μM NH_3 treatment group. Similar morphological impairments were observed in an Anatolian khramulya (*Capoeta tinca*) population living in a stream with high concentrations of water pollutants, including NH_3 (58 μM; Erkmen and Kolankaya, 2000). These histopathological changes may underlie a disrupted ion transport (Ip et al, 2004a). The epithelium from the filaments is known to be more permeable than the lamellar epithelium, due to the presence of 'tight junctions' that require strict regulation to guarantee epithelial permeability to water and ions (Evans, 1987). One should further consider that the filamental epithelium harbors the chloride cells that facilitate the major part of ion transports in the gills, and that the inherent cellular make-up involves specific junctional complexes to seal the epithelium as required for the transports taking place; in control lamellar epithelium chloride cells are normally absent and thus one may predict a differential permeability in filamental and lamellar epithelium. Hypertrophy of the filament epithelium may represent an adaptive response to increase a barrier to reduce the inflow of NH_3. Hypertrophy of the epithelium associated with lamellar fusions, as well as mucous cells proliferation can be interpreted as adaptations to increase the distance between the water and the blood flow, reducing the permeability of the gills.

Whereas chloride cell migration towards the lamellae expands the transport capacity to facilitate ion exchange, it may simultaneously increase branchial permeability, as it potentially extends the leakiness of the branchial epithelium as a whole. Clearly a balance needs to be made up to weigh contributions of extra ion transport capacity (more chloride cells) and an enhanced branchial surface with increased permeability (and thus passive movement of water and ions) to the adaptive response seen.

Gills are the predominant place for NH_3 excretion in freshwater fish (Evans et al, 2005; Wilkie, 2002). Chloride cells in the branchial epithelium are the site of active excretion of NH_4^+ against an inwardly directed electrochemical gradient of ammonia, through the Na^+/K^+-ATPase (1 ATP: $2K^+$ or NH_4^+: 3 Na^+) (reviewed by Heisler, 1984; Evans, 1987; Evans et al, 2005; Ip et al, 2004a) at the basolateral plasma membrane of the chloride cells; NH_4^+ may be exchanged at the apical membrane for waterborne chloride. As described before, African catfish are able to maintain a relatively low plasma NH_4^+ concentration despite the high external ammonia concentration (Ip et al, 2004b). In this condition, it is assumed that as the external ammonia concentration increases, the active excretion of NH_4^+ is enhanced. This is supported by an increased, energized Na^+/K^+-ATPase mediated export in the 1084 μM treatment group.

4.5 Feed intake, specific growth rate, feed conversion rate and mortality

The absence of mortality in this experiment shows that the lethal concentration for NH_3 for chronic exposure lies above 1084 µM. The 96 h LC_{50} for African catfish is reported to be as high as 380 mM for T_{Amm} (Britz, 1988 in Ip et al, 2004b). However the pH at which the acute toxicity was tested is not reported, and this hinders comparison just based on NH_3 of our study with these studies.

In the first week of the experiment feed intake was similar among treatments (Fig. 2). This we attribute to the experimental design where we have chosen to gradually built-up the water ammonia during the first 4 days of the experiment.

Surprisingly, a higher total feed intake was observed in the 14 and 38 µM NH_3 treatments compared to the control treatment. We hypothesize that the fish overcompensated for the extra energy demand associated to the active excretion of NH_4^+ by increasing their feed intake, a phenomenon described as hormesis (Calabrese, 2005).

The cumulative feed intake in the 176 µM NH_3 treatment and the control were equal until approximately day 25 of the experiment and after day 25, cumulative feed intake was lower in the 176 µM NH_3 treatment (Fig. 2). This suggests that the fish exposed to 176 µM NH_3 were aiming to maintain normal feed intake, but were unable to sustain this during prolonged ammonia exposure. Overall this resulted in a lower total feed intake in the 176 µM NH_3 treatment compared to the control (Table 3).

Total feed intake and SGR were lower in the 176 µM NH_3 treatment compared to the 14 and 38 µM NH_3 treatments. Since the feed conversion rate was equal among these treatments, the lower SGR in the 176 µM NH_3 treatment must be attributed to reduced feed intake rather than reduced feed utilization. This notion is corroborated by studies on turbot, where it was found that NH_3 reduces growth as a result of reduced feed intake (Person-Le Ruyet et al, 1997).

Total feed intake and SGR were negatively affected in the fish exposed to the highest NH_3 concentration: exposure to 1084 µM NH_3 resulted in a 58% lower total cumulative feed intake and a 57% lower SGR compared to the control treatment. Fish exposed to 1084 µM NH_3 showed a higher FCR compared to the fish in all other treatments. This suggests a high energy demand for the maintenance of low plasma NH_4^+ concentrations at high water ammonia at the cost of growth.

4.6 NH_3 threshold concentrations

African catfish chronically exposed to NH_3 concentrations as high as 176 µM (2.5 mg NH_3-N/L) did not show major physiological disturbances, except for gill morphology. This suggests that the threshold NH_3 concentration for physiological disturbance is at least 176 µM. However, both feed intake and specific growth rates were found to be reduced at much lower NH_3 concentrations: the EC_{10} for NH_3 was found to be 89 µM for feed intake and 122 µM for SGR.

The physiological parameters measured in this experiment include both primary (plasma cortisol) and secondary (plasma glucose and plasma lactate) stress responses. Feed intake and growth rate are tertiary stress responses (Wendelaar Bonga, 1997). The observed effects on feed intake and growth in the absence of major physiological disturbances are therefore surprising, but indicate that the priority for the fish apparently lies in proper stress regulation over regulation of growth. Feed intake and growth are thus good indicators for negative effects of high water ammonia on African catfish, in particular when evaluating chronic suboptimal conditions. The threshold concentration for chronic NH_3 exposure of African catfish is in our view best based on NH_3 effects on feed intake and growth. Considering that the lowest EC_{10} value was obtained for feed intake and taking into account the lower limit of its 95% confidence interval, the NH_3 threshold concentration should preferably be set at 24 µM (0.34 mg NH_3-N/L). At this NH_3 concentration the risk of a 10% reduction in feed intake is 5% maximally and growth will not be compromised.

5. Conclusions

This study clearly demonstrates that plasma levels of NH_4^+, cortisol, glucose and lactate, as well as plasma osmolality are not indicators of first choice for chronic toxicity of high water ammonia in African catfish, *Clarias gariepinus*. We advise for African catfish not to exceed a water NH_3 concentration of 24 µM (0.34 mg NH_3-N/L) to reduce the risk of reduced growth and feed intake. Below this NH_3 threshold concentration less obvious, but potentially dangerous disturbances such as deteriorated gill fine structures are avoided.

Acknowledgements

This research project has been carried out within the Policy Supporting Research for the Ministry of Agriculture, Nature and Food Quality (BO-07-011-028).

The authors would like to express their gratitude to Edwin Foekema, IMARES Wageningen UR, for assisting in the construction of the concentration–effect curves, to Femke Geurds for technical assistance and to Fleuren-Nooijen BV, Someren, The Netherlands, for supplying the experimental fish.

Chapter 6

The impact of elevated water nitrate concentration on physiology, growth and feed intake of African catfish *Clarias gariepinus* (Burchell 1822)

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In: Aquaculture Research, *in press*. DOI: 10.1111/are.12098

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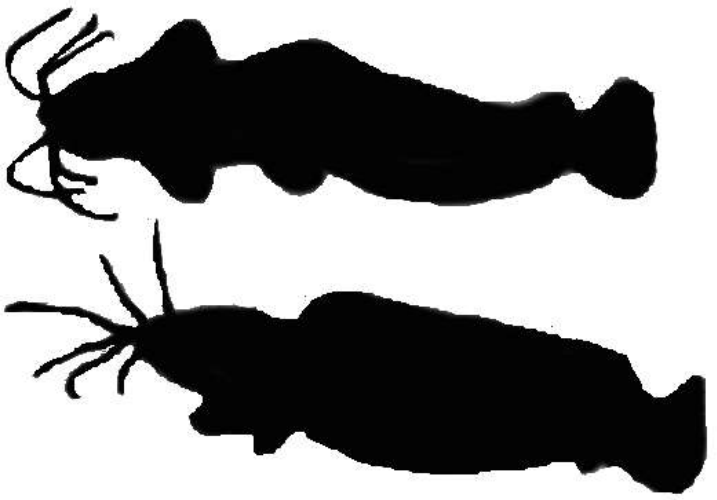

Abstract

The nitrate threshold concentration in rearing water of African catfish (*Clarias gariepinus*) was assessed. Female African catfish with an initial mean (SD) weight of 154.3 (7.5) g were exposed to 0.4 (Control), 1.5, 4.2, 9.7 and 27.0 mM nitrate for 42 days. Mean (SD) plasma concentrations of nitrate increased from 71 (29) to 6623 (921) μM at the highest ambient nitrate level. Mean (SD) plasma nitrite concentration ranged from 1.2 (0.5) to 7.9 (9.0) μM. Hematocrit, plasma concentrations of non-esterified fatty acids (NEFA), cortisol, glucose, lactate, osmolality, gill morphology and branchial Na^+/K^+-ATPase activity were not affected. Feed intake and specific growth rate were significantly reduced at the highest nitrate concentration. We advise not to exceed a water nitrate concentration of 10 mM (140 mg/L NO_3^--N) to prevent the risk of reduced growth and feed intake in African catfish aquaculture.

1. Introduction

Aquatic organisms risk exposure to toxic levels of nitrate in their natural environment due to agricultural application of fertilizers (Bouchard et al, 1992) and in intensive aquaculture when recirculation systems (RAS) are used (Van Rijn, 2010). Nitrate is toxic to teleosts. Camargo and colleagues (2005) suggested, in analogy to the mechanism of nitrite toxicity (Eddy and Williams, 1987; Williams et al, 1993), that nitrate toxicity is due to conversion of hemoglobin to methemoglobin and the inherent loss of oxygen carrier capacity. Uptake of nitrate via the integument from the water by aquatic animals is low compared to that of nitrite. In fish this is attributed to an apparent low branchial permeability to nitrate (Stormer et al, 1996); similar conclusions were reached for gill-breathing crustacean species (Jensen, 1996; Cheng and Chen, 2002). Nitrate is therefore considered to be less toxic than nitrite. Acute and chronic effects of nitrate exposure were studied in freshwater channel catfish (*Ictalurus punctatus*): the lethal nitrate concentration (96h LC_{50} at 26°C) was as high as 105 mM (Colt and Tchobanoglous, 1976). No obvious adverse effects were observed during 164 days exposure of channel catfish to 6.4 mM nitrate (Knepp and Arkin, 1973).

African catfish (*Clarias gariepinus*) is commercially farmed in intensive RAS in The Netherlands. In RAS ammonia is converted to nitrate in aerobic biological filters. Nitrate accumulates in the system (Bovendeur et al, 1987; Eding et al, 2006) and fish farmed in RAS may be chronically exposed to nitrate levels ranging from 7 to 70 mM (100-1000 mg N/L) depending on RAS design and management (Van Rijn, 2010). African catfish are typically exposed to nitrate levels around 7 mM (Verreth and Eding, 1993). The effects of this chronic nitrate exposure on African catfish physiology are not known. We exposed African catfish to increased water nitrate levels for 42 days and the nitrate threshold concentration was assessed.

2. Materials and methods

2.1 Experimental conditions

Female African catfish (*Clarias gariepinus*) were obtained from Fleuren-Nooijen BV, Someren, The Netherlands. Fish (n =208) were randomly divided over 16 30-L rectangular glass, dark covered, tanks and acclimatized to the experimental tanks for 7 days. At the start of the 42 day experiment, the overall initial mean (SD) individual weight was 154.3 (7.5) g. The resulting mean stocking density was 66.9 kg/m^3, well below fish densities found at

commercial farms for this size class (100 to 300 kg/m^3, Van de Nieuwegiessen et al, 2009).

The treatment of the fish was in accordance with Dutch law concerning animal welfare, as tested by the ethical committee for animal experimentation of Wageningen UR Livestock Research (number 2011015.c).

The experiment consisted of 8 duplicated treatments. Treatments were assigned randomly to the tanks. Treatment 1 was included to collect blood and plasma at the start of the experiment (t = 0). Fish in treatments 2 to 6 were exposed to one of five different nitrate concentrations in the water: 0.4 (control), 1.5, 4.2, 9.7 and 27.0 mM. Fish in treatment 7 (pair-fed) were kept at 0.4 mM nitrate and pair-fed to the fish kept in 27.0 mM nitrate (treatment 6) to discriminate between effects caused by low feed intake and exposure to a high nitrate concentration in the water. Fish in treatment 8 (chloride) were exposed to high nitrate (22.5 mM) in the presence of sodium-chloride (2.4 g/L) to evaluate a potential attenuating effect of chloride (and sodium) on nitrate toxicity, similar to nitrite toxicity (Eddy et al, 1983).

During acclimatization and experimental periods, all tanks were supplied with local tap water via a header tank at a flow of 185 L per day. Experimental nitrate concentrations were realized by infusion of $NaNO_3$ stock solutions prepared in tap water (Table 1), which were pumped into the tanks by a peristaltic pump (Watson Marlow 505 S; Rotterdam, The Netherlands) at a flow of 4.75 L per day per tank. Each tank was equipped with an air stone to guaranty good mixing of the infused stock solution with the tank water. Flow rates were monitored daily and adjusted when necessary to reach the desired nitrate concentrations. Nitrate concentrations were gradually increased to the designated concentrations during the first 4 days of the experimental period. Fresh stock solutions (Table 1) were prepared daily during the first 11 days of the experimental period and in this period nitrate, nitrite and total ammonia (NH_4^+-N plus NH_3-N) concentrations were monitored daily (Spectroquant cell tests for NO_3^--N, NO_2^--N and NH_4^+-N, Merck, Darmstadt, Germany, in a Hach Lange DR2800 spectro-photometer, Germany). During the remainder of the experimental period fresh stock solutions were prepared weekly and nitrate, nitrite (Table 1) and total ammonia concentrations (0.33- 0.57 mM) monitored twice per week. Water temperature (25.4-25.6°C), pH (Table 1) and dissolved oxygen concentrations (0.27-0.39 mM) were monitored daily prior to feeding in all tanks (Hach Lange HQ 40 multimeter, Germany) throughout the entire experiment.

Treatment	$NaNO_3$ stock	NaCl stock	Pred. tank $[NO_3^-]$	Pred. tank $[Na^+]$	Pred. tank $[Cl^-]$	Pred. tank salinity	$[NO_3$-N]	$[NO_3$-N]	$[NO_2$-N]	pH range
	(g/L)	(g/L)	(mM)	(mM)	(mM)	(g/L)	(mg/L)	(mM)	(µM)	
2-control	0	0	0	0	0	0	5.5 (0.31)	0.39 (0.02)	4.6 (1.6)	7.30-8.06
3	3.5	0	1.07	1.07	0	0.02	20.5 (0.51)	1.46 (0.04)	8.5 (2.9)	7.35-7.75
4	10.6	0	3.21	3.21	0	0.05	59.0 (8.86)	4.21 (0.63)	7.0 (3.6)	7.36-7.92
5	31.9	0	9.64	9.64	0	0.14	135.8 (10.2)	9.69 (0.73)	7.3 (2.6)	7.35-7.76
6	95.6	0	28.9	28.9	0	0.41	378.8 (32.8)	27.04 (2.34)	10.2 (1.9)	7.40-7.89
7-pair-fed	0	0	0	0	0	0	5.6 (0.23)	0.40 (0.02)	7.1 (3.3)	7.39-7.69
8-chloride	95.6	77.8	28.9	63.2	34.2	2.4	315.0 (25.3)	22.49 (1.80)	3.5 (1.3)	7.55-7.99

Table 1. Composition of the treatment specific stock solutions, the predicted[a] nitrate, sodium and chloride concentrations and the mean (SD) values for measured nitrate and nitrite concentrations in the tanks for all treatments.
[a] Based on equal flow rates per tank of 4.75 L/d for the stock solutions and 185 L/day for the tap water flow.

2.2 Blood and plasma sampling

One day before exposure to nitrate started (day 0), fish in treatment 1 were sampled. After 42 days exposure to nitrate, the fish from the seven remaining treatments were sampled (13 fish per tank). Fish were rapidly netted and anaesthetized in 0.1% (v/v) 2-phenoxyethanol (Sigma, St. Louis, USA). Within 2 min, blood (2 x 1.0 mL) was taken by puncture of the caudal vessels by the use of a heparinized syringe fitted with a 25-gauge needle. One 50 µl aliquot was used for the hematocrit measurement, the remainder was immediately centrifuged for 10 min (14,000 g, 4°C) and the plasma so obtained stored at -20°C until further analyses.

2.3 Plasma nitrate and nitrite concentration

NO_x (the sum of NO_2^- and NO_3^-) was measured with the nitrate/nitrite colorimetric assay kit from Cayman Chemical Company (Ann Arbor, Michigan, USA). Prior to measurement, plasma samples were ultra-filtrated using Millipore Ultra-free MC centrifugal filter device (0.1 µm pore size) to reduce background absorbance due to the presence of hemoglobin and improve color formation using the Griess reagents. Samples of 80 µL (in duplicate) were diluted in the assay buffer and then incubated for 3 h at room temperature with 10 µL of Enzyme Co-factor mixture and 10 µL of Nitrate reductase mixture. Fifty

µL of the first Griess reagent (R1) followed by the second Griess reagent (R2) were added and absorbance was read at 530 nm using a Wallac 1420 VICTOR^2 counter (Turku, Finland). The nitrite (NO_2^-) fraction within NO_x was measured using 100 µL of undiluted plasma together with the Griess reagents combination. The NO_3^- fraction was calculated as the difference between the plasma NO_x and the plasma NO_2^- concentrations.

2.4 Plasma concentrations of cortisol, glucose, lactate, plasma osmolality and branchial Na^+/K^+-ATPase activity

Plasma concentration of cortisol was determined by radioimmunoassay as described in detail by Metz and colleagues (2005). Plasma osmolality was measured using a cryoscopic osmometer (Osmomat 030, Gonotec, Germany). Plasma concentrations of glucose, lactate were measured with commercially available enzymatic kits adapted to 96 wells plates as described recently (Schram et al, 2010). Branchial Na^+/K^+-ATPase activity was measured as described by Metz and colleagues (2003).

2.5 Plasma NEFA concentration

Plasma non-esterified fatty acid (NEFA) concentration was measured with a commercial kit (Wako Chemicals GmbH, Neuss, Germany), with a protocol adapted to a 96 wells microplate. Four µL of sample or standard (0, 0.25, 0.50, 0.75 and 1 mM) were mixed with 225 µL of first reagent (Acyl-CoA synthetase (ACS), Coenzyme A (CoA) and ATP) and incubated 3 min at 37°C and absorbance was measured at 595 nm (sample blank measurement), followed by addition of 75 µL of the second reagent containing Acyl-CoA oxidase (ACOD), peroxidase (POD), Methyl-Ethyl-Hydroxymethyl-Alanine (MEHA); the plate was incubated for another 15 min and final absorbance read at 595 nm.

2.6 Gill morphology

From each sampled fish the second gill arch was removed immediately after blood sampling and placed overnight in Bouin's fixative (75 volumes saturated picric acid, 25 volumes saturated formaldehyde, and 5 volumes acetic acid) and embedded in paraffin. Gill sections were made to include the trailing edge of the filament where the chloride cells reside. Gill sections were immune-stained according to Dang and colleagues (2000) as described in detail for African catfish (Schram et al, 2010).

2.7 Blood hematocrit levels

Immediately after blood puncture, subsamples were drawn into glass capillaries and centrifuged (13,600 g; 3 min) to assess hematocrit values. Results were rounded to the closest 0.5%.

2.8 Specific growth rate, feed intake and feed conversion ratio

On day 0 and day 42, the fish in each tank were individually weighed (Mettler PM 34 Delta range) to the nearest 1 g, to calculate the specific growth rate (SGR) as follows:

$$SGR = (\ln(W_t) - \ln(W_0)) \times \frac{100}{t}$$

Where *SGR* = specific growth rate (% per day), W_t = mean weight at day 42 (g), W_0 = mean weight at day 0 (g) and *t* = number of days.

Floating feed (Catfish type Me-3; Skretting, Boxmeer, The Netherlands) with 49% crude protein and 11% crude lipids was given twice daily at 9:00 and 15:00 until apparent satiation (no more feed taken for at least five min following administration of the feed). Feed loads per tank were recorded daily. All uneaten pellets were collected from each tank 1 h after each of the two daily feeding sessions. Feed loss per tank was calculated as the total number of uneaten feed pellets multiplied by 0.0966 g per pellet, the average weight of a pellet, determined by weighing 100 feed pellets. Daily feed intake per tank resulted from the difference between daily feed load and daily feed loss. Daily feed intake per tank was divided by the number of fish in the tank to calculate the daily feed intake per fish in each tank. For each tank the total feed intake per fish (TFI) was determined by summation of daily feed intake per fish in each tank. Total feed intake per fish and biomass increase per fish were used to calculate feed conversion ratio (FCR) as follows:

$$FCR = \frac{TFI}{(W_t - W_0)}$$

Where *FCR* = feed conversion ratio (g/g), *TFI* = total feed intake (g/fish), W_t = mean individual weight at day 42 (g) and W_0 = mean individual weight at day 0 (g).

2.9 Statistics

Physiological parameters. Physiological parameters are expressed as mean (SD) of the individual measurements per treatment. For each treatment, 26 fishes

had been sampled; in some instances not all samples were analyzed because of insufficient plasma volume. When necessary, data were log-transformed to obtain residuals that were approximately normally distributed and to obtain homogeneity of variance of residuals across treatment levels. Mean values for physiological parameters were tested for differences among the treatments using linear mixed models (REML) with treatments as fixed effects and tank as a random effect (F-tests with Kenward-Roger approximation to the residual degrees of freedom (Kenward and Rogers, 1997)). Statistical analyses were performed in SAS 9.2 (SAS Institute Inc., Cary, North Carolina, USA). Only when significant treatment effects were detected, a least significance difference (LSD) post-hoc analysis was used to estimate the level of significance between mean values. For both REML and LSD analysis the fiducial limit was set at 5%.

Plasma nitrate concentrations and plasma nitrate to water nitrate ratios were related to water nitrate concentrations by linear regression analyses with water nitrate concentration as fixed effect and tank as a random effect. Plasma nitrite concentrations were related to water nitrite and plasma nitrate concentrations by linear regression analyses with either water nitrite or plasma nitrate concentration as fixed effect and tank as a random effect. Plasma chloride concentrations were related to plasma nitrate concentrations by linear regression analysis with plasma nitrate concentration as fixed effect and tank as a random effect. In all regression analyses F-tests with Kenward-Roger approximation to the residual degrees of freedom were used (Kenward and Rogers, 1997). The pair-fed and sodium chloride groups were not considered in regression analyses.

Feed intake and growth. Initial and final individual weight, total feed intake per fish (TFI), specific growth rate (SGR) and feed conversion ratio (FCR) are presented as means per treatment (N = 2). Mean values per treatment were tested for significant differences among the treatments by one-way ANOVA in SAS 9.2 (SAS Institute Inc., Cary, North Carolina, USA). Only in case significant treatment effects were detected, a least significance difference (LSD) post-hoc analysis was used to estimate the level of significance between mean values. For both ANOVA and LSD analysis the fiducial limit was set at 5%.

Concentration-effect curves and NOEC. Nitrate concentration-effect curves were fitted for specific growth rate (SGR) and total feed intake per fish (TFI) using a log-logistic model (Seefeldt et al*,* 1995). As a blank could not be included, the effects are expressed as absolute values. Curve-fitting was carried out with the Marquadt and Levenberg algorithm (Moré, 1978) as provided in the PRISM 4.00 software package (Graphpad Software, Inc.). The 10% effect concentrations

(EC_{10}) and their 95% confidence limits were calculated (Miller and Miller, 2000). No observed effect concentrations (NOEC) were determined for SGR and TFI as the highest nitrate concentrations in the experiment at which no significant difference with the control treatment were observed.

3. Results

3.1 Plasma nitrate and nitrite concentrations.

Nitrate concentrations in the water had a strong effect on plasma nitrate (NO_3^-) concentrations. Plasma nitrate concentrations were significantly different among all treatments (Table 2) and found to increase linearly with increasing nitrate concentrations in the water (Table 3). Pair-feeding nor (sodium) chloride addition to the water affected plasma nitrate levels (Table 2). Plasma nitrate to water nitrate ratios ranged from 0.15 to 0.25 among the nitrate treatments. The highest value (0.31) was observed in the high nitrate exposure level (22.5 mM) combined with sodium chloride. However, treatments effects on the plasma to water nitrate ratios were not detected (Tables 2 and 3). Plasma nitrite (NO_2^-) differed among treatments (Table 2) but at 8 μM the highest value observed is still low and only slightly higher than control values (3.4 μM).

The increase in plasma nitrite could not be attributed to differences in water nitrite concentrations (Table 3). Instead, the plasma nitrite concentration was found to increase linearly with increasing water nitrate and plasma nitrate concentrations (Table 3).

3.2 Plasma chloride and plasma osmolality

Significantly different plasma chloride concentrations were detected among treatments (Table 2). Plasma chloride concentrations were found to decrease linearly with increasing plasma nitrate concentration (Table 3). Plasma osmolality was unaffected by the treatments (Table 2).

3.3 Hematocrit, plasma cortisol, glucose, lactate and NEFA and branchial Na^+/K^+-ATPase activity.

As shown in Table 4, no significant differences in plasma concentrations of cortisol, glucose, lactate and NEFA, branchial Na^+/K^+-ATPase activity and hematocrit were observed. All values were within normal ranges previously reported for African catfish, *Clarias gariepinus* (Schram et al, 2010).

Treatment	Water NO_3 (mM)	Plasma NO_3 (µM)	n	Plasma NO_3/ Water NO_3	n	Plasma NO_2 (µM)	n	Plasma Cl^- (mM)	n	Na^+/K^+-ATPase activity (µM $Pi.h^{-1}$.mg $prot^{-1}$)	n	Plasma osmolality (mOsmol.kg^{-1})	n
1-t = 0	-	59 (14)	10	-	-	3.4 (1.0)	9	111.6 (12.8)	26	1.2 (0.4)	10	268.6 (5.5)	25
2-control	0.39	71 (29)^a	10	0.18 (0.07)	10	3.4 (2.8)^{ac}	10	108.7 (6.8)^a	26	1.4 (0.7)	9	269.3 (6.4)	26
3	1.46	282 (99)^b	10	0.19 (0.07)	10	2.3 (1.2)^{ab}	10	108.9 (11.5)^a	26	0.9 (0.5)	10	271.5 (6.6)	25
4	4.21	623 (202)^c	10	0.15 (0.05)	10	1.2 (0.5)^b	9	102.6 (7.6)^a	26	1.2 (0.5)	10	269.2 (6.3)	26
5	9.69	2076 (872)^d	12	0.21 (0.08)	12	4.9 (3.8)^{ac}	11	101.0 (9.8)^{ac}	26	1.0 (0.5)	9	267.6 (4.8)	26
6	27.04	6623 (921)^e	13	0.25 (0.05)	13	7.9 (9.0)^c	17	86.5 (9.7)^b	22	1.7 (1.0)	10	264.9 (7.2)	23
7-pair-fed	0.4	85 (26)^a	10	0.21 (0.06)	10	4.5 (2.3)^{ac}	10	113.5 (9.4)^a	23	1.2 (0.5)	9	267.6 (5.0)	23
8-chloride	22.49	7011 (996)^e	14	0.31 (0.03)	14	5.1 (4.3)^{ac}	14	89.1 (11.1)^{bc}	24	1.0 (0.7)	10	269.1 (5.7)	26
P-value		< 0.0001		0.2		0.049		0.01		0.7		0.47	

Table 2. Mean (SD) values at the start (t =0) and per treatment for the end (t = 42 days) of the experiment for plasma NO_3^-, plasma NO_3^- to water NO_3^- ratio, plasma NO_2^-, plasma Cl^-, branchial Na^+/K^+-ATPase activity and plasma osmolality. Mean values with different superscripts are significantly different (REML, P values as shown). SD = standard deviation of mean values per treatment, n as indicated in the table. t = 0 values were not considered in the statistical analysis.

Response variable	Explanatory variable	Regression coefficient		Intercept	
		Estimate	P value*	Estimate	P-value
Plasma nitrate (mM)	Water nitrate (mM)	0.2442	< 0.0001	-0.1857	0.52
Plasma chloride (mM)	Plasma nitrate (mM)	-3.601	0.0005	108.06	<0.0001
Plasma nitrate to water nitrate ratio	Water nitrate	0.0026	0.14	0.171	<0.0001
Plasma nitrite (µM)	Water nitrite (µM)	0.152	0.56	2.51	0.25
Plasma nitrite (µM)	Plasma nitrate (µM)	0.000624	0.0075	2.42	0.0020
Plasma nitrite (µM)	Water nitrate (µM)	0.000130	0.026	2.48	0.0037

*) equals model P value

Table 3. Results of linear regression analyses with tanks as random effect.

Treatment	Water NO_3 (mM)	Cortisol (nM)	n	Glucose (mM)	n	Lactate (mM)	n	NEFA (mM)	n	Hematocrit (%)	n
1-t = 0	-	15.2 (25.2)	22	3.28 (1.1)	26	4.79 (1.0)	26	0.17 (0.07)	21	-	
2-control	0.39	9.6 (6.2)	22	3.54 (1.0)	26	4.97 (1.2)	26	0.17 (0.11)	18	32.1 (4.3)	24
3	1.46	14.2 (9.8)	24	3.72 (0.9)	26	5.02 (1.1)	26	0.22 (0.11)	18	29.0 (5.1)	26
4	4.21	20.4 (16.3)	25	2.89 (0.9)	26	4.84 (0.9)	26	0.23 (0.14)	20	27.0 (3.9)	25
5	9.69	10.8 (12.8)	23	3.00 (0.4)	26	4.96 (0.9)	26	0.15 (0.07)	19	28.8 (5.3)	26
6	27.04	19.5 (16.7)	23	2.92 (0.5)	23	5.51 (1.5)	23	0.22 (0.13)	16	32.4 (5.4)	23
7-pair-fed	0.4	17.6 (11.0)	23	3.36 (0.8)	20	4.10 (0.7)	23	0.23 (0.12)	22	25.7 (4.8)	22
8-chloride	22.49	20.3 (12.7)	25	2.58 (0.4)	25	5.61 (1.2)	25	0.24 (0.11)	25	31.7 (4.5)	23
P-value		0.71		0.43		0.3		0.54		0.08	

Table 4. Mean (SD) values at the start (t =0) and per treatment for the end (t = 42 days) of the experiment for plasma cortisol, plasma glucose, plasma lactate and NEFA concentrations and hematocrit. Mean values with different superscripts are significantly different (REML, P values as shown). SD = standard deviation of mean values per treatment, n as indicated in the table. t = 0 values were not considered in the statistical analysis.

Treatment	Water NO_3 (mM)	Initial weight (g)	Final weight (g)	TFI (g/fish)	SGR (%BW/d)	FCR	Survival (%)
1-t = 0	-	152.6 (3.1)	-	-	-	-	-
2-Control	0.39	153.6 (9.0)	495.5 (5.6)^{a}	262.4 (0.6)^{a}	2.93 (0.18)^{a}	0.77 (0.03)^{a}	100 (0)
3	1.46	155.1 (12.4)	480.3 (12.8)^{a}	251.4 (4.4)^{a}	2.83 (0.13)^{a}	0.77 (0.01)^{ab}	100 (0)
4	4.21	157.2 (5.7)	483.3 (28.6)^{a}	252.9 (17.9)^{a}	2.81 (0.06)^{a}	0.78 (0.00)^{ab}	100 (0)
5	9.69	151.6 (7.2)	464.7 (48.9)^{a}	246.9 (23.6)^{a}	2.80 (0.14)^{a}	0.79 (0.03)^{ab}	100 (0)
6	27.04	149.4 (9.6)	303.5 (91.7)^{b}	127.3 (60.8)^{b}	1.72 (0.60)^{b}	0.84 (0.05)^{b}	88 (16)
7-pair-fed	0.4	155.0 (5.4)	330.9 (16.5)^{b}	136.0 (12.9)^{b}	1.90 (0.21)^{b}	0.77 (0.02)^{ab}	88 (16)
8-chloride	22.49	158.3 (13.4)	278.9 (31.6)^{b}	111.4 (19.5)^{b}	1.41 (0.07)^{b}	0.92 (0.02)^{c}	100 (0)
P-value		0.96	0.004	0.002	0.002	0.009	0.58

Table 5. Mean (SD) values per treatment (N = 2) for initial weight, final weight, total feed intake, specific growth rate (SGR), feed conversion ratio (FCR) and survival rate. Mean values with different superscripts are significantly different (one-way ANOVA, P values as shown). SD = standard deviation of mean values per treatment.

3.4 Gill morphology.

Gill morphology, presented for controls (0.39 mM NO_3^-), pair-fed (0.40 mM NO_3^-), highest (27.0 mM NO_3^-) and chloride (22.5 mM NO_3^-), was not affected by nitrate exposure (Fig. 1). The number of chloride cells, nor their location within the gills were affected (data not shown).

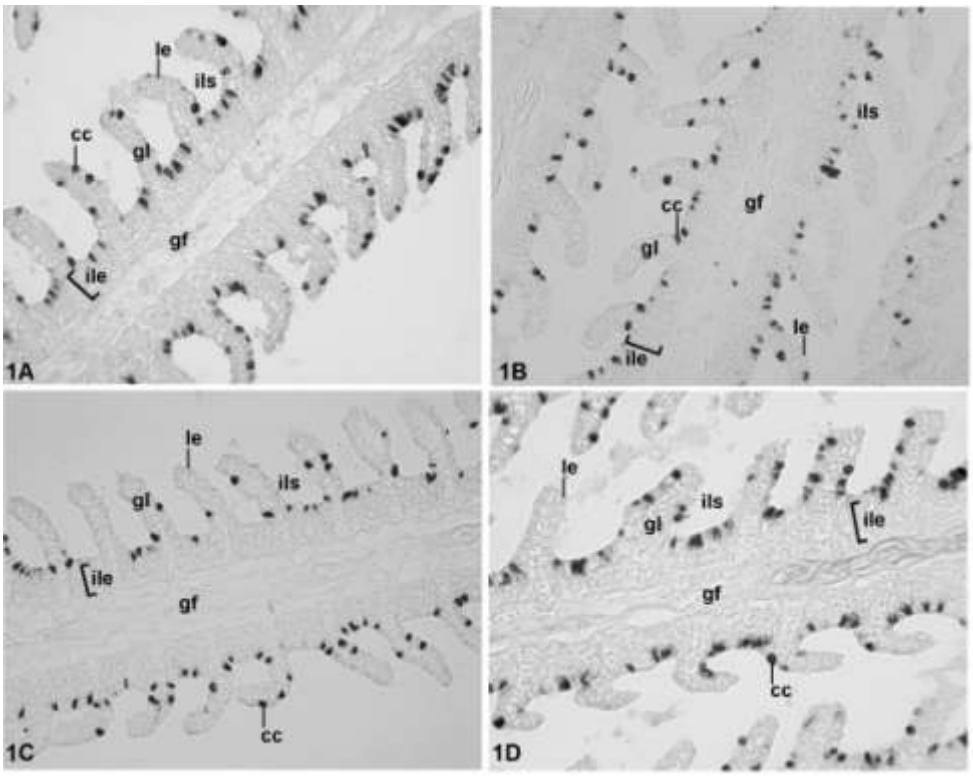

Fig. 1. Histology of gill epithelium immunohistochemically stained for Na^+/K^+ ATPase-rich cells (chloride cells) of the control (3A), pair fed (3B), 27.0 mM NO_3 (3C) and 22.5 mM NO_3 in addition with NaCl (3D) treatment groups. No effects on the gill's morphology with increasing water nitrate level were observed. Legend: ile = inter-lamellar epithelium, le = lamellar epithelium, ils = inter-lamellar space, cc = chloride cell, gf = gill filament, gl = gill lamellae.

3.5 Feed intake, specific growth rate, feed conversion rate and mortality.

Four fish died during the experiment out of a total of 208 fish. Death did not result in significantly different survival rates among treatments (Table 5). Total feed intake per fish differed among treatments (Table 5). At the highest nitrate level (27.0 mM) and the high nitrate exposure level (22.5 mM) combined with sodium chloride, the total feed intake per fish was reduced by

over 50% compared to the groups exposed to nitrate levels of 9.7 mM or lower. The differences in feed intake among treatments became apparent already 1 day after the highest nitrate exposure concentration had been reached (Fig. 2).

Specific growth rate (SGR) differed among treatments (Table 5). At the highest nitrate exposure level (27.0 mM), in the pair-fed group and at the high nitrate exposure level (22.5 mM) combined with sodium chloride, SGR was significantly reduced by at least 35% compared to the groups exposed to nitrate levels of 9.7 mM and lower. The highest value for feed conversion ratio was seen in the 22.5 mM-NO_3^- with sodium-chloride group (0.92); the FCR of 0.84 at the highest nitrate exposure level (27.0 mM) was not different from the values calculated for pair-fed controls.

3.6 EC_{10} and NOEC for total feed intake and SGR

The concentration-effect curves for TFI and SGR in relation to the water NO_3 concentration (Figs. 3A and 3B), yield and EC_{10} for NO_3^- of 22 mM (312 mg/L NO_3^--N), with a 95% confidence interval from 20 to 25 mM when read against TFI. For SGR, a very similar EC_{10} for NO_3^- of 23 mM (321 mg/L NO_3^--N), with a 95% confidence interval from 21 to 26 mM was calculated. For both SGR and TFI the highest test concentration at which no significant difference with the lowest nitrate exposure concentration was observed (NOEC) was 9.7 mM.

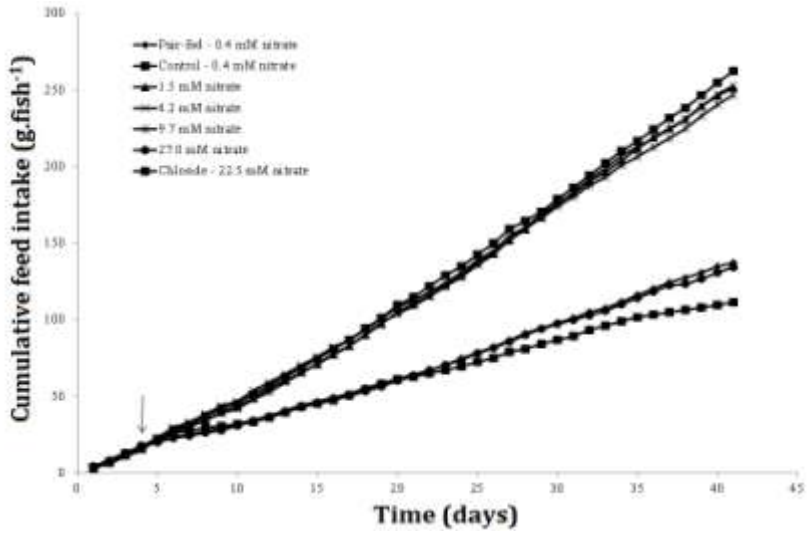

Fig. 2. Mean (N=2) cumulative feed intake per fish during the experimental nitrate exposure. The arrow indicates the first day at which all treatments reached their designated nitrate concentrations.

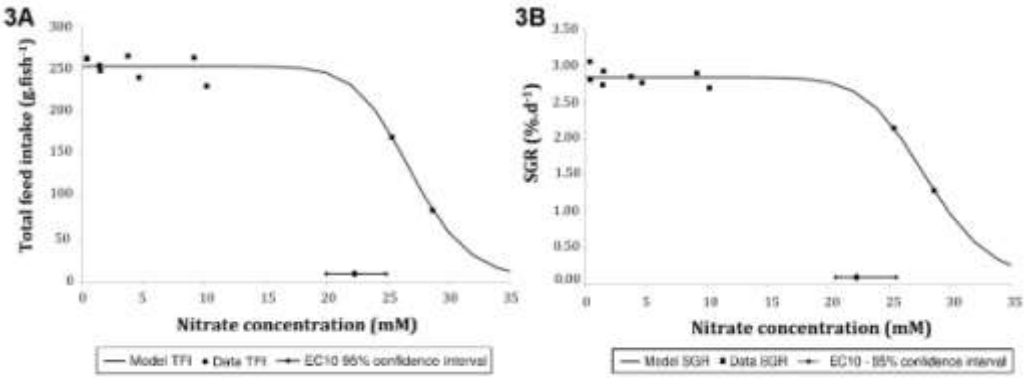

Fig 3. Concentration-effect curves for Total feed intake (TFI) (A) and specific growth rate (SGR) (B) in relation to the water NO_3 concentration($[NO_3]$, mM). TFI = $253.4\text{-}253.4/(1+10^{((1.432\text{-}\log[NO3])/0.0869)})$ and SGR = $2.84 - 2.84/(1+10^{((1.450\text{-}\log[NO3])/0.0936)})$. Pair-fed and chloride treatments were not included.

4. Discussion

4.1 Plasma nitrate and nitrite concentrations

In *Clarias gariepinus* plasma nitrate concentrations increase with increasing nitrate concentrations in the water. Nitrate transport mechanisms have not been established in fish to date. It has been suggested that nitrate is only passively moving across the gills of rainbow trout, *Oncorhynchus mykiss* (Stormer et al, 1996). In that study on trout, an ambient nitrate concentration of 1 mM resulted in a 5 times lower plasma concentration (0.2 mM). We observed very similar ambient to plasma nitrate ratio in our African catfish study, suggesting similar nitrate handling in both species. These low molar ratios between plasma nitrate and ambient nitrate suggest that that the integument forms a significant barrier to waterborne nitrate.

Nitrate has, *in vitro*, low, millimolar, affinity for branchial chloride carriers (Stormer et al, 1996). This is confirmed *in vivo* in our study as addition of (sodium) chloride to the water (next to high nitrate) did not affect plasma nitrate levels nor nitrate toxicity in African catfish.

As branchial nitrate permeability appears to be low, other nitrate uptake routes also need to be considered. Gastro-intestinal nitrate exposure via water ingestion may become significant when the fish starts drinking or ingests water during feeding. Intestinal uptake of nitrate has not yet been examined in fish but for nitrite this pathway is well documented in European flounder, *Platichthys flesus* (Grosell and Jensen 2000). Intestinal uptake may explain why turbot, a marine and therefore drinking species, is relatively sensitive to waterborne nitrate (Van Bussel et al, 2012).

From our studies we cannot discriminate a contribution of ingested nitrate to nitrate accumulation in the plasma but the reduced feed intake we observed in our fish exposed to high nitrate may be a behavioral adaptation aimed at reducing nitrate exposure via ingested water. Fish moisten dry feed pellets with water and this may contribute to intake of nitrate. Increased ionic levels in fresh water could further induce drinking (Hirano, 1974). It should be noted that the addition of the highest concentration of nitrate in this experiment was less than 30 mM $NaNO_3$, equivalent to an increase in water osmolality of about 60 mOsmol/kg. In media of such low osmolality passive/diffusional water influx may be significant and therefore drinking an unwanted behavior. Independently of what the route of entry is, apparently millimolar plasma concentrations of nitrate are tolerated by African catfish. This is in accordance with the consensus on the robustness of this fish in aquaculture.

4.2 Plasma osmolality, plasma chloride and gill morphology and Na^+/K^+-ATPase activity

High levels of nitrate in the water did not affect plasma osmolality in this catfish. Since Cl^- (with Na^+) is a major determinant of plasma osmolality, the hypochloremia (86.5 (9.7) mM *vs.* 108.7 (6.8) mM in controls) observed at the highest ambient nitrate levels could have induced a 20 mOsmol/kg decrease in osmolality of the plasma compartment. The high plasma nitrate levels (> 6 mM) may have compensated, at least partly, hypochloremic effects on plasma osmolality. Whereas nitrate transport is well known in plants and yeast (Orsel et al, 2002; Machín et al, 2004), to date, no literature seems to exist on nitrate transport proper in animal tissues. Nitrate may interfere and reduce chloride reabsorption in the distal mammalian nephron (Kahn et al, 1975), but specific nitrate transporter mechanisms have, to the best of our knowledge, not been demonstrated in animal osmoregulatory organs. The hypochloremia seen at the highest ambient nitrate levels and the coinciding millimolar plasma nitrate levels do suggest that plasma nitrate interferes with chloride handling. In a freshwater fish, branchial and intestinal chloride uptake from water and food and renal reabsorption are key to chloride homeostasis (Fuentes et al, 1997) and these are the sites where nitrate could interfere to explain the hypochloremia.

Nitrate exposure did not affect branchial Na^+/K^+-ATPase activity. The enzymatic activities measured in this experiment are somewhat lower than the values reported recently for the same species (Schram et al, 2010), which we

attribute to salinity differences between the current (0.4-2.4 g/L) and the previous ammonia study (4 g/L).

Apparently a significant hypernitratemia does not alter the activity if this enzyme. This corresponds to our observation that the number of Na^+/K^+-ATPase rich chloride cells was not affected by nitrate exposure. Nitrate exposure also did not cause morphological changes nor anomalies of the branchial epithelium. Morphological adaptations to reduce the permeability of the gills were observed when this fish species was exposed to increasing ambient ammonia concentrations (Schram et al, 2010). The results jointly taken indicate that high levels of nitrate do not affect permeability of the gills, neither to water or ionic species central to osmotic homeostasis, nor to nitrate itself (Stormer et al, 1996) as we conclude from the insignificant increase in molar ratios for nitrate in water and plasma; clearly, nitrate is not very toxic for this fish species.

4.3 Stress physiology

None of the stress and energy metabolite parameters (plasma cortisol, glucose, lactate and NEFA) were affected by nitrate. All parameters showed values within the range considered normal and reported before for the same species (Schram et al, 2010). Even the highest nitrate exposure concentration apparently did not impose distress.

4.4 Feeding

During the first 4 days of the experiment when nitrate concentrations were building up, feed intake was similar among all treatments. On day 5 however, when maximal nitrate levels had been reached , significant differences in feed intake showed up more or less instantaneously at the highest nitrate levels (22.5 and 27.0 mM). Feed intake instantly drops above 10 millimolar nitrate in the water; apparently a threshold is surpassed where appetite is lost. We conclude from this that nitrate is apparently 'sensed' by this fish. The effect of high ambient nitrate on feed intake appears direct and persistent, as feed intake dropped within 24 h and remained low for up to 42 days.

A pair-fed group was introduced to discriminate effects of nitrate exposure from potential effects of reduced feed intake. No significant differences were observed in physiological parameters for any treatment. However, feed intake, feed conversion ratio and specific growth rate were equal for catfish exposed to the highest nitrate level and their pair-fed controls. We conclude from this observation that high nitrate exposure must inflict upon appetite or feeding behavior. The highest feed conversion ratio (0.92) seen in

the group exposed to high nitrate concentration (22.5 mM) together with sodium chloride suggests that in these fish osmoregulatory activity imposes an extra energy demand.

4.5 Nitrate toxicity

Camargo and colleagues (2005) suggested that conversion of hemoglobin to methemoglobin and loss of oxygen carrying capacity is at the basis of nitrate toxicity, similar to the well-documented mechanism of nitrite toxicity (Eddy and Williams, 1987; Williams et al, 1993). Direct evidence for this mode of action for nitrate toxicity in fish is limited to one paper on rainbow trout (Grabda et al, 1974) and, for a similar action on hemocyanin in a penaeid shrimp (Cheng and Chen, 2002). In contrast, elevated methemoglobin was not observed in nitrate exposed rainbow trout in a study by Stormer and colleagues (1996). According to Bodansky (1951), nitrate is first reduced to nitrite before oxidizing hemoglobin to methaemoglobin. Unfortunately, we failed to determine methemoglobin in our nitrate-exposed catfish. However, the findings of Stormer and colleagues (1996), Bodansky (1951) together with the plasma nitrate and hematocrit data from our study do suggest though that other mechanisms for nitrate toxicity than direct hemoglobin oxidation need to be considered. Plasma nitrate levels of 7mM did not cause severe adverse effects in African catfish, while very similar plasma nitrite levels are reported to be lethal in fish (5 to 9 mM in channel catfish (Lewis and Morris, 1986), 8 mM in salmonids (Eddy et al, 1983). We conclude from this that it is unlikely that nitrate significantly converted hemoglobin to methaemoglobin. This notion is supported by our observation that nitrate exposure did not affect hematocrit levels. Decreased hematocrit was observed in nitrite exposed rainbow trout (Stormer et al, 1996) and common carp (*Cyprinus carpio*) (Jensen, 1990), an effect attributed to increased red blood cell turnover, due to nitrite-induced methemoglobinemia.

The slightly elevated plasma nitrite we observed in African catfish was related to plasma nitrate rather than water nitrite. Elevated plasma nitrite could originate from plasma nitrate when cells reduce nitrate to nitrite (Lundberg et al, 2008) which then appears in the plasma compartment, but mechanisms for such route have not been documented in fish to date (Sandvik et al, 2011). Our results indicate that nitrate-nitrite conversion could be present in African catfish. It is however not likely that nitrite induced methemoglobinemia is at the basis of treatment effects rather than the nitrate exposure. The presence of methemoglobin reductase inside the red blood cells (Cameron, 1971) would keep pace with any slightly elevated hemoglobin

oxidation rate caused by this slightly elevated plasma nitrite, maintaining methemoglobin low.

4.6 Nitrate threshold concentrations

African catfish chronically exposed to 27.0 mM ambient nitrate did not show major physiological disturbances. The threshold concentration for chronic nitrate exposure of African catfish is in our view therefore best based on nitrate effects on feed intake and growth. As only the two highest tested concentrations (22.5 and 27.0 mM) induced a significant effect, we propose to use the highest test concentration that showed no significant effect (no observed effect concentration, NOEC) as a safe threshold concentration for nitrate (9.7 mM, 140 mg/L NO_3^--N) and not the calculated EC_{10}.

5. Conclusions

Nitrate may be considered an end product and stable non-toxic form of nitrogenous waste, and this notion fits in with millimolar plasma levels of nitrate. Yet, ambient levels above 10 millimolar nitrate affected food intake and reduced growth, and rather instantly. We therefore advise for African catfish, *Clarias gariepinus* not to exceed a water nitrate concentration of 10 mM (140 mg/L NO_3^--N). Below this nitrate concentration physiological and growth disturbances are avoided. We further advise to avoid incidental high nitrate as African catfish may immediately stop feed intake. Mildly elevated salinity (by sodium-chloride addition) does not reduce effects of high nitrate exposure in African catfish.

Acknowledgements

The authors would like to express their gratitude to Marnix Gorissen, Jeroen Boerrigter, Remy Manuel and Erik de Vrieze for their technical assistance. This study was funded by the Dutch Ministry of Economic Affairs, Agriculture and Innovation (contract number BO-12.02-002-041.06.).

Chapter 7

The impact of elevated water nitrite concentration on physiology, growth and feed intake of African catfish, *Clarias gariepinus* (Burchell 1822)

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Aquaculture Research, in press. DOI: 10.1111/are.12292

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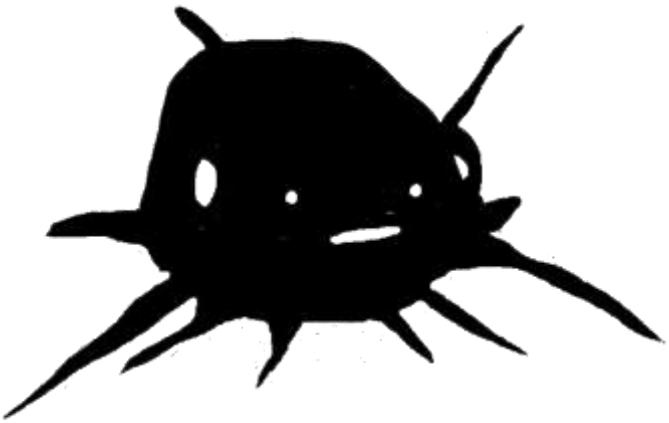

Abstract

The nitrite threshold concentration in rearing water of African catfish (*Clarias gariepinus*) was assessed. African catfish with an initial mean (SD) weight of 219.7 (57.8) g were exposed to a increasing range of water nitrite from 6 μM (Control) to 928 μM nitrite for 28 days. Mean (SD) plasma nitrite concentrations increased from 5.0 (3.6) to 32.5 (12.6) μM at 928 μM ambient nitrite. The increase in nitrite was accompanied by gradual increase in plasma nitrate from 41.6 (28.4) μM to 420.2 (106.4) μM. Hematocrit, hemoglobin, methemoglobin, plasma concentrations of cortisol, glucose, lactate, osmolality, gill morphology and branchial Na^+/K^+-ATPase activity were not affected. Feed intake, final weight, SGR, FCR and mortality were not affected. We advise not to exceed a water nitrite concentration of 43 μM (0.6 mg/L NO_2^--N) to prevent the risk of reduced growth and feed intake in African catfish aquaculture.

1. Introduction

The aquaculture industry has been expanding rapidly over the past four decades (FAO, 2012). In (intensive) recirculation systems (RAS), water is treated to allow the reuse of over 90% of the rearing water (Bovendeur et al, 1987; Eding et al, 2006). In RAS fish risk exposure to toxic levels of nitrite. Nitrogenous wastes in RAS are managed by nitrification (biological oxidation of ammonia to nitrate) and denitrification (biological reduction of nitrate to nitrogen gas) (Bovendeur et al, 1987; Eding et al, 2006; Van Rijn, 2010). Both processes can cause nitrite accumulation in the rearing water (Van Rijn and Rivera, 1990).

Nitrite is very toxic to organisms, as it converts hemoglobin (Hb) into methemoglobin (MethHb) that does not carry oxygen (Kiese, 1974). At high external nitrite levels, fish reduce their overall activity to reduce their oxygen requirement; this goes however with the cost of impaired growth and a series of vital functions (reviewed by Lewis and Morris 1986). In freshwater, nitrite enters the organism primarily via the chloride cells in the gills (Bath and Eddy 1980). Nitrite has an affinity for this branchial Cl^-/HCO_3^- exchanger and therefore can compete with the normal Cl^- uptake mechanism. This can lead to (partial) shift from Cl^- uptake to NO_2^- uptake when nitrite is present at high concentrations in the environment (Jensen, 2003). Therefore, fish with high branchial chloride uptake rates are more sensitive to nitrite toxicity than those with low uptake rates (Williams and Eddy, 1986). This pertains in particular to freshwater species, where active branchial uptake is the main route of entry of anions (Jensen, 2003). Elevated chloride levels in the water is known to alleviate nitrite toxicity, due to competitive effects on branchial nitrite uptake (Crawford and Allen, 1977). This protective effect is not uniform between species (reviewed by Lewis and Morris, 1986).

African catfish (*Clarias gariepinus*) is commercially farmed in intensive RAS in The Netherlands (Van Duijn et al, 2010). The global aquaculture production of African catfish has been increasing rapidly since 2000. In 2011 194.000 tons were farmed globally (FAO, 2011). The effects of chronic nitrite exposure on African catfish physiology and food intake are not known. Most of the studies on nitrite toxicity determined the median lethal dose, LD_{50} for several fish species with exposure times up to 120 h. Chronic exposure studies are scarce and mostly limited to purely toxicological effects, with no attention to effects on growth parameters, of interest to farmers (reviewed by Lewis and Morris, 1986; Kroupova et al, 2005). Hilmy and coworkers (1987) exposed African catfish for 6 months to 228 μM of nitrite ($1/10^{th}$ of their 96 median tolerance limit). They observed a decrease in erythrocytes counts, haemoglobin

content and production of methemoglobin. In our experimental design, we exposed African catfish to a range of nitrite concentrations that allowed us to see subtle but yet important effect on parameters of interest for fish farmers, without causing mortalities nor serious toxic effect.

For channel catfish, *Ictalurus punctatus*, the lowest concentration nitrite leading to growth suppression after 31 days was 115 μM (44% of the minimum concentration to induce mortality) (Colt et al, 1981). The minimum concentration causing mortality for this species is about half of the 96 h LC_{50} (Bowser et al, 1983). Therefore the minimum ambient concentration to of nitrite leading to detectable growth was set up at about one-fifth of the 96 h LC_{50} (Lewis and Morris, 1986). This information was taken into account when designing the experimental concentrations range. We exposed African catfish to increasing water nitrite concentrations (6 (Control), 111, 280, 459 and 928 μM) for 28 days to assess the nitrite threshold concentration for physiology, growth and food intake. In addition, we exposed two aquaria to high ambient nitrite concentrations (921 μM) in the addition on sodium-chloride (350 mg/L; 6 mM) to investigate the potential attenuating effect of chloride on nitrite toxicity.

2. Materials and methods

2.1 Experimental conditions

African catfish (*Clarias gariepinus*) were obtained from Fleuren-Nooijen BV, Someren, The Netherlands. The treatment of the fish in the laboratory was in accordance with Dutch law concerning animal welfare, as tested and approved by the ethical committee for animal experimentation of Wageningen UR Livestock Research.

Fish (n =192) were randomly divided over sixteen 30-L rectangular glass (12 fish per tank), dark covered tanks and acclimatized to the experimental tanks for 15 days. During this acclimatization period, some fish with skin damage (due to aggressive behavior; n=6) were removed, resulting in some variation in number of fish per tank (10 to 12). After this period of 15 days, the behavior of the fish had stabilized, and nitrite exposure commenced. At the start of the 28 days experiment, the overall initial mean (SD) weight was 219.7 (57.8) g. The mean stocking density was 84.7 kg/m^3, a value below densities practiced at commercial farms for this size class (100 to 300 kg/m^3, Van de Nieuwegiessen, et al, 2009).

The experiment consisted of 8 treatments, in duplicate. Treatments were assigned randomly to the 16 tanks. Treatment 1 was included to collect blood at the start of the experiment (t = 0). Fish in treatments 3 to 7 were

exposed to one of 5 different nitrite concentrations in the water: 6, 111, 280, 459 and 928 μM. Fish in treatment 2 (pair-fed) were kept at control nitrite levels and pair-fed to the fish kept at 928 μM nitrite (treatment 7) to discriminate between effects caused by low feed intake due to high nitrite in the water. Fish in treatment 8 (chloride) were exposed to high nitrite (921 μM) in the presence of sodium-chloride (350 mg/L; 6 mM) to evaluate a potential attenuating effect of chloride (and sodium) on nitrite toxicity (Eddy et al, 1983; Stormer et al, 1996; reviewed by Lewis and Morris 1986 and Kroupova et al, 2005). During acclimatization and experimental periods, all tanks were supplied with local tap water via a header tank at a flow of 185 L per day (chloride concentrations range: 0.4-0.7 μM, Vitens Watertechnologie, The Netherlands). Experimental nitrite concentrations were realized by infusion of $NaNO_2$ (Merk, Hohenbrunn, Germany) stock solutions prepared in tap water (Table 1), which were pumped into the tanks by a peristaltic pump (Watson Marlow 505 S; Rotterdam, The Netherlands) at a flow of 4.75 L per day per tank; each tank was equipped with an air stone positioned at the point of sodium nitrite inflow to guaranty good mixing of the infused stock solution with the tank water. Flow rates were monitored daily and adjusted when necessary to reach the desired nitrite concentrations. Nitrite concentrations were gradually increased to the desired concentrations during the first 4 days of the experimental period. Fresh stock solutions (Table 1) were prepared daily during the first 11 days of the experimental period and in this period nitrite (NO_2^--N), nitrate (NO_3^--N) and total ammonia (NH_4^+-N plus NH_3-N) concentrations were monitored daily (NitriVer 3 TNT Reagent Set, NitraVer X Nitrogen-Nitrate Reagent Set, Nitrogen-Ammonia TNT, AmVer Reagent Set, tests for NO_3^-, NO_2^- and NH_4^+, Hach Lange, Düsseldorf, Germany, in a Hach DR/890 colorimeter, Hach Lange, Düsseldorf, Germany). During the remainder of the experimental period fresh stock solutions were prepared weekly and nitrite, nitrate and total ammonia concentrations monitored twice per week. Water temperature, pH and dissolved oxygen concentrations were monitored daily prior to feeding in all tanks (Hach Lange HQ 40 multimeter, Düsseldorf, Germany) throughout the entire experiment. Mean (SD) water temperature was 25.7 (0.5) °C throughout the experimental period. Conductivity was measured with a WTW Cond 315i (WTW GmbH, Weilheim in Oberbayern, Germany), and presented in Table 1.

2.2 Blood sampling

On the day nitrite exposure started (day 0), fish in treatment 1 (t=0) were sampled. After 28 days of exposure to nitrite, the fish from the seven

remaining treatments were sampled (10-12 fish per tank). Fish were rapidly netted and anaesthetized in 0.1% (v/v) 2-phenoxyethanol (Sigma, St. Louis, USA). Within 2 min, blood (2 x 1.0 mL) was taken by puncture of the caudal vessels with a tuberculin syringe fitted with a 25-gauge needle; Na_2EDTA was used as anticoagulant. One 150 microtiter aliquot was immediately used for the hematocrit determination and hemoglobin/methemoglobin measurement. The remainder of blood was immediately centrifuged for 10 min (14,000 g, 4°C) and the plasma so obtained stored at -20°C until further analyses.

NO_2^- Treatment	Stock solutions	Predicted[a] water quality			Measured water quality			
	[$NaNO_2$] (g/L)	[NO_2^-] (µM)	[Na^+] (µM)	[Cl^-] (µM)	[NO_2^--N] (mg/L)	[NO_2^-] (µM)	Conductivity (mS/cm)	pH range
2 - Pair fed	0	0	0	0	0.93	66	448.4 (9.9)	7.27-7.80
3 – Control	0	0	0	0	0.10	6	488.0 (23.2)	7.06-7.73
4 – NO_2^-	2.58	89	89.3	0	1.55	111	492.5 (21.5)	7.01-7.80
5 – NO_2^-	5.15	179	178.6	0	3.92	280	484.5 (23.1)	7.28-7.79
6 – NO_2^-	10.30	357	357.1	0	6.43	459	514.4 (30.1)	7.40-7.87
7 – NO_2^-	20.60	715	714.3	0	13.0	928	545.8 (14.4)	7.28-7.80
8 – NO_2^- + NaCl	20.60	715	6361.1	5646.8	12.9	921	1300.3 (78.3)	7.01-7.88

Table 1. Composition of the treatment specific stock solutions, the predicted[a] nitrite and sodium concentrations, the predicted salinity in all treatments and the measured values per treatment for nitrite concentration, conductivity and the pH range.
[a] Based on equal flow rates per tank of 5 L/day for the stock solutions and 500 L/day for the tap water flow.

2.3 Blood hematocrit, hemoglobin and methemoglobin levels

Immediately after blood puncture, subsamples were drawn into (heparinized) glass capillaries and centrifuged (13,600 g; 2 min) to measure hematocrit values. Results were rounded to the closest 0.5%. Blood hemoglobin and methemoglobin were measured with commercially available kits (Instruchemie, Delfzijl, The Netherlands; FAR diagnostics, Verona, Italy).

2.4 Plasma concentrations of nitrite, nitrate, cortisol, glucose, lactate, chloride, plasma osmolality and branchial Na^+/K^+-ATPase activity

Plasma concentration of cortisol was determined by radioimmunoassay as described in detail by Gorissen and colleagues (2012). Plasma osmolality was measured using a cryoscopic osmometer (Osmomat 030,

Gonotec, Germany). Plasma concentrations of glucose, lactate, chloride, nitrate and nitrite were measured with commercially available enzymatic kits adapted to 96 wells plates as described recently (Schram, et al, 2010; Schram et al, 2012). Branchial Na^+/K^+-ATPase activity was measured as described by Metz and colleagues (2003).

2.5 Gill morphology

From each fish a second gill arch was removed immediately after blood sampling and placed overnight in Bouin's fixative (75 volumes saturated picric acid, 25 volumes saturated formaldehyde, and 5 volumes acetic acid) and then embedded in paraffin. Gill sections were made to include the trailing edge of the filament where in this species the chloride cells reside. Gill sections were immune-stained according to Dang and colleagues (2000) as described in detail for African catfish (Schram et al, 2010; 2012).

2.6 Specific growth rate, feed intake and feed conversion rate

On day 0 and day 28, the fish in each tank were individually weighed (Mettler PM 34 Delta range) to the nearest 1 g, to calculate the specific growth rate (SGR) as follows:

$$SGR = (\ln(W_t) - \ln(W_0)) \times \frac{100}{t}$$

Where SGR = specific growth rate (%/d), W_t = mean weight at day 28, W_0 = mean weight at day 0 (g) and t = number of days.

Floating feed (Catfish type Me-3; Skretting, Boxmeer, The Netherlands) with 49% crude protein and 11% crude lipids was given twice daily at 9:00 and 15:00 until apparent satiation (no more feed taken for at least 5 min following administration of the feed). Feed loads per tank were recorded daily. All uneaten pellets were collected from each tank 1 h after each of the two daily feeding sessions. Feed loss per tank was calculated as the total number of uneaten feed pellets multiplied by 0.0966 g per pellet, the average weight of a pellet, determined by weighing 100 feed pellets. Daily feed intake per tank resulted from the difference between daily feed load and daily feed loss. Daily feed intake per tank was divided by the number of fish in the tank to calculate the daily feed intake per fish in each tank (to account for different numbers of fish per tank). For each tank the total feed intake per fish (TFI) was determined by summation of daily feed intake per fish in each tank. Total feed intake per fish and biomass increase per fish were used to calculate feed conversion rate (FCR) as follows:

$$FCR = \frac{TFI}{(W_t - W_0)}$$

Where *FCR* = feed conversion rate (g/g), *TFI* = total feed intake (g/fish), W_t = mean individual weight at day 28 and W_0 = mean individual weight at day 0 (g).

2.7 Statistics

2.7.1 Physiological parameters

Physiological parameters are expressed as mean (SD) of the individual measurements per treatment. For each tanks, 10-12 fishes had been sampled; in some instances not all samples were analyzed because of insufficient volume or fish mortality. Data were log-transformed (if required) to obtain residuals that were normally distributed and to obtain homogeneity of variance of residuals across treatment levels. Mean values for physiological parameters were tested for differences among the treatments using linear mixed models (REML) with treatments as fixed effects and tank as a random effect (F-tests with Kenward-Roger approximation to the residual degrees of freedom (Kenward and Rogers, 1997)). Statistical analyses were performed in SAS 9.2 (SAS Institute Inc., Cary, North Carolina, USA). Only when significant treatment effects were detected, a least significance difference (LSD) post-hoc analysis was used to estimate the level of significance between mean values. For both REML and LSD analysis the fiducial limit was set at 5%.

Plasma nitrite and plasma nitrate concentrations were related to water nitrite concentrations by linear regression analyses (Table 2).

2.7.2 Feed intake and growth

Initial and final individual weight, total feed intake per fish (TFI), specific growth rate (SGR) and feed conversion rate (FCR) are presented as mean values per treatment (Table 5). Mean values per treatment were log-transformed to obtain residuals that were approximately normally distributed and to obtain homogeneity of variance of residuals across treatment levels and then tested for significant differences among the treatments by one-way ANOVA in SAS 9.2 (SAS Institute Inc., Cary, North Carolina, USA). For both ANOVA and LSD analysis the fiducial limit was set at 5%.

2.7.3 Concentration-effect curves

Nitrite concentration-effect curves were fitted for specific growth rate (SGR) and total feed intake per fish (TFI) using a log-logistic model (Seefeldt et al, 1995). As a blank could not be included, the effects are expressed as absolute

values. Curve-fitting was carried out with the Marquadt and Levenberg algorithm (Moré, 1978) in R (the 10% effect concentrations (EC_{10}) and their 95% confidence limits were calculated (Miller and Miller, 2000).

3. Results

3.1 Plasma nitrite and nitrate concentrations

We observed a significant (linear increase in plasma nitrite concentration with increasing ambient nitrite concentrations (Tables 2 and 3). Basal values for the control group were 5.0 (3.6), increasing up to 32.5 (12.6 µM) in the highest NO_2^- treatment. Similarly, a significant linear increase of plasma nitrate was observed with increasing ambient nitrite concentrations (Tables 2 and 3). Basal plasma nitrate concentrations for the control group were 41.6 (28.4 µM), increasing up to 420.2 (106.4 µM) in the highest NO_2^- treatment. The addition of sodium chloride to high ambient NO_2^- (treatment 8) did not show an attenuating effect on both plasma NO_2^- and NO_3^- concentrations.

3.2 Blood hematocrit, hemoglobin and methemoglobin

No significant differences in hematocrit and methemoglobin levels after 28 days of exposure to any of the ambient nitrite concentrations were detected (Table 4). Significant differences in hemoglobin were detected among treatments (Table 4), but within the same (narrow) biological range, marginal differences could not be related to treatments.

Response variable	Explanatory variable	Regression coefficient		Intercept		
		Estimate	P value*	Estimate	P value	95% CI**
Plasma nitrite (µM)	Water nitrite (µM)	0.026	<0.0001	8.94	<0.0001	6.0 – 11.8
Plasma nitrate (µM)	Water nitrate (µM)	0.41	<0.0001	59.9	<0.0001	34.6 – 85.1

Table 2. Results of linear regression analyses.
*) equals model P-value
**) CI = confidence interval

NO_2^- Treatment	Water NO_2^- (µM)	Plasma NO_2^- (µM)	n	Plasma NO_2^- to water NO_2^- ratio	n	Plasma NO_3^- (µM)	n	Plasma Cl^- (mM)	n	Plasma osmolality (mOsmol/kg)	n	Na^+/K^+-ATPase activity (µM Pi/h/mg protein)	n
1 - T = 0	-	1.6 (1.0)	12	-	12	52.6 (19.1)	13	113.6 (8.5)	16	269.3 (11.1)	22	-	-
2 - Pair fed	-	8.8 (3.8)^{ab}	16	0.13 (0.06)^{a}	16	108.4 (21.6)^{ab}	16	118.2 (12.6)	24	269.7 (6.6)	24	-	-
3 - Control	6	5.0 (3.6)^{a}	19	0.71 (0.51)^{b}	19	41.0 (28.4)^{c}	18	114.4 (10.0)	23	275.0 (13.2)	23	3.11 (0.83)	10
4 – NO_2^-	111	12.0 (5.1)^{abc}	16	0.11 (0.05)^{a}	16	88.6 (44.9)^{bcd}	16	112.0 (12.5)	24	272.3 (17.8)	24	-	-
5 – NO_2^-	280	23.7 (9.7)^{abc}	16	0.08 (0.03)^{ac}	16	220.2 (71.9)^{ae}	16	112.9 (14.8)	21	266.0 (5.4)	21	-	-
6 – NO_2^-	459	18.8 (7.2)^{abc}	13	0.04 (0.02)^{ac}	13	266.1 (112.1)^{ae}	11	113.4 (20.9)	22	269.6 (28.2)	22	-	-
7 – NO_2^-	928	32.5 (12.6)^{c}	17	0.04 (0.01)^{cd}	17	420.2 (106.4)^{e}	9	111.1 (14.0)	22	266.3 (8.9)	22	2.52 (0.57)	10
8 – NO_2^- + NaCl	921	12.5 (10.7)^{abc}	15	0.01 (0.01)^{d}	15	198.4 (98.6)^{bde}	13	118.0 (11.8)	23	271.7 (12.5)	23	3.28 (0.76)	9
P-value		0.049		0.003		0.0053		0.41		0.63		-	

Table 3. Mean (SD) values at the start (t =0) and per treatment for the end (t = 28 days) of the nitrite experiment for plasma NO_2^-, plasma NO_2^- to water NO_2^- ratio, plasma NO_3^-, plasma Cl^-, plasma osmolality and branchial Na^+/K^+-ATPase activity. Mean values with different superscripts are significantly different (REML, P values as shown). SD = standard deviation of mean values per treatment, n as indicated in the table. T = 0 values were not considered in the statistical analysis.

NO_2^- Treatment	Water NO_2^- (µM)	Htc (%)	n	Hb (g/Dl)	n	MetHb %	n	Plasma cortisol (nM)	n	Plasma glucose (mM)	n	Plasma lactate (mM)	n
1 - T = 0	-	32.3 (4.0)	22	12.2 (2.7)	13	8.8 (5.9)	14	91.6 (46.4)	13	2.9 (0.9)	22	3.4 (0.8)	18
2 - Pair fed	-	39.1 (2.7)	22	9.2 (1.7) ^{abcd}	19	8.1 (2.6)	16	83.9 (63.9)	24	4.5 (1.0)	24	5.5 (1.3)	24
3 - Control	6	38.7 (2.9)	18	8.5 (2.4) ^{acd}	13	6.6 (4.3)	15	67.7 (53.0)	23	3.8 (1.1)	23	4.7 (1.2)	23
4 – NO_2^-	111	37.7 (3.8)	22	8.2 (1.6) ^{ac}	15	7.6 (3.5)	15	43.7 (35.9)	24	5.7 (1.0)	24	6.7 (1.7)	23
5 – NO_2^-	280	35.8 (4.3)	21	8.7 (1.1) ^{ce}	18	8.7 (4.3)	15	88.7 (71.0)	21	3.6 (0.8)	21	4.5 (1.2)	21
6 – NO_2^-	459	38.8 (4.2)	22	10.0 (0.9) ^{bd}	14	9.3 (4.3)	16	65.7 (89.0)	22	5.7 (1.4)	22	6.1 (0.7)	22
7 – NO_2^-	928	37.5 (3.1)	22	9.0 (2.0) ^{cd}	20	10.8 (3.8)	18	55.9 (27.1)	21	4.4 (1.3)	22	4.7 (1.1)	21
8 – NO_2^- + NaCl	921	38.2 (3.5)	21	9.6 (1.7) ^{de}	13	9.8 (4.8)	15	56.6 (32.3)	22	4.4 (1.3)	23	5.2 (1.2)	23
P-value		0.14		0.041		0.37		0.71		0.12		0.12	

Table 4. Mean (SD) values at the start (t =0) and per treatment for the end (t = 28 days) for blood, hematocrit (Htc), hemoglobin (Hb) and methemoglobin (MetHb), plasma cortisol, plasma glucose and plasma lactate concentrations. Mean values with different superscripts are significantly different (REML, P values as shown). SD = standard deviation of mean values per treatment, n as indicated in the table. t = 0 values were not considered in the statistical analysis.

3.3 Plasma cortisol, glucose, lactate, chloride, plasma osmolality and Na^+/K^+-ATPase activity

No significant differences in plasma concentration of cortisol, glucose, lactate, chloride, plasma osmolality and branchial Na^+/K^+-ATPase activity were observed (Table 4). All values were within normal ranges previously reported for African catfish, *Clarias gariepinus* (Schram et al, 2010; 2012).

3.4 Gill morphology.

Gills stained for Na^+/K^+ ATPase-rich cells (chloride cells) are presented for the control group (Fig. 1A), 928 μM NO_2^- (Fig. 1B) and 921 μM NO_2^- in addition with NaCl (Fig. 1C). Gill morphology was not affected by elevated NO_2^- exposure.

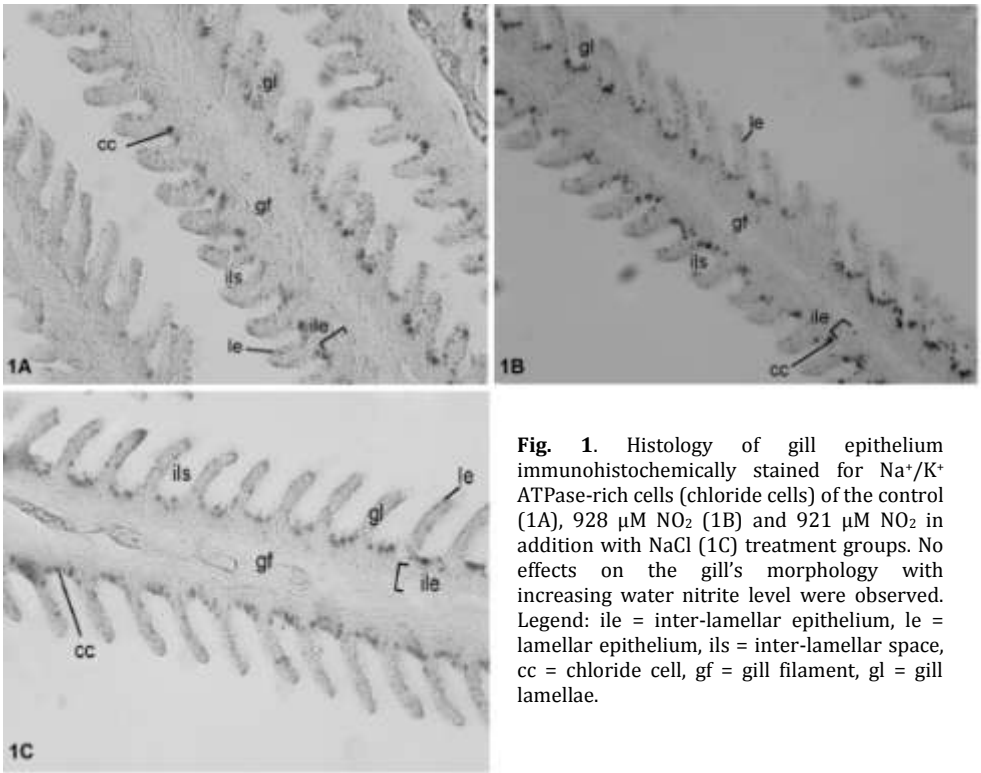

Fig. 1. Histology of gill epithelium immunohistochemically stained for Na^+/K^+ ATPase-rich cells (chloride cells) of the control (1A), 928 μM NO_2 (1B) and 921 μM NO_2 in addition with NaCl (1C) treatment groups. No effects on the gill's morphology with increasing water nitrite level were observed. Legend: ile = inter-lamellar epithelium, le = lamellar epithelium, ils = inter-lamellar space, cc = chloride cell, gf = gill filament, gl = gill lamellae.

3.5 Total feed intake, specific growth rate, feed conversion rate and mortality.

Daily feed intake appeared to cumulate to marked treatment effects on total feed intake (TFI) (Fig. 2). However, no significant treatment effect on TFI could be detected. Also no differences among mean values for specific growth rate (SGR), final weight and feed conversion ratio (FCR) could be detected after 28 days of exposure to nitrite (Table 5). Two fish out of a total 186 died during the course of the experiment. Two fishes were euthanized at day 4 (treatment 7) and day 10 (treatment 6) reaching the humane endpoint. Those 2 fish had low hematocrit levels (20%), high levels of methemoglobin (96% and 76% respectively) and high plasma nitrite levels (1540 and 510 μM respectively), accompanied by reduced activity (Personal observation).

NO_2^- Treatment	Water NO_2^- (μM)	Initial weight (g)	Final weight (g)	TFI (g/fish)	SGR (%BW/d)	FCR	Survival (%)
1 – t = 0	-	234.4 (17.9)	-	-	-	-	-
2 - Pair fed control	-	221.8 (4.8)	334.2 (25.0)	87.6 (6.9)	1.46 (0.19)	0.79 (0.08)	100
3 - Control	6	233.3 (19.9)	445.4 (2.6)	156.9 (8.4)	2.32 (0.28)	0.74 (0.02)	100
4 – NO_2^-	111	226.2 (4.0)	409.1 (40.4)	131.8 (18.4)	2.11 (0.29)	0.72 (0.04)	100
5 – NO_2^-	280	223.5 (17.0)	390.8 (33.1)	128.7 (5.7)	1.99 (0.03)	0.77 (0.04)	100
6 – NO_2^-	459	210.0 (13.8)	349.5 (64.1)	111.8 (40.3)	1.79 (0.42)	0.80 (0.00)	91.7
7 – NO_2^-	928	187.2 (1.2)	307.4 (6.1)	92.9 (2.2)	1.77 (0.09)	0.77 (0.03)	91.7
8 – NO_2^- + NaCl	921	220.8 (28.5)	360.3 (83.6)	114.8 (39.7)	1.71 (0.37)	0.83 (0.04)	100
P-value		0.17	0.19	0.19	0.19	0.34	-

Table 5. Mean (SD) values per treatment (N = 2) for initial weight, final weight, total feed intake (TFI), specific growth rate (SGR), feed conversion ratio (FCR) and survival. SD = Standard deviation of mean values per treatment.

3.6 EC_{10} for total feed intake and SGR

The concentration-effect curves for total feed intake and specific growth rate in relation to water nitrite concentration (Fig. 3A and 3B) reveal a significant effect of nitrite. For total feed intake the EC_{10} for nitrite is 84 μM with a 95% confidence interval from 2 μM to 3.7 mM. For specific growth rate the EC_{10} for nitrite is 43 μM with a 95% confidence interval from 0 to 68 M (Not shown on the figures).

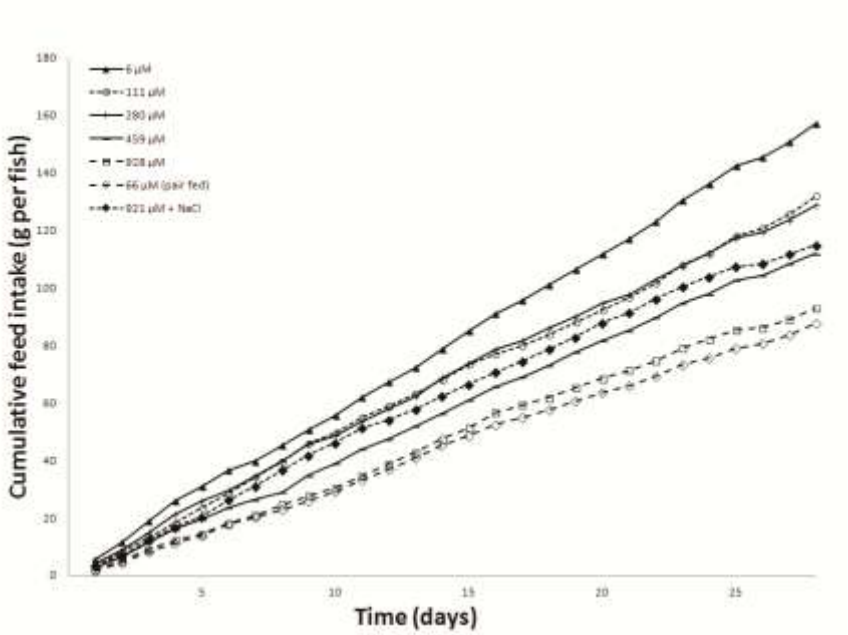

Fig. 2. Mean (N=2) cumulative feed intake per fish during the experimental nitrite exposure. The arrow indicates the first day at which all treatments reached their designated nitrite concentrations.

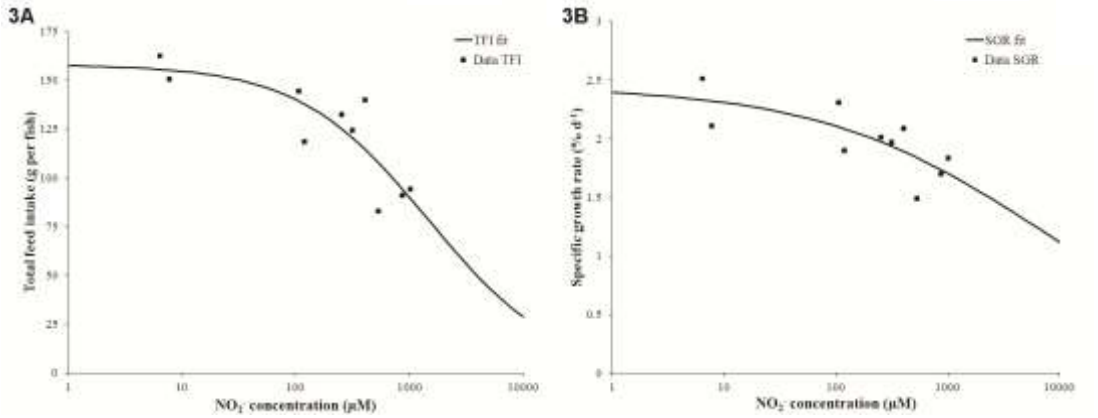

Fig. 3. Concentration-effect curves for total feed intake (TFI) (3A) and specific growth rate (SGR) (3B) in relation to the water NO_2^- concentration. TFI = 158*(1-1/(-log[NO2] – 3.1574)/1.2906) (r^2 = 0.72). SGR = 2.4466*(1-1/(-log[NO2] - 3.8324)/2.3018) (r^2 = 0.55).

4. Discussion

4.1 Blood hematocrit, hemoglobin and methemoglobin levels

A primary toxic action of nitrite is the conversion of hemoglobin to methemoglobin, which is not able to carry oxygen (Bodansky, 1951). Basal levels of methemoglobin in some fish species can reach 10%, and as a rule of thumb, and levels below 50% generally do not result in mortality (Lewis and Morris, 1986). At higher levels of methemoglobin (70-80%), the behavior of fish is affected as they become less active to reduce their oxygen demand. In some species, defense mechanisms exist and acclimatization can occur after a certain exposition time (reviewed by Lewis and Morris, 1986; Kroupova et al, 2005). Pikeperch (*Sander lucioperca*) appears unable to acclimatize when chronically exposed to 250 µM NO_2^-, as mean methemoglobin levels remained at 67% after 32 days of exposure (Wuertz et al, 2013). African catfish (*Clarias gariepinus*; 166 g) exposed to 2.3 mM NO_2^- for 96 h showed blood methemoglobin levels to increase from 10% to 50% after 24 h, and reaching 90% after 96 h (Hilmy et al, 1987). Moreover, chronic exposure of African catfish to $1/10^{th}$ of this dose (0.23 mM) over a period of 6 months led to a slight increase in methemoglobin measured after 1 month (below 15%), reaching a peak after 4 months (40%) followed by a decrease to 25% after 6 months exposure (Hilmy et al, 1987). Our data indicate similar acclimatization process occurring in our fishes, with acute formation of methemoglobin at high ambient nitrite concentrations (seen in the euthanized fish) and basal levels reached after 28 days of exposure.

4.2 Plasma nitrite and nitrate concentrations

Plasma nitrite concentrations gradually increased with increasing ambient nitrite concentrations (ranging from 1.0-13.7 µM in the control groups and 13.1-56.6 µM in the 928 µM group).

Plasma nitrite concentration varies among species (Table 6). In some species, plasma nitrite concentration can reach build up to levels up to 10 times higher than ambient (Eddy et al, 1983). Juvenile African catfish exposed to 1736 µM NO_2^- for 24 h had plasma concentrations on NO_2^- reaching almost 5.5 mM (Ekwe et al, 2012). Exposure of adult African catfish to high ambient nitrite concentration seems to elicit a strong initial increase of plasma nitrite, but fish seem to acclimatize when chronically exposed to high levels of ambient nitrite. This is confirmed by the fact that addition of sodium chloride (6 mM) does not show a significant attenuating effect on plasma nitrite concentration. African catfish seem to acclimatize to the range of concentration studied; therefore the addition of sodium chloride does not add more protective effect.

Plasma nitrate concentrations gradually increased with increasing ambient nitrite concentrations. This can be explained by the intrinsic defense mechanism of conversion of plasma nitrite into less toxic nitrate (Doblander and Lackner, 1996; 1997). African catfish are very tolerant to nitrate exposure, as only their growth was affected when ambient concentrations reached 27.04 mM (plasma: 6.6 (0.9) mM) (Schram et al, 2012). In the current experiment, plasma nitrate concentrations were well below the millimolar level, the toxic effects observed should therefore be attributed to nitrite rather than nitrate.

Species	Water NO_2^- (μM)	Water Cl^- (mM)	Mean plasma NO_2^- (μM)	Exposure time (days)	Reference
African catfish *Clarias gariepinus*	1736	0.62	5456	1	Ekwe et al, 2012
Nile Tilapia *Oreochromis niloticus*	1736	0.62	4361	1	Ekwe et al, 2012
Goldfish *Carassius auratus*	0.43	0.28	0.75	2	Hansen and Jensen, 2010
Largemouth bass *Micropterus salmoides*	6929	0.62	484	1	Palacheck and Tomasso, 1984
	13865	0.62	1971	1	
	865	0.62	76	1	Tomasso, 1986
Channel catfish *Ictalurus punctatus*	1736	0.62	5471	1	Palacheck and Tomasso, 1984
	865	0.62	2784	1	Tomasso, 1986
Tilapia *Tilapia aurea*	1736	0.62	4361	1	Palacheck and Tomasso, 1984
Pike-perch *Sander lucioperca*	0	1.13	7.1	32	Wuertz et al, 2013
	249	1.13	540	32	
	711	1.13	3629	42	
	711	12.41	22.1	42	
Rainbow trout *Onchorhynchus mykiss*	0.2 – 2.2	0.28	Not detectable	28	Kroupova et al, 2008
	13	0.28	0.65	28	
	65	0.28	8.2	28	
Walleye *Sander vitreus*	900	0.15	2100	1	Madison and Wang, 2006
			3000	2	

Table 6. Overview of nitrite plasma concentrations in several fish species under different acute and chronic nitrite exposures. When original data were presented in mg/L NO_2^--N or mg/L, we converted them in the international system unit (molar). Data presented in mg/L NO_2^- were multiplied by 21.63 and data presented in mg/L NO_2^--N were multiplied by 71.14 to obtain result in micromolar.

During the whole experimental period, no abnormal swimming behavior was observed as in other nitrite exposed fish species (Personal

observations; Lewis and Morris, 1986). The absence of abnormal swimming behavior may be explained by the air-breathing nature of African catfish. Branchial nitrite uptake could then be limited by limiting oxygen uptake from the water in favor of air-breathing. Quantitative observation of air-breathing behavior of nitrite exposed African catfish would be required to assess this hypothesis.

4.3 Stress physiology, plasma osmolality, plasma chloride and gill morphology and Na^+/K^+-ATPase activity

Plasma glucose, lactate and cortisol levels were in the normal biological range measured in previous studies for this species (Schram et al, 2010; 2012). A chronic exposure of 28 days to elevated ambient nitrite concentrations did not affect these parameters, underpinning once again the tolerance of the African catfish towards high nitrogenous waste compounds. Plasma osmolality and plasma chloride, a major determinant of plasma osmolality, were not affected by high nitrite concentrations, The Na^+/K^+-ATPase activity was in the range previously observed for this species exposed to ammonia (Schram et al, 2010). The Na/K-ATPase activity was assessed only in the control group (treatment 3), high nitrite (treatment 7) and high nitrite in presence of sodium chloride (treatment 8). Since no differences between those extreme groups were observed, the intermediate groups were not measured. This enzyme does not play a role in the nitrite uptake or removal of nitrite as it was observed for ammonia (Schram et al, 2010). The branchial Cl^-/HCO_3^- exchanger which normally is involved in chloride uptake and may be disrupted when ambient nitrite concentrations are high, causing a (partial) shift to NO_2^- uptake (Jensen, 2003) was not investigated in this study.

In rainbow trout exposed to several increasing nitrite concentrations for 28 days, severe morphological alterations of the gills were observed already from the lowest 0.22 µM NO_2^-, culminating in the highest concentration (65.2 µM NO_2^-) (Kroupova et al, 2008). Over 32 days of exposure to different nitrite concentrations (0 - 250 µM NO_2^-), 40 to 60% of the gills of juvenile pikeperch showed abnormalities. Nevertheless, no changes could be related to treatment effects (Wuertz et al, 2013). Toxic effect of gill morphology thus depends on the species.

4.4 Feed intake

During the first 4 days of the experiment, when nitrite concentrations were building up, feed intake was similar among all treatments. However, when the desired concentrations of nitrite had been reached (day 5), different pattern

in feed intake showed up almost instantly (Fig. 2), with intermediate patterns related to the different concentrations, the minimum feed intake being reached in the highest nitrite concentration group, and a potential attenuating effect of the addition of sodium chloride. Feed intake and SGR and final weight patterns seem to be gradually affected by raising ambient nitrite concentrations (Table 5; Fig. 2). However, differences are not significant. A pair fed group was introduced to discriminate effects of high nitrite exposure from potential effects of reduced feed intake. No significant differences were observed in physiological parameters for any treatment.

4.5 Nitrite toxicity

The main mechanism of nitrite toxicity is well documented with the conversion of hemoglobin to methemoglobin, incapable of carrying oxygen (Bodansky, 1951). The comparable methemoglobin levels among treatments after 28 days of nitrite exposure show that African catfish is able to acclimatize to relatively high ambient nitrite concentrations. Similar methemoglobin concentrations were observed in this species exposed to $1/10^{th}$ of the 96 median tolerance limit (228 μM NO_2^-; Hilmy et al, 1987). Plasma nitrite concentrations gradually increased with increasing ambient nitrite concentrations. The increase of plasma nitrate with increasing water nitrite concentrations indicates that African catfish successfully detoxify internally nitrite to less toxic nitrate as described earlier in trout hepatocytes (Doblander and Lackner 1996). The addition of sodium chloride (6 mM) does not show a further attenuating effect both regarding plasma nitrite concentration and growth parameters; African catfish appears to acclimatize to chronic nitrite exposure already without sodium chloride; the addition of this compound has no real beneficial effect in the concentration range studied.

The nitrite concentration range was designed based on actual nitrite exposure in commercial African catfish aquaculture combined with values from the literature and the aim of this experiment was to measure subtle changes that could impair the fish welfare. In our opinion, the set of data obtained allows to draw conclusions regarding the nitrite toxicity to African catfish and the threshold concentrations for safe aquaculture production. Previous studies on nitrite toxicity mainly focused on acute LC_{50} up to 96 h (reviewed by Kroupova et al, 2005). The parameters traditionally measured are mainly related to the nitrite effect on blood hemoglobin and methemoglobin formation. In this study, we investigated in addition to those parameters, the effect of chronic nitrite exposure on stress physiology and growth, parameters of interest from the welfare and commercial aquaculture points of view.

4.6 Nitrite threshold concentrations

African catfish chronically exposed to 1 mM ambient nitrite appears capable of mitigating the adverse effects of nitrite. The concentration effect curves revealed a significant effect of nitrite exposure on total feed intake and specific growth rate. The concentration-effect curves do not provide a clear cut nitrite threshold concentration for African catfish as the calculated EC_{10} values for nitrite of 84 µM for feed intake and 43 µM for growth both have very large 95% confidence intervals. Therefore the reported EC_{10} values should be treated as indicative. All observations jointly taken we advise for African catfish not to exceed a nitrite concentration of 43 µM. As stated earlier, several studies investigated the acute lethal concentration of nitrite for numerous species, but data regarding nitrite toxicity for chronic exposure, combining physiology and growth are scare. As an example, the NOEC and LOEC for juvenile rainbow trout after 28 days of nitrite exposure was estimated at 0.22 µM NO_2^- (0.01 mg/L NO_2^-) and 4.34 µM NO_2^- (0.2 mg/L NO_2^-) respectively (Kroupova et al, 2008); which indicates that this species is more sensitive to nitrite than African catfish.

5. Conclusions

This study demonstrates that African catfish, *Clarias gariepinus*, is tolerant to high ambient nitrite concentrations over a period of 28 days. Nitrite accumulates mildly in the plasma with increasing ambient nitrite concentrations. The greater increase in plasma nitrate with increasing ambient nitrite concentrations provide a good indirect evidence for internal nitrite detoxification into less toxic nitrate. Stress physiology and ionic balance are not affected by high ambient nitrite concentrations. Growth and feed intake show a differential pattern from the first day of exposure, but differences are not significant.

We advise for African catfish not to exceed a water nitrite concentration of 43 µM (0.6 mg/L NO_2^--N). Below this nitrite concentration physiological and growth disturbances are avoided.

Acknowledgements

The authors would like to express their gratitude to Kim Roelofs, Corrina Hinrichs, Mark Westerink, Sven Würtz and Lauriane Michel for their help and technical assistance. This study was funded by the Dutch Ministry of Economic Affairs, Agriculture and Innovation.

Chapter 8

The impact of elevated water ammonia and nitrate concentrations on physiology, growth and feed intake of pikeperch (*Sander Lucioperca*)

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Aquaculture, accepted

Abstract

The ammonia (NH_3) and nitrate (NO_3^-) threshold concentrations in rearing water of juvenile pikeperch (*Sander lucioperca*) were assessed.

Pikeperch with an initial mean (SD) weight of 17.7 (4.2) g were exposed to 0.9 (control), 3.6, 5.2, 7.1, 11.2 and 18.9 µM NH_3 in the water for 42 days. Plasma NH_4^+ concentrations stayed at control levels (~ 650 µM) up to 11.2 µM NH_3 in the water. At the highest water NH_3 concentration tested, plasma NH_4^+ had more than doubled to 1400 µM. Based on the specific growth rate, the EC_{10} value for NH_3 was 5.7 µM.

When pikeperch (initial mean (SD) weight of 27.0 (4.9) g) were exposed to 0.1 (control), 1.5, 2.3, 3.7, 6.1, 10.2, 15.8 and 25.6 mM NO_3 for 42 days, mean (SD) plasma NO_3 concentrations increased linearly from 88 (47) to 5993 (899) µM at the highest ambient NO_3 level. Feed intake, specific growth rate and feed conversion ratio were not affected.

Neither NH_3 nor NO_3^- exposure significantly affected hematocrit, plasma concentrations of cortisol, glucose, lactate, osmolality, gill morphology or branchial Na^+/K^+-ATPase activity in pikeperch. For juvenile pikeperch we advise not to exceed a water NH_3 concentration of 3.4 µM (0.05 mg NH_3-N/L), the lower limit of the 95% confidence interval of the EC_{10} value for SGR, to ensure proper physiology and growth. For NO_3^- we advise not to exceed 25 mM (350 mg NO_3^--N/L). This criterion is based on the highest NO_3^- concentration tested (25.6 mM). As no negative effects were detected at the highest concentration tested, the actual NO_3^- threshold is probably greater than 25.6 mM.

1. Introduction

Aquaculture of pikeperch (*Sander lucioperca*) is important for the diversification of freshwater aquaculture production in Northern and Central Europe, and an attractive alternative for common carp (*Cyprinus carpio*) and rainbow trout (*Oncorhynchus mykiss*). Among aquaculturists interest increases to intensify aquaculture of pikeperch (Muller-Belecke, 2008). In intensive recirculating aquaculture systems (RAS) fish run the risk of significant exposure to nitrogenous wastes including ammonia (NH_3) and nitrate (NO_3^-). Nitrogenous waste is produced by fish through protein catabolism (Wood, 1993). The majority of teleostean fish, including pikeperch, is ammonotelic and excrete most of their nitrogenous waste as ammonia across the gills to the water (Wilkie, 2002). High water ammonia leads to rapid accumulation of ammonia in plasma and tissues (Wright et al, 2007), where, at physiological pH, it is mainly present as NH_4^+ (Wilkie, 2002). High internal ammonia is neurotoxic (Cooper and Plum, 1987 in Wilkie, 2002). High water ammonia is an important limiting factor for intensive aquaculture (Boeuf et al, 1999) and should therefore be kept below a defined species-specific threshold.

In RAS, ammonia in the culture water is controlled by conversion of ammonia to NO_3^- in aerobic biofilters. NO_3^- subsequently accumulates in the culture water (Bovendeur et al, 1987; Eding et al, 2006) and fish farmed in RAS may be chronically exposed to NO_3^- levels up to 70 mM (1000 mg N/L; Van Rijn, 2010). High water NO_3^- results in the appearance of NO_3 in the plasma compartment (Schram et al, 2012; Stormer et al, 1996). Uptake of NO_3^- via the gills is low compared to that of ammonia and nitrite due to an apparently low branchial permeability for NO_3^- (Stormer et al, 1996). NO_3^- is less toxic than nitrite and ammonia (Scott and Crunkilton, 2000). Chronic exposure to high NO_3 however, can lead to reduced feed intake, and growth (Schram et al, 2012). In addition, nitrate is potentially associated with health problems, as observed in rainbow trout reared in RAS with near zero water exchange (Davidson et al, 2011).

For juvenile pikeperch, neither NH_3 nor NO_3^- threshold concentrations have been established. As a result it is unclear whether intensive farming of pikeperch at high water NH_3 or NO_3^- results in physiological disturbance and reduced growth. We exposed juvenile pikeperch to increased water NH_3 and NO_3^- levels for 42 days to establish threshold concentrations.

2. Materials and methods

2.1 Experimental fish

Juvenile pikeperch (*Sander lucioperca*) were obtained from Excellence Fish BV, Horst, The Netherlands. Husbandry and experimentation was in accordance with the Dutch law on animal welfare, and approved by the ethical committee for animal experimentation of Wageningen UR Livestock Research (number 2012053.b for the NH_3 experiment, number 2012021.b for the NO_3^- experiment).

2.2 Experimental exposure system

During acclimatization and the experimental period of both experiments, all aquaria were supplied with local tap water at a flow rate of 500 L per day. Experimental NH_3 concentrations were realized by infusion of NH_4Cl stock solutions (Table 1). Sodium bicarbonate ($NaHCO_3$) was added to the NH_4Cl stock solutions to adjust the pH (overall pH range: 7.00-8.18; Table 1). In addition, sodium chloride (NaCl) was added to the NH_4Cl stock solutions to compensate for the differences in chloride concentrations in the aquaria arising from NH_4Cl addition. Total predicted sodium concentrations in the aquaria from $NaHCO_3$ and NaCl combined were equal among treatments (Table 1). Fresh stock solutions were prepared daily during the first 6 days of the experimental period. During the remainder of the experimental periods fresh stock solutions were prepared twice per week. To prevent evaporation of NH_3, stock solutions were stored in closed vessels and inside the vessel the surface of the stock solution was covered by a floating plastic sheet. NH_3 concentrations were gradually increased to the designated concentrations during the first 6 days of the experimental period.

Experimental NO_3^- concentrations were realized by infusion of $NaNO_3$ stock solutions (Table 2). Fresh stock solutions were prepared daily during the first 10 days of the experimental periods. During the remainder of the experimental periods fresh stock solutions were prepared twice per week for both experiments. NO_3^- concentrations were gradually increased to the designated concentrations during the first 10 days of the experimental period.

All stock solutions were prepared in tap water and pumped into the aquaria by a peristaltic pump (Watson Marlow 505 S; Rotterdam, The Netherlands) at a flow rate of 5 L per day per aquarium.

2.3 NH_3 experiment

Juvenile pikeperch (n =192) with a mean (SD) weight of 17.7 (4.2) g were randomly divided over sixteen 30-L rectangular aquaria and acclimatized to the experimental aquaria for 7 days. The experiment lasted for 42 days and consisted of eight, duplicated treatments which were randomly assigned to the aquaria. Two extra aquaria were included to collect blood and plasma of untreated fish at the start of the experiment (treatment 1, T = 0). Fish in treatments 3 to 8 were exposed to one of six different NH_3 concentrations in the water: 0.9 (control), 3.6, 5.2, 7.1, 11.2 and 18.9 µM. Fish in treatment 2 (pair-fed control) were kept at control (0.5 µM) NH_3 levels and fed the same feed ration as the fish kept at the highest (18.9 µM) NH_3 level to discriminate between effects caused by low feed intake and exposure to a high NH_3 concentration in the water.

NH_3 Treatment	Composition of stock			Predicted concentrations in the				Measured water quality				
	$[NH_4Cl]$ (g/L)	$[NaHCO_3]$ (g/L)	$[NaCl]$ (g/L)	$[NH_3]^b$ (µM)	$[Na^+]$ (mM)	$[Cl^-]$ (mM)	Total diss. solids (g/L)	$[NH_3\text{-}N]$ (mg/L)	$[NH_3]$ (µM)	$[T_{Amm}]$ (mM)	Cond. (µS/cm)	pH range
2 - Pair fed	0	0	50.5	0	8.7	5.2	0.5	0.01	0.46	0.01	1752	7.34-8.18
3 – Control	0	0	50.5	0	8.7	5.2	0.5	0.01	0.9	0.03	1668	7.60-8.05
4 - NH_3	2.36	3	48.5	5.7	8.7	5.2	0.53	0.05	3.55	0.18	1557	7.00-7.96
5 - NH_3	3.27	4.3	47.5	7.7	8.7	5.2	0.55	0.07	5.16	0.24	1544	7.14-7.97
6 - NH_3	4.63	6	46.4	11.1	8.7	5.3	0.56	0.1	7.12	0.35	1579	7.14-7.95
7 - NH_3	6.55	8.5	44.7	15.8	8.7	5.3	0.59	0.16	11.2	0.46	1531	7.18-7.96
8 - NH_3	9.18	12	42.3	22.1	8.7	5.4	0.63	0.26	18.9	0.73	1643	7.33-8.01

Table 1. Composition of the treatment specific stock solutions in the ammonia experiment, the predicted[a] total ammonia, ammonia, sodium and chloride concentrations and salinity and the measured values per treatment for NH_3 and total ammonia (T_{Amm}) concentration, conductivity and the pH range in the aquaria.
[a] Based on equal flow rates per tank of 5 L/day for the stock solutions and 500 L/day for the tap water flow.
[b] Based on a pH of 7.4 and a water temperature of 24°C.

2.4 NO_3^- experiment

Juvenile pikeperch (n =240) with an individual mean (SD) weight of 27.0 (4.9) g were randomly divided over twenty 30-L rectangular aquaria and acclimatized to the experimental aquaria for 14 days. The NO_3^- experiment lasted for 42 days and consisted of ten duplicated treatments, which were assigned randomly to the aquaria. Two extra aquaria (treatment 1, T = 0) were included to collect blood and plasma of untreated fish at the start of the experiment. In treatments 3 to 10 fish were exposed to one of eight different

NO_3^- concentrations in the water: 0.1 (control), 1.5, 2.3, 3.7, 6.1, 10.2, 15.8 and 25.6 mM. Fish in treatment 2 (pair fed control) were kept at control (0.1 mM) NO_3^- levels and pair-fed to the fish kept in 25.6 mM NO_3^-.

NO_3^- Treatment	Stock solutions	Predicted[a] water quality			Measured water quality			
	[$NaNO_3$] (g/L)	[NO_3^-] (mM)	[Na^+] (mM)	Total dissolved solids (g/L)	[NO_3^--N] (mg/L)	[NO_3^-] (mM)	Conductivity (µS/cm)	pH range
2 - Pair fed	0	0	0	0	1.4	0.1	684	7.63-7.96
3 - Control	0	0	0	0	1.4	0.1	685	7.39-7.97
4 - NO_2^-	12.1	1.4	1.4	0.12	20	1.5	841	7.61-7.95
5 - NO_3^-	20	2.4	2.4	0.2	32	2.3	932	7.15-8.03
6 - NO_6^-	33.1	3.9	3.9	0.33	52	3.7	1101	7.10-7.97
7 - NO_8^-	54.5	6.4	6.4	0.54	85	6.1	1377	7.63-7.98
8 - NO_9^-	90	10.6	10.6	0.89	143	10.2	1832	7.59-7.94
9 - NO_5^-	148.5	17.5	17.5	1.47	221	15.8	2418	7.55-7.95
10 - NO_0^-	245	28.8	28.8	2.42	359	25.6	3504	7.64-7.93

Table 2. Composition of the treatment specific stock solutions in the nitrate experiment, the predicted[a] nitrate and sodium concentrations, the predicted salinity in all treatments and the measured values per treatment for nitrate concentration, conductivity and the pH range.
[a] Based on equal flow rates per tank of 5 L/day for the stock solutions and 500 L/day for the tap water flow.

2.5 Water quality measurement

All aquaria were equipped with an air stone to guaranty good mixing of the infused stock solutions with the aquarium water. Flow rates were monitored daily and adjusted when necessary to reach the desired NH_3 or NO_3 concentrations. Total ammonia (T_{Amm} = NH_3+NH_4^+) and NO_3^- concentrations were monitored in the respective experiments (Spectroquant cell tests for total ammonia and NO_3^--N, Merck, Darmstadt, Germany, Hach Lange DR2800 spectro-photometer, Germany). In both experiments water samples were collected twice per week from all aquaria at approximately 11:00, 1 h after the first feeding session of the day. NH_3 concentrations were calculated from the temperature, pH and salinity dependent molar fraction of NH_3 and the measured T_{Amm} concentrations (Emerson et al, 1975), effectively accounting for any variation in pH among NH_3 treatments. In both experiments water temperature, pH and dissolved oxygen concentrations (Hach Lange HQ 40 multimeter, Germany) and conductivity (WTW Cond 315i) were monitored daily in all aquaria prior to the first daily feeding session (Table 1 and 2). Dissolved oxygen ranged 6.7 to 8.1 mg/L in the NH_3 experiment and from 6.7 to 7.0 mg/L in the NO_3^- experiment. Water temperature was 23.8°C in the NH_3 experiment and 23.0°C in the NO_3^- experiment. pH ranged from 7.00 to 8.18 in the NH_3 experiment and from 7.10 to 7.98 in the NO_3^- experiment.

2.6 Sample measurements
2.6.1 Blood and plasma sampling

One day before exposure to NH_3 or NO_3^- started (day 0), fish in treatment 1 (T = 0) were sampled. After 42 days exposure, the fish from the remaining treatments were sampled (12 fish per aquarium). Fish were rapidly netted and anaesthetized in 0.1% (v/v) 2-phenoxyethanol (Sigma, St. Louis, USA). Within 2 min, blood was taken by puncture of the caudal vessels with a syringe fitted with a 25-gauge needle. Na_2EDTA (NH_3 experiment) or heparin (NO_3^- experiment) were used as anti-coagulants. A 10 µl aliquot blood was used to assess hematocrit, the remainder was immediately centrifuged for 10 min (14,000 g, 4°C) and plasma was stored at -20°C until further analyses.

2.6.2 Plasma NO_3^- concentration – NO_3^- experiment

NO_x (the sum of NO_2^- and NO_3^-) was measured with a commercial nitrate/nitrite colorimetric assay kit (Cayman Chemical Company, Ann Arbor, Michigan, USA). Prior to measurement, plasma samples were filtered using a Millipore Ultra-free MC filter device (0.1 µm pore size) to remove haemoglobin and reduce background absorbance and improve color formation with Griess reagents. Samples of 80 µL (in duplicate) were diluted in the assay buffer and then incubated for 3 h at room temperature with 10 µL of Enzyme Co-factor mixture and 10 µL of NO_3^--reductase mixture. Fifty µL of the first Griess reagent (R1) and of the second Griess reagent (R2) were added and absorbance read at 530 nm in a Wallac 1420 VICTOR^2 counter (Turku, Finland). Due to low plasma volumes and interference with heparin plasma NO_2^- could be determined in only a subset of samples. All NO_2^- values were below 35 µM, which we interpret to indicate that plasma NO_x refers to NO_3^-.

2.6.3 Plasma NH_4^+ - ammonia experiment

Plasma NH_4^+ was determined using a commercial kit (Instruchemie, Delfzijl, The Netherlands), with a protocol adapted for a 96-well microplate.

2.6.4 Plasma concentrations of cortisol, glucose, lactate, plasma osmolality and branchial Na^+/K^+-ATPase activity

Plasma cortisol was determined by radioimmunoassay as described in detail by Metz and colleagues (2005). Plasma osmolality was measured using a cryoscopic osmometer (Osmomat 030, Gonotec, Germany). Plasma glucose and

lactate were measured with commercial enzymatic test kits (Instruchemie, Delfzijl, The Netherlands), with protocols adapted to a 96-wells microplate. For glucose, 10 µL sample or standard (5.55 mM glucose) was mixed with 200 µL reagent and incubated for 10 min at 25°C. Absorbance was read within 60 min at 495 nm. For lactate, 10 µL sample or standard (4.44 mM lactate) or blank (8% perchloric acid) was mixed with 290 µL of lactate reagent and incubated for 20 min at 37°C. Absorbance was read at 355 nm. Branchial Na^+/K^+-ATPase activity was measured as described by Metz and colleagues (2003).

2.6.5 Gill morphology

From each sampled fish the second gill arch was removed immediately after blood sampling and placed overnight in Bouin's fixative (75 volumes saturated picric acid, 25 volumes saturated formaldehyde, and 5 volumes acetic acid) and embedded in paraffin. Gill sections through the trailing edge of the filament, where the chloride cells reside, were immunostained according to Dang and colleagues (2000). After removal of the paraffin, blocking of endogenous peroxidase with 2% (v/v) H_2O_2 and blocking of non-specific sites with 10% (v/v) normal goat serum, slides were incubated overnight with a monoclonal antibody against chicken Na^+/K^+- ATPase (final dilution of 1:500; IgGα5, Developmental Studies Hybridoma Bank, Department of Biological Sciences, University of Iowa, USA). Goat anti-mouse (Nordic Immunology, Tilburg, The Netherlands) was used as a second antibody (1:150). The slides were subsequently incubated with mouse peroxidase anti-peroxidase (1:150) (M-PAP, Nordic Immunology). In the peroxidase reaction 0.025% (w/v) 3,3'-diaminobenzidine (DAB) was used as chromogen in the presence of 0.0005% (v/v) H_2O_2. Finally, sections were dehydrated and mounted. As a control for specificity the procedure was carried out as above, with the omission of the first antiserum.

2.6.6 Hematocrit levels

Immediately after blood puncture, subsamples were drawn into heparinized glass capillaries and centrifuged (13,600 g; 3 min) to assess hematocrit values. Results were rounded to the closest 0.5 %.

2.7 Specific growth rate, feed intake and feed conversion ratio

On day 0 and day 42, fish were individually weighed to the nearest 1 g (Mettler PM 34 Delta range), to calculate the specific growth rate (SGR) as follows:

$$SGR = (\ln(W_t) - \ln(W_0)) \times \frac{100}{t}$$

Where *SGR* = specific growth rate (%/d), W_t = mean weight at day 42 (g), W_0 = mean weight at day 0 (g) and *t* = number of days.

In both experiments floating feed (Skretting R-2 15F) was given twice daily at 10:00 and 14:00 pm until apparent satiation (no more feed taken for at least 5 min following administration of the feed). Feed loads per aquarium were recorded daily. All uneaten pellets were collected from each aquarium 1 h after each of the two daily feeding sessions. Feed loss per aquarium was calculated as the total number of uneaten feed pellets multiplied by 11.15 mg per pellet, the average weight of a pellet, determined by weighing 100 feed pellets. Daily feed intake per aquarium resulted from the difference between daily feed load and daily feed loss. To account for mortalities, daily feed intake per aquarium was divided by the number of fish in the aquarium, yielding the daily feed intake per fish in each aquarium. Cumulative daily feed intake per fish was calculated from the daily feed intake per fish in each aquarium. Total feed intake per fish (TFI) was determined as the cumulative feed intake at the last day of the experiment. Total feed intake per fish and biomass increase per fish were used to calculate feed conversion ratio (FCR) as follows:

$$FCR = \frac{TFI}{(W_t - W_0)}$$

Where *FCR* = feed conversion ratio (g/g), *TFI* = total feed intake (g/fish), W_t = mean individual weight at day 42 (g) and W_0 = mean individual weight at day 0 (g).

2.8 Statistics

2.8.1 Physiological parameters

Physiological parameters are expressed as mean (SD) of the individual measurements per treatment. For each treatment, 24 fishes were sampled; in some instances less samples were analyzed due to mortalities or because of insufficient plasma volume. When necessary, data were log-transformed to obtain residuals that were normally distributed and to obtain homogeneity of

variance of residuals across treatment levels. Mean values for physiological parameters were tested for differences among the treatments using linear mixed models (REML) with treatments as fixed effects and aquarium as a random effect (F-tests with Kenward-Roger approximation to the residual degrees of freedom (Kenward and Rogers, 1997)). Statistical analyses were performed in SAS 9.2 (SAS Institute Inc., Cary, North Carolina, USA). Only when significant treatment effects were detected, a least significance difference (LSD) post-hoc analysis was used to estimate the level of significance between mean values. For both REML and LSD analysis the fiducial limit was set at 5%. Linear regression analyses was performed with response variables as fixed effects and aquaria as random effects. In addition, F-tests with Kenward-Roger approximation to the residual degrees of freedom were used (Kenward and Rogers, 1997). Pair-fed groups were not considered in regression analyses.

2.8.2 Feed intake and growth

Initial and final individual weight, specific growth rate (SGR) and feed conversion ratio (FCR) are presented as means per treatment (N=2). Mean values per treatment were tested for significant differences among the treatments by one-way ANOVA. Only in case significant treatment effects were detected, a least significance difference (LSD) post-hoc analysis was used to estimate the level of significance between mean values.

Mean (N=2) cumulative daily feed intake per treatment was tested for significant differences among the treatments by repeated measures ANOVA.

Only in case significant treatment effects were detected, a post-hoc analysis (LSD for one-way ANOVA, Tukey for repeated measures ANOVA) was used to estimate the level of significance between mean values. All analyses were performed in SAS 9.2. For all analyses the fiducial limit was set at 5%.

2.9 Concentration-effect curves and NOEC.

NH_3 concentration-effect curves were fitted for specific growth rate (SGR) and total feed intake per fish (TFI) using a log-logistic model (Seefeldt et al, 1995). As a blank could not be included, the effects are expressed as absolute values. Curve-fitting was carried out with the Marquadt and Levenberg algorithm (Moré, 1978) as provided in the PRISM 4.00 software package (Graphpad Software, Inc.). The 10% effect concentrations (EC_{10}) and their 95% confidence limits were calculated (Miller and Miller, 2000). In the NO_3^- experiment, no observed effect concentrations (NOEC) were determined for

SGR and TFI as the highest NO_3^- concentrations in the experiment at which no significant difference with the control treatment were observed.

3. Results

3.1 Plasma NH_4^+

Up to the second highest water NH_3 concentration (11.2 μM) the mean plasma NH_4^+ concentrations ranged between 614 and 762 μM without significant differences among treatments. However, at 18.6 μM water NH_3, the plasma NH_4^+ concentration had significantly increased to a nearly double level of 1399 μM compared to all other treatments (Table 3).

3.2 Plasma NO_3^-

Water NO_3^- concentration had a strong effect on plasma NO_3^- concentration. Plasma NO_3^- concentrations differed among all treatments (Table 4) and increased linearly with increasing water NO_3^- concentration ([Plasma NO_3^-] = 0.23 * [Water NO_3^-]-0.1(mM); $P < 0.0001$). In pair-fed fish plasma NO_3^- levels (Table 4) were not affected.

3.3 Plasma chloride and osmolality and branchial Na^+/K^+-ATPase activity

The increase in plasma NH_4^+ at the highest NH_3 concentration concurred with a significant decrease in plasma chloride to 107 mM. Plasma chloride ranged between 136 and 150 mM in all other NH_3 treatments without significant differences among these treatments (Table 3). For plasma osmolality significant differences were detected among NH_3 treatments (Table 3). No differences in plasma chloride concentration or osmolality were detected among NO_3^- treatments (Table 4). No significant differences in branchial Na^+/K^+-ATPase activity were detected among NH_3 treatments (Table 3) or NO_3^- treatments (Table 4).

3.4 Hematocrit, methaemoglobin, plasma cortisol, glucose and lactate

No significant differences in hematocrit, plasma concentrations of cortisol, glucose and lactate were detected among NH_3 treatments (Table 5) and NO_3 treatments (Table 6). Brown coloration of sampled blood, indicative of methaemoglobin formation, was not observed.

NH_3 Treatment	Water NH_3 (µM)	Plasma NH_4^+ (µM)	n	Plasma Cl^- (mM)	n	Plasma osmolality (µOsmol/kg)	n	Na^+/K^+-ATPase activity (µmol Pi/h/mg protein)	n
1 - T = 0	-	716 (348)	11	153 (27)^a	22	304 (10)	23	-	-
2 - Pair fed	0.46	614 (175)^a	8	136 (24)^a	18	281 (9)^{ad}	20	1.44 (0.73)	12
3 – Control	0.90	657 (250)^a	12	150 (24)^a	13	296 (14)^{bc}	14	1.42 (0.45)	12
4 - NH_3	3.55	696 (222)^a	15	149 (19)^a	17	301 (18)^b	20	1.44 (0.38)	12
5 - NH_3	5.16	656 (131)^a	13	138 (22)^a	15	296 (9)^{bc}	17	1.40 (0.53)	12
6 - NH_3	7.12	759 (155)^a	16	149 (28)^a	17	283 (23)^a	21	1.17 (0.30)	11
7 - NH_3	11.2	762 (183)^a	15	140 (25)^a	18	286 (20)^{ac}	19	1.42 (0.51)	12
8 - NH_3	18.9	1399 (361)^b	13	107 (26)^b	12	270 (22)^d	16	1.52 (0.53)	11
P value		0.04		0.0001		<0.0001		0.91	

Table 3. Mean (SD) values at the start (t =0) and per treatment for the end (t = 42 days) of the ammonia experiment for plasma NH_4^+, plasma Cl^-, plasma osmolality and branchial Na^+/K^+-ATPase activity. Mean values with different superscripts are significantly different (REML, P values as shown). SD = standard deviation of mean values per treatment, n as indicated in the table. t = 0 values were not considered in the statistical analysis.

NO_3^- Treatment	Water NO_3^- (mM)	Plasma NO_3^- (µM)	n	Plasma NO_3^- to water NO_3 ratio	n	Plasma Cl^- (mM)	n	Plasma osmolality (µOsmol/ kg)	N	Na^+/K^+-ATPase activity (µmol Pi/h/mg protein)	n
1 - T = 0	-	-	-	-	-	121 (21)	20	320 (9)	20	-	-
2 - Pair fed	0.1	75 (27)^a	22	1.05 (0.38)^a	22	142 (24)	24	309 (13)^{ac}	24	1.2 (0.4)	8
3 – Control	0.1	88 (47)^a	19	0.90 (0.48)^a	19	152 (24)	24	303 (6)^{ab}	24	1.4 (0.3)	8
4 - NO_3^-	1.5	380 (52)^{ab}	20	0.26 (0.03)^b	20	149 (19)	23	303 (6)^{ab}	23	1.4 (0.7)	9
5 - NO_3^-	2.3	552 (90)^{ab}	18	0.24 (0.04)^b	18	141 (29)	24	299 (3)^b	24	1.2 (0.4)	9
6 - NO_3^-	3.7	820 (151)^b	20	0.22 (0.04)^b	20	152 (11)	22	308 (8)^{ac}	22	1.4 (0.3)	8
7 - NO_3^-	6.1	1378 (197)^c	19	0.23 (0.03)^b	19	150 (14)	24	308 (6)^{ac}	23	1.4 (0.7)	8
8 - NO_3^-	10.2	2136 (642)^d	14	0.20 (0.05)^b	14	133 (21)	22	307 (7)^{ac}	23	1.2 (0.3)	8
9 - NO_3^-	15.8	3493 (553)^e	19	0.22 (0.04)^b	19	144 (25)	23	303 (7)^{ab}	22	1.2 (0.4)	8
10 - NO_3^-	25.6	5993 (899)^f	21	0.23 (0.04)^b	21	135 (27)	24	312 (10)^c	23	0.8 (0.4)	8
P value		< 0.0001		< 0.0001		0.64		0.06		0.40	

Table 4. Mean (SD) values at the start (t =0) and per treatment for the end (t = 42 days) of the nitrate experiment for plasma NO_x, plasma Cl^- , plasma osmolality and branchial Na^+/K^+-ATPase activity. Mean values with different superscripts are significantly different (REML, P values as shown). SD = standard deviation of mean values per treatment, n as indicated in the table. T = 0 values were not considered in the statistical analysis.

NH_3 Treatment	Water NH_3 (μM)	Plasma cortisol (nM)	N	Plasma glucose (mM)	n	Plasma lactate (mM)	n	Hematocrit (%)	n
1 - T = 0		14.3 (8.8)	23	5.63 (3.94)	22	7.56 (4.24)	23	23.1 (3.6)	22
2 - Pair fed	0.46	12.6 (15.5)	21	7.94 (2.19)	20	4.11 (1.09)	20	32.3 (10.2)	21
3 - Control	0.90	14.3 (8.8)	15	5.99 (2.49)	16	5.34 (2.41)	14	40.8 (12.1)	17
4 - NH_3	3.55	12.4 (13.0)	20	6.65 (2.36)	20	5.73 (1.52)	20	31.8 (12.3)	20
5 - NH_3	5.16	11.3 (9.1)	18	7.17 (1.08)	17	5.77 (2.45)	17	33.7 (12.2)	23
6 - NH_3	7.12	17.7 (13.6)	21	7.15 (2.64)	21	4.51 (1.65)	18	38.0 (12.9)	23
7 - NH_3	11.2	16.0 (10.7)	19	7.27 (2.17)	19	4.34 (2.08)	18	37.3 (12.1)	19
8 - NH_3	18.9	10.4 (10.4)	18	6.99 (2.55)	18	5.30 (2.44)	16	29.5 (16.1)	19
P value		0.87		0.85		0.63		0.08	

Table 5. Mean (SD) values at the start (t =0) and per treatment for the end (t = 42 days) of the ammonia experiment for plasma cortisol, plasma glucose and plasma lactate concentrations and hematocrit. Mean values with different superscripts are significantly different (REML, P values as shown). SD = standard deviation of mean values per treatment, n as indicated in the table. T = 0 values were not considered in the statistical analysis.

NO_3^- Treatment	Water NO_3^- (mM)	Plasma cortisol (nM)	n	Plasma glucose (mM)	n	Plasma lactate (mM)	n	Hematocrit (%)	n
1 - T = 0	-	13 (22)	20	5.4 (2.8)	22	7.5 (3.5)	22		12
2 - Pair fed	0.1	134 (106)	24	7.5 (1.9)	24	7.3 (3.3)	13	37 (4)	24
3 – Control	0.1	210 (150)	23	7.5 (1.6)	24	6.8 (2.6)	20	36 (4)	24
4 - NO_3^-	1.5	119 (123)	23	7.5 (1.9)	23	7.0 (2.5)	18	38 (7)	24
5 - NO_3^-	2.3	166 (162)	24	7.3 (1.8)	24	5.4 (3.1)	19	37 (1)	24
6 - NO_3^-	3.7	123 (132)	22	7.7 (1.9)	24	8.0 (3.0)	18	31 (6)	24
7 - NO_3^-	6.1	186 (107)	24	7.9 (1.3)	24	8.2 (4.0)	18	36 (5)	24
8 - NO_3^-	10.2	155 (123)	22	7.5 (1.6)	24	6.8 (3.5)	14	36 (2)	24
9 - NO_3^-	15.8	115 (132)	21	7.5 (2.4)	24	7.3 (3.1)	15	38 (5)	23
10 - NO_3^-	25.6	47 (31)	23	7.9 (1.5)	24	8.6 (3.7)	17	35 (6)	24
P value		0.76		0.99		0.99		0.86	

Table 6. Mean (SD) values at the start (t =0) and per treatment for the end (t = 42 days) of the nitrate experiment for plasma cortisol, plasma glucose and plasma lactate concentrations and hematocrit. Mean values with different superscripts are significantly different (REML, P values as shown). SD = standard deviation of mean values per treatment, n as indicated in the table. t = 0 values were not considered in the statistical analysis.

3.5 Gill morphology.

Gill morphology (Fig. 1) was not affected by water NH_3, nor NO_3^-.

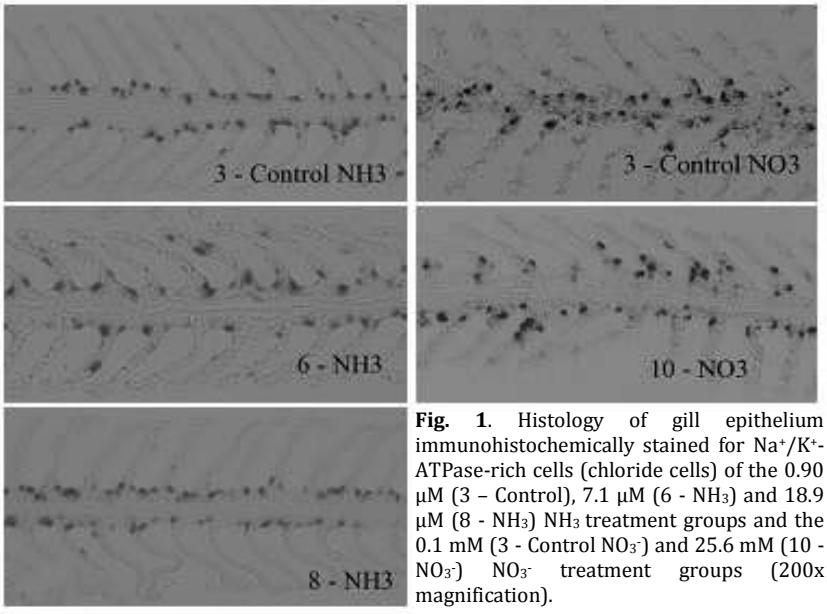


Fig. 1. Histology of gill epithelium immunohistochemically stained for Na^+/K^+-ATPase-rich cells (chloride cells) of the 0.90 µM (3 – Control), 7.1 µM (6 - NH_3) and 18.9 µM (8 - NH_3) NH_3 treatment groups and the 0.1 mM (3 - Control NO_3^-) and 25.6 mM (10 - NO_3^-) NO_3^- treatment groups (200x magnification).

3.6 Feed intake, specific growth rate, feed conversion ratio and mortality.

In the NH_3 experiment a total of 19 out of 168 fish had died across NH_3 treatments, resulting in a survival rate ranging between 71 and 100% among treatments. Mortality was probably a consequence of low feed intake as mortalities occurred mainly towards completion of the NH_3 experiment and, without exception, dead fish appeared emaciated (data not shown). Mortality could not be related to the NH_3 treatments (Table 7), leaving the underlying reason for emaciation unclear.

NH_3 exposure had a strong effect on total feed intake (TFI) and specific growth rate (SGR). For both TFI and SGR differences were detected among NH_3 treatments (Table 7). At the highest NH_3 concentration (18.9 µM) the TFI had decreased by 69% and SGR by 75% compared to control. The differences in TFI developed over time (Fig. 2). Mean feed conversion ratios (FCR) did not differ among NH_3 treatments (Table 7).

No fish died in the NO_3^- experiment. Differences in final weight, total feed intake (TFI), specific growth rate (SGR) and feed conversion ratio (FCR) were not detected among NO_3^- treatments (Table 8).

NH_3 Treatment	Water NH_3 (μM)	Initial weight (g)	Final weight (g)	TFI (g/fish)	SGR (%BW/d)	FCR	Survival (%)
1 - T = 0	-	16.1 (0.57)	-	-	-	-	-
2 - Pair fed	0.46	17.3 (0.41)	25.3 (0.20) ^{ab}	7.5 (0.21)^{a}	0.95 (0.04)^{a}	0.94 (0.00)	92 (0.0)
3 - Control	0.90	18.7 (3.19)	53.4 (12.6)^{c}	21.7 (0.55)^{bc}	2.61 (0.17)^{b}	0.65 (0.16)	71 (18)
4 - NH_3	3.55	18.2 (0.58)	46.9 (1.37)^{cd}	23.0 (1.78)^{b}	2.36 (0.01)^{bc}	0.80 (0.04)	88 (6)
5 - NH_3	5.16	17.7 (0.04)	46.0 (4.89)^{cd}	24.1 (1.73)^{b}	2.38 (0.26)^{bc}	0.86 (0.09)	96 (6)
6 - NH_3	7.12	17.9 (0.38)	40.4 (0.62)^{cd}	19.8 (1.04)^{c}	2.04 (0.09)^{cd}	0.88 (0.01)	100 (0)
7 - NH_3	11.2	18.1 (0.45)	36.7 (6.16)^{ad}	15.4 (0.13)^{d}	1.75 (0.36)^{d}	0.87 (0.26)	88 (18)
8 - NH_3	18.9	17.3 (0.27)	22.5 (1.07)^{b}	6.7 (0.32)^{a}	0.65 (0.08)^{a}	1.31 (0.27)	88 (6)
P value		0.91	0.007	<0.0001	<0.0001	0.07	0.27

Table 7. Mean (SD) values per treatment (N=2) in the ammonia experiment for initial weight, final weight, total feed intake (TFI), specific growth rate (SGR) and feed conversion ratio (FCR). Mean values with different superscripts are significantly different (ANOVA, P values as shown).SD = Standard deviation of mean values per treatment.

NO_3^- Treatment	Water NO_3^- (mM)	Initial weight (g)	Final weight (g)	TFI (g/fish)	SGR (%BW/d)	FCR
1 - T = 0	-	26.4 (0.3)	-	-	-	-
2 - Pair fed control	0.1	26.6 (0.5)	67.9 (3.3)	34.7 (2.1)	2.29 (0.16)	0.81 (0.02)
3 - Control	0.1	27.5 (2.0)	72.5 (0.5)	35.2 (1.1)	2.31 (0.16)	0.78 (0.00)
4 - NO_3^-	1.5	26.3 (3.7)	70.4 (2.1)	35.4 (1.0)	2.35 (0.26)	0.80 (0.01)
5 - NO_3^-	2.3	27.4 (0.0)	70.8 (3.5)	35.8 (1.4)	2.26 (0.12)	0.82 (0.03)
6 - NO_3^-	3.7	27.7 (0.3)	65.7 (3.7)	30.7 (3.1)	2.05 (0.11)	0.81 (0.01)
7 - NO_3^-	6.1	27.4 (0.3)	72.8 (3.7)	35.5 (1.7)	2.33 (0.10)	0.78 (0.02)
8 - NO_3^-	10.2	27.5 (0.2)	70.2 (4.2)	34.5 (2.7)	2.23 (0.13)	0.81 (0.01)
9 - NO_3^-	15.8	25.4 (0.5)	68.2 (6.1)	33.8 (3.8)	2.35 (0.17)	0.79 (0.02)
10 - NO_3^-	25.6	28.2 (1.8)	69.5 (3.2)	33.6 (0.5)	2.15 (0.04)	0.82 (0.02)
P value		0.58	0.81	0.42	0.46	0.25

Table 8. Mean (SD) values per treatment (N=2) in the nitrate experiment for initial weight, final weight, total feed intake (TFI), specific growth rate (SGR) and feed conversion ratio (FCR). SD = Standard deviation of mean values per treatment.

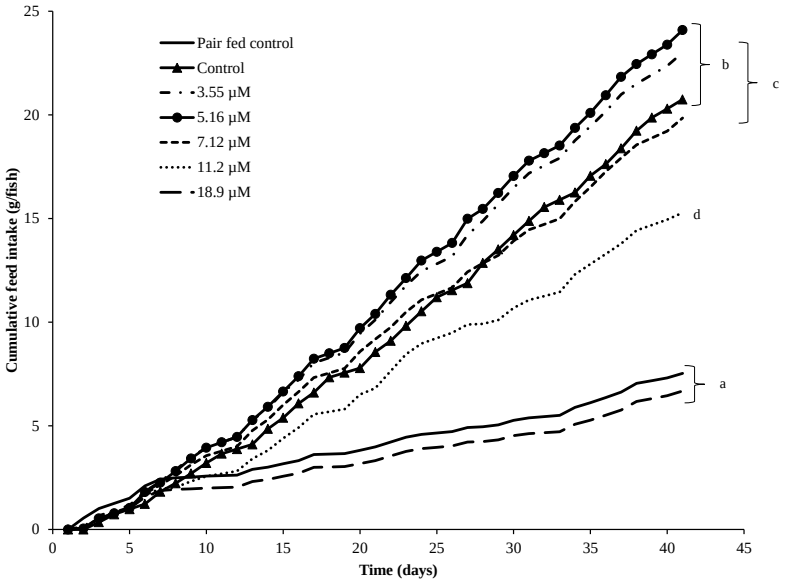


Fig. 2 Mean (N = 2) cumulative feed intake of juvenile pikeperch exposed to ammonia. For series marked with different letters the cumulative feed intake resulted in significantly different total feed intake (Repeated measures ANOVA, $P < 0.05$).

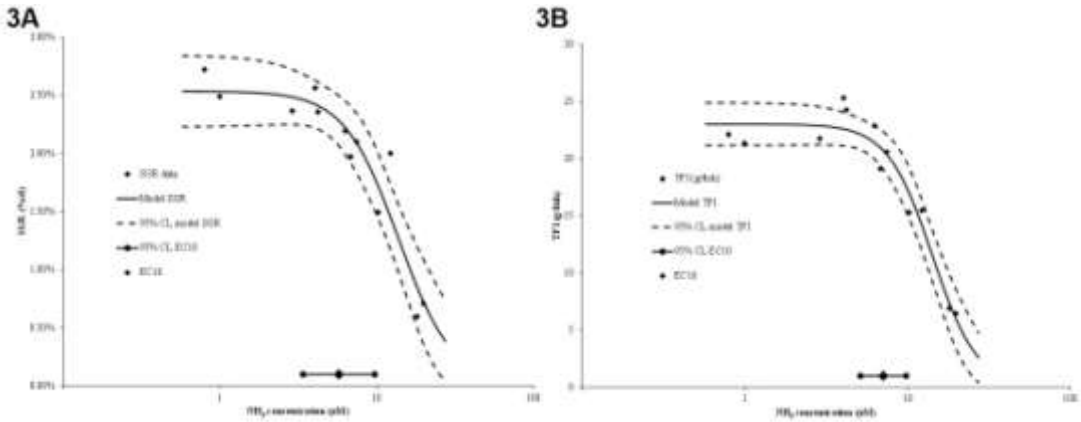


Fig. 3. Concentration-effect curves for specific growth rate (SGR, 3A) and total feed intake (TFI, 3B) in relation to the water NH_3 concentration. SGR = $0.025\text{-}0.025/(1+10^{(-\log[NH3]-1.14)/0.40)})$, ($r^2 = 0.90$) and TFI = $23.1 - 23.1/(1+10^{(-\log[NH3]-1.15)/0.32)})$, ($r^2 = 0.94$). EC_{50} for SGR = 13.7 µM NH_3, EC_{50} for TFI = 14.2 µM NH_3.

3.7 EC_{10} and NOEC for total feed intake and SGR

The concentration-effect curves for TFI and SGR in relation to the water NH_3 concentration (Figs. 3A and 3B), yield an EC_{10} for NH_3 of 7.1 µM (0.1 mg NH_3-N/L), with a 95% confidence interval from 5.1 to 9.8 µM when read against TFI. For SGR, a somewhat lower EC_{10} for NH_3 of 5.7 µM (0.08 mg NH_3-N/L), with a 95% confidence interval from 3.4 to 9.7 µM was calculated. For both SGR and TFI the NOEC was 5.2 µM NH_3.

For both SGR and TFI the NO_3^- NOEC was 25.6 mM, coinciding with the highest NO_3^- concentration the fish were exposed to.

4. Discussion

4.1 NH_3 experiment

Juvenile pikeperch (*Sander Lucioperca*) successfully control plasma NH_4^+ up to a water NH_3 concentration of 11.2 µM. However, water NH_3 levels below 11.2 µM lead to reduced feed intake and growth and thus appear superior parameters to assess NH_3-tolerance.

4.1.1 Plasma NH_4^+, feed intake and growth

Fish produce ammonia as main end product of the catabolism of ingested proteins (Handy and Poxton, 1993). Ammonia then appears in the plasma compartment, which shows postprandial peaks (Wicks and Randall, 2002a). Self-intoxication by NH_3 is avoided in fish by up-regulating muscle glutamine synthetase activity (Wicks and Randall, 2002b) and by excreting ammonia across the gills to the water (Wilkie, 2002). High external (water) ammonia leads to an influx of ammonia in plasma and tissues (Wright et al, 2007). To avoid toxic plasma ammonia levels during an influx of external ammonia, fish reduce their own ammonia production by reduction of food intake (Randall and Tsui, 2002). This ammonia defense mechanism explains the reduced feed intake we observed in ammonia exposed pikeperch. Differences in feed intake showed up more or less instantaneously at the two highest NH_3 levels (11.2 and 18.9 µM), resulting in significant differences in total feed intake among treatments at the end of the experiment. We conclude from this observation that the reduced growth in pikeperch in response to NH_3 is mainly an effect of reduced feed intake, which corresponds to previous observations on NH_3 exposed turbot (*Scophthalmus maximus*) (Person-Le Ruyet et al, 1997) and African catfish (*Clarias gariepinus*) (Schram et al, 2010).

A higher ability of fish to cope with high external ammonia has been related to lower plasma ammonia concentrations in these fish (Wicks and Randall, 2002a). The basal plasma ammonium concentrations of around 650 µM observed in pikeperch seem high compared to values reported for other fish species (~160 µM NH_4^+ in African catfish (Schram et al, 2010); ~200 µM T_{amm} in Atlantic salmon (*Salmo salar*) (Knoph and Thorud, 1996); ~300 µM T_{amm} in European seabass (*Dicentrarchus labrax*) (Lemarié et al, 2004), may reflect an adaptation to piscivory, and may be related to increased tolerance to NH_4^+ through protonation of NH_3 in the plasma compartment.

We advise to set the water NH_3 threshold concentration for growth at 3.4 µM, the lower limit of the 95% confidence interval of the EC_{10} we calculated for growth. This indeed classifies juvenile pikeperch as significantly more sensitive to water ammonia levels than for instance African catfish (EC_{10} of 24 µM). Atlantic salmon appears more sensitive than pikeperch, with growth being affected already above 1.4 µM NH_3 (Arillo, 1981); also, basal plasma total ammonium values in Atlantic salmon are relatively low (~200 µM T_{amm}, Knoph and Thorud, 1996). From that perspective pikeperch seems relatively robust and tolerates high water ammonia through the ability to maintain acceptable plasma NH_4^+ values over a rather wide range of water ammonia levels. The prediction would be that this tolerance increases at lower water temperatures assuming similar chemistry of NH_3 in water and blood plasma (Emerson et al, 1975). In Atlantic salmon (Knoph and Thorud, 1996) and several other marine species such as European seabass, gilthead sea bream (*Sparus aurata*) and turbot (Person-Le Ruyet et al, 1995) this ability seems absent as plasma ammonia has been observed to increase linearly with ammonia in the water. Unfortunately the possibilities to explore the relation between basal plasma ammonia and ammonia sensitivity are limited as data on plasma ammonia and detailed threshold concentrations for chronic NH_3 exposure of fish are scarce.
The ability of pikeperch to buffer plasma NH_3 to NH_4^+ at higher water NH_3 is limited yet considerable as the plasma NH_4^+ concentration in pikeperch exposed to 18.9 µM NH_3 in the water doubled to almost 1.4 mM compared to control values. Apparently a threshold is surpassed between 11.2 and 18.9 µM NH_3 above which pikeperch can no longer maintain low plasma NH_4^+ concentrations.

4.1.2 Plasma osmolality, plasma chloride, Na^+/K^+-ATPase activity and gill morphology

Freshwater fish continuously lose ions via diffusion across the mucous epithelium of gills and skin to the surrounding less saline water (Evans et al,

2005). For homeostasis of bodily fluids freshwater fish tightly regulate plasma osmolality by active Na^+ and Cl^- uptake (McDonald and Wood, 1981). Hypochloremia was detected in pikeperch exposed to 18.9 µM NH_3 (concurrent with a significant increase in plasma NH_4^+) revealing a disturbance of chloride-homeostasis and interference of plasma NH_4^+ with chloride handling. Branchial and intestinal chloride uptake from water and food and renal reabsorption are key to chloride homeostasis in freshwater fish (Fuentes et al, 1997). It would seem then that the significant decrease in food intake seen at high water NH_3 also interferes with chloride regulation in this fish. Further, significant differences in osmolality were detected among NH_3 treatments. Significantly reduced osmolality as compared to the control treatment was also observed in pair-fed controls. Thus the decrease in osmolality may indeed be an effect of reduced feed intake rather than NH_3 exposure *per se*.

At high ambient ammonia several active NH_4^+ excretion pathways could facilitate ammonia efflux (Wright and Wood, 2009). Na^+/K^+-ATPase is a driving force in active NH_4^+ excretion when internal NH_4^+ (replacing K^+ on the enzyme) is exchanged for waterborne Na^+ (reviewed by Heisler, 1984; Evans, 1987; Evans et al, 2005). Increased branchial Na^+/K^+-ATPase activity in response ammonia exposure has been described for several fish species (Alam and Frankel, 2006; Sinha et al, 2012). However, in pikeperch branchial Na^+/K^+-ATPase activity did not increase in response to increased ambient NH_3 nor plasma NH_4^+. An extensive (re-)analysis of NH_3 chemistry at the (sub-)cellular level and consideration of NH_3 transporter (Rhesus) proteins (Nakada et al, 2007) seems indicated. At least 4 Rhesus protein species were described in branchial epithelium of zebrafish (Braun et al, 2009), but analysis of these is beyond the scope of this study.

There appears to be no consensus on the effect of ammonia on fish gill morphology. In the past gill hyperplasia has been proposed as a common indicator for ammonia toxicity in fish (Smith and Piper, 1975; Redner and Stickney, 1979 in Mitchell and Cech, 1983) based on various ammonia toxicity studies presenting evidence of gill epithelial damage (citations in Mitchell and Cech, 1983). However, no evidence of gill damage could be detected in ammonia exposed rainbow trout (*Oncorhynchus mykiss*) (Smart, 1976), Dover sole (*Solea solea*) and turbot (*Scophthalmus* maximus) (Alderson, 1979). The validity of attributing gill hyperplasia to ammonia alone was questioned by Mitchell and Cech (1983) once they showed that gill hyperplasia was absent in ammonia exposed channel catfish (*Ictalurus punctatus*), except when low levels of chlorine compounds, residuals from municipal water treatment, were present next to ammonia. Indeed, for many ammonia toxicity studies in the past the presence of chlorine compounds cannot be excluded. Gill damage observed in

past ammonia toxicity studies should however not be automatically attributed to chlorine rather ammonia exposure as in African catfish gill morphology gradually changed with increasing NH_3 exposure concentration in water that had not been chlorinated (Schram et al, 2010).

Morphological changes of the gills may be interpreted as adaptations to increase the diffusion distance between the water and the blood flow, reducing the permeability of the gills and subsequently the influx of NH_3. No clear effects of ammonia exposure on gill morphology were observed in pikeperch. The absence of such a morphological response in pikeperch gills surprised us as pikeperch were clearly affected by the highest NH_3 exposure concentration (18.9 μM). However, the absence of morphological changes in the gills is not exceptional and has been previously reported in other ammonia exposed fish species (see above).

4.1.3 Stress physiology

Plasma cortisol levels below 50 ng/mL or 138 nM are considered as stress free levels (typical basal levels for common carp, *Cyprinus carpio*; below 15 nM; Metz et al, 2005). Increases up to 166 nM are generally referred to as a mild response, while rapid increases above 276 nM are generally considered to reflect a severe stress response (Wendelaar Bonga, 1997). The stress and energy metabolite parameters (plasma cortisol, glucose and lactate) in pikeperch were not affected by NH_3 exposure. Plasma cortisol values found in this study (10-18 nM) are very much lower than values reported in other studies on pikeperch (> 700 nM, Falahatkar et al, 2012; 124-180 nM, Sarameh et al, 2012) and probably the best representation of basal cortisol levels in pikeperch to date. Plasma glucose and lactate levels observed in the NH_3 experiment are slightly higher than control values reported in other pikeperch studies (5mM lactate, 4.5-6 mM glucose, Falahatkar et al, 2012) but lie in the range that can be considered normal. We conclude form these observations that ammonia even at its highest exposure concentration apparently did not impose distress.

4.2 NO_3^- experiment

NO_3^- exposed juvenile pikeperch accumulated nitrate in the plasma compartment, but no effects on physiology and growth were detected even at water nitrate as high as 26 mM, commensurate with the notion that the end product of the nitrogen waste cycle is relative harmless to fish. The high NO_3^- tolerance allows for low water exchange of RAS used for juvenile pikeperch

culture. However it should be noted that, next to apparently relatively harmless NO_3^-, other, potentially harmful compounds may then accumulate in the rearing water.

4.2.1 Plasma NO_3^-, feed intake and growth

Nitrate can become toxic to fish (Camargo et al, 2005). Chronic exposure to high water nitrate leads to nitrate accumulation in the plasma compartment and reduced feed intake and growth in African catfish (Schram et al, 2012). In pikeperch plasma NO_3^- concentrations increased linearly with increasing external NO_3 concentrations, seemingly unaffected by the differences in sodium concentration and conductivity among treatments. Plasma NO_3^- increased in a very similar manner to what we observed in NO_3^- exposed African catfish (Schram et al, 2012). Surprisingly, whereas African catfish showed reduced growth and feed intake upon nitrate exposure, pikeperch was refractory to this treatment. Clearly species-specific differences in NO_3^- toxicity exist and are not related to differences in the capability to maintain low plasma NO_3^- when external NO_3^- is high. Instead the internal NO_3^- handling seems much more important. Differences in sodium concentration and conductivity arising from the sodium nitrate addition to the aquaria apparently did not affect NO_3^- accumulation in the plasma given the linear increase in plasma NO_3^- with increasing external NO_3^- concentrations.

The growth performance of the pikeperch in our NO_3^- experiment (overall mean (SD) specific growth rate of 2.26 (0.15) %/d) corresponds well to growth performances of similar sized pikeperch at the same water temperature in commercial pikeperch farming (Vestergaard, personal communication).

Previously observed molar ratios (plasma:water) of approximately 0.2 in nitrate exposed rainbow trout (Stormer et al, 1996) and African catfish (Schram et al, 2012) suggest that the integument forms a significant barrier to waterborne nitrate. The molar ratios we observed in pikeperch range from 0.23 to 0.26 and are in good agreement with these observations, suggesting similar nitrate handling in rainbow trout, African catfish and pikeperch. Daily plasma sampling of catheterized, nitrate exposed rainbow trout over a period (8 days) revealed that an apparent chemical equilibrium was reached within a day (Stormer et al, 1996). We have no data that describe the time-kinetics of plasma nitrate levels in pikeperch. However, considering Stormer's (1996) observations on rainbow trout it seems unlikely that after 42 days of nitrate exposure, nitrate further accumulates in the plasma to levels that are eventually not tolerated by pikeperch. We therefore consider the current experiment representative for chronic nitrate exposure.

The millimolar plasma NO_3^- concentrations that are apparently tolerated by pikeperch support the notion that NO_3^- is not very toxic to fish and may be considered an end product and stable non-toxic form of nitrogenous waste.

4.2.2 NO_3^- effects on physiology and gill morphology

High levels of NO_3^- in the water did not affect plasma osmolality and plasma chloride. NO_3^- exposure did not affect branchial Na^+/K^+-ATPase activity. Apparently a significant hypernitratemia does not alter the activity if this enzyme. This corresponds to our observation that the number of Na^+/K^+-ATPase rich chloride cells was not affected by NO_3^- exposure.

NO_3 exposure did not cause morphological changes nor anomalies of the branchial epithelium of pikeperch. The results taken jointly indicate that high levels of NO_3^- do not affect permeability of the gills, neither to water or ionic species central to osmotic homeostasis, nor to NO_3^- itself (Stormer et al, 1996) as we conclude from the linear mild increase in molar ratios for NO_3^- in water and plasma; clearly, NO_3^- is not very toxic for this species.

Considering the mildly elevated plasma cortisol values (just over 100 nM) observed in all NO_3^- treatments, it seems likely that treatment effects (if any) on plasma cortisol were overridden by cortisol release due to sampling procedures. However, the normal values observed for plasma glucose and plasma lactate in pikeperch suggest that NO_3^- exposure did not chronically stress the fish (Wendelaar Bonga, 1997).

5. Conclusions

Toxicity of nitrogenous compounds to fish depends on several biotic parameters, including size and life-stage (McGurk et al, 2006; Brinkman et al, 2009). The here reported NH_3 and NO_3^- threshold concentrations may therefore be exclusively applicable to juvenile pikeperch, *Sander lucioperca*. Juvenile pikeperch chronically exposed to NH_3 as high as 11.2 µM NH_3 did not show major physiological disturbances. However, feed intake and growth decreased already at very much lower NH_3 concentrations: the EC_{10} was found to be 5.7 µM for SGR and 7.1 µM for TFI. Feed intake and growth are thus good and easily assessed indicators for negative effects of high NH_3 on pikeperch. Considering the lower limit of the 95% confidence interval of the lowest EC_{10} value, the NH_3 threshold concentration for juvenile pikeperch should be set at 3.4 µM (0.05 mg NH_3-N/L).

Juvenile pikeperch chronically exposed to the highest NO_3^- test concentration (25.6 mM) did not show major physiological disturbances or reduced growth performance. The threshold concentration for chronic NO_3^- exposure of juvenile pikeperch thus seems to lie outside the NO_3^- range investigated in the current experiment. We propose to use the highest test concentration that (still) showed no significant effect as a safe threshold concentration for NO_3^-: 25.6 mM (358 mg NO_3^--N/L).

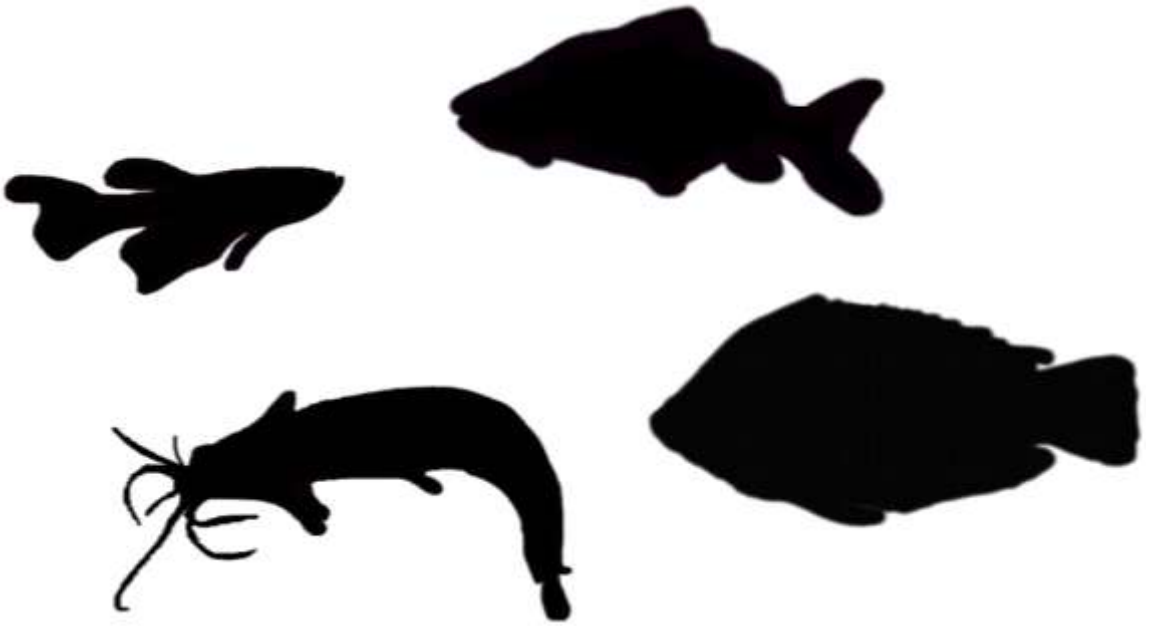
Acknowledgements

This study was funded by the Dutch Ministry of Economic Affairs, Agriculture and Innovation, contract number BO-12.02-002-041.06.

The authors would like to express their gratitude to Tony Coenen and Margot Bekhuis for their technical assistance.

Chapter 9

General discussion



General discussion

In this thesis acute and chronic discomfort of cultured fish is addressed. After a brief summary of the most important findings a more in-depth discussion of the main findings is given.

1. Summary of the results

The overall aim of this thesis research is to increase knowledge on welfare of farmed fish through study of the effects of relevant and well-defined external stimuli on physiology and behavior.

In **chapters 2-4**, we investigated the effects of acute, potentially painful stimuli that are likely to cause acute discomfort in fish. We used ultrastructural, physiological and behavioral analyses as read-out parameters for this acute discomfort.

The presence of nerve fibers involved in pain perception (A-δ and C-fibers) was established in the tailfin of the teleost common carp, *Cyprinus carpio* (**chapter 2**); very similar fibers could also be demonstrated in the tail fin of Mozambique tilapia, *Oreochromis mossambicus* (**chapter 3**) and zebrafish, *Danio rerio* (**chapter 4**). When Nile tilapia, *Oreochromis niloticus*, received a tailfin clip, the fish responded by active mucus secretion from their gills 1 h after the stimulus, and after 6 h, branchial chloride cells migrated to lamellar positions and enhanced swimming activity was seen that could be ascribed to the clip proper (compared to controls, in unhandled and handled test groups). Plasma parameters (cortisol, glucose and lactate concentrations) did not allow discrimination between the clipping procedure and the handling stress, showing the limitations of these parameters in this field of research (**chapter 2**).

Chapter 3 deals with the effects of another acute potentially pain-inducing stimulus, presumed non-damaging, applied to the tail region of Mozambique tilapia, *i.e.* a standardized electric shock. This stimulus was selected because it can induce pain without inflicting the apparent physical damage inherent to clipping. We observed decreased swimming activity up to 4 h and a latency in resuming chafing behavior. Plasma glucose had significantly increased 6 h after the shock compared to handling, indicative of an enhanced adrenergic activity. In contrast to the fin clip, no effect on branchial mucus release or chloride cell migration/proliferation was found. The results show that exposure of Mozambique tilapia to even a mild electric shock has an effect on the fish. In our view this study supports the EFSA recommendations on electro-stunning conditions for fish, *i.e.* electrical stunning should provoke

immediate loss of consciousness and sensibility in fish (EFSA, 2009b). The differential responses to the two types of noxious stimuli studied in chapters **2** and **3** indicate that these noxious stimuli are discriminated by the fish from the stress stimuli inherent to the handling procedures to apply a noxious stimulus.

Behavioral parameters such as swimming and light-dark preference were affected by both clipping and electro-shock. In **chapter 4**, the effect of fin clipping on habituation in zebrafish, using a novel tank test was studied. Habituation to novelty can be demonstrated by a reduction in behavior related to anxiety (freezing, erratic movements) and increased exploration. In our study we found no differences in these 'classical' behavioral parameters on the days the fin clip was given (day 1 and day 5). Habituation was affected by the painful stimulus when applied on the 5th day, while the novelty-induced anxiety may override the fin clip effect on the 1st day, as a result of motivational conflict. Therefore we concluded that both fin clipping and mild electro-shocking are noxious stimuli that induce acute discomfort, but discomfort the fish can overcome.

In **chapters 5-8** of this thesis, we investigated effects of ambient nitrogenous waste products on stress parameters and growth of two species during on-growing in European RAS conditions: African catfish, *Clarias gariepinus* and pikeperch *Sander lucioperca.*

Chapter 5, African catfish face severely decreased food intake and growth rate upon exposure to ambient concentrations of ammonia above 90 μM. Gill morphology was gradually affected with increasing ambient ammonia concentrations. At the highest concentration tested, severe epithelium hypertrophy and lamellar fusion was seen, which was interpreted as a defense mechanism to increase the distance between the ambient water and the 'milieu intérieur' of the fish and thereby limit passive ammonia influx. Remarkably, the fish managed to keep plasma ammonium concentrations constant, even at the highest ambient ammonia levels. Other plasma parameters remained unaffected or were (mildly) affected, at the highest ambient ammonia concentration only. We advise, based on a concentration dependence study, not to exceed a water concentration of 24 μM NH_3, to avoid the risk of impaired growth, reduced feed intake and deteriorated gill morphology in this species.

Chapter 6 reveals a concomitant rise in the plasma nitrate concentrations of African catfish when exposed to increasing ambient nitrate levels. We show a concomitant rise in plasma nitrate concentrations. Food intake and specific growth rate were only affected at the highest ambient nitrate concentration tested. Hematocrit, plasma composition, gill morphology and cellular make-up remained unaffected regardless of the ambient and

plasma nitrate concentrations. We advise not to exceed a water concentration of 10 millimolar nitrate to avoid the risk of impaired growth in African catfish.

In **chapter 7**, it is demonstrated that African catfish exposed to increasing ambient nitrite concentrations have plasma nitrite concentrations that increased concomitantly. In addition, plasma nitrate increased concomitantly as well, revealing a defense mechanism of internal detoxification (nitrite conversion to the less toxic nitrate in the plasma). Blood hematocrit, hemoglobin and methemoglobin, plasma composition, gill morphology remained unaffected regardless of the ambient nitrite concentrations. We advise not to exceed a water concentration of 43 μM nitrite to prevent risks of impaired growth in this species kept in RAS.

In **chapter 8** we report on juvenile pike perch exposed to different ambient concentrations of NH_3 and NO_3^-. Plasma concentrations of NH_4^+ appeared to be tightly regulated and increased only at the highest concentration of NH_4^+ studied. Plasma NO_3^- however increased linearly with increasing ambient nitrate concentrations. In both studies, blood hematocrit, plasma composition, and gill morphology were unchanged. Growth became affected by an ammonia concentration of 11.2 μM, but was not affected by nitrate exposure. For juvenile pikeperch in RAS we advise not to exceed a water NH_3 3.4 μM (0.05 mg NH_3-N/L), the lower limit of the 95% confidence interval of the EC10 value for ammonia on the specific growth rate (SGR). The threshold concentration for chronic NO_3^- exposure of juvenile pikeperch thus seems to lie outside the NO_3^- range investigated in the current experiment. We propose to use the highest test concentration that (still) showed no significant effect as a safe threshold concentration for NO_3^-: 25.6 mM (358 mg NO_3^--N/L).

2. Nociception and pain in fish

In fish, nerve fiber types that we know are involved in nociception in mammals were first identified in the trigeminal nerve of rainbow trout, *Onchorhynchus mykiss* (Sneddon, 2002). Furthermore, some invertebrates also possess nociceptors (Smith and Lewin, 2009; Elwood, 2011) and there is convincing evidence that some invertebrate species respond to a stimulus known to induce pain in mammals, by behavioral changes (Elwood, 2011). Thus nociception is (at least) a vertebrate trait. But the possession of such fibers, nociceptive reflex circuitry, does not automatically imply pain perception/interpretation of painful stimuli, as this part is done in the brain. In this thesis, we identified and quantified the nociceptive fibers in the tailfin of common carp, Nile tilapia, and zebrafish. The fiber types and composition was similar to those found in the head of rainbow trout (Sneddon, 2002). The

relative abundance of specific nerve fibers is not universal among vertebrates, nor within an organism (Lynn, 1994; Lamont et al, 2000). The differences between mammals and teleosts regarding the relative abundance of A-δ and C-fibers may relate to the water-to-land transition (Sneddon, 2002). A terrestrial niche with increased chances of injuries due to gravity, extreme differences in temperature (day *vs*. night) and noxious gases (Sneddon, 2002) may have had impact on the more complex development of nociceptive system of terrestrial vertebrates. Acute pain stimuli, mediated through A-δ fibers, may have an important survival value as they drive the organism away from the location of the noxious stimulus and will facilitate the healing process (Lamont et al, 2000).

The types of pain mediated by C-fibers in mammals (pathologic and chronic pains) are by their very nature linked to higher cognitive brain centers where noxious signals are interpreted as such. The presence of C-fibers in teleost fish would suggest then an analogy. It seems reasonable to propose that fish, the earliest vertebrates, developed two nociceptive systems to discriminate chronic from acute noxious stimuli as these stimuli are not restricted to terrestrial niches.

For non-human vertebrates, not able to express verbally their subjective experience of pain and distress, scientists have to rely on indirect parameters, including behavior as readout for pain (Chapman et al, 1985; Keefe et al, 1991; Lamont et al 2000). Behavioral changes can be used as indicators for pain studies in fish; rainbow trout injected in their lips with either bee venom or acetic acid, substances used in mammalian pain research, displayed significant delayed resume feeding and increased opercular beat rate for up to 2h 30 min in comparison to control and saline-injected fish (Sneddon et al, 2003a; 2003b); unanaesthetized goldfish, *Carassius auratus*, displayed escape responses to noxious increase in heat (Nordgreen et al, 2009). In our studies we used simple behavioral tests such as swimming activity and place preference in tilapia subspecies receiving a tailfin clip or a tail electric shock as potentially painful stimuli. In both studies, the behavior of the animals was more profoundly affected by these stimuli than the groups that were handled only. After an electric shock plasma glucose had significantly increased at 6 h post-shock compared to handling, which is indicative of enhanced/stronger adrenergic activity; for fin clipping no significant differences in levels of cortisol, glucose, lactate were observed. However, fin clipping resulted in a remarkable migration of Na^+/K^+-ATPase-rich chloride cells in the gills to the lamellar epithelium 6 h after clipping. In the clipped fish the gill mucus cells released their content at 1 h after the clip; this response is transient, at 6 h post clipping mucus cells had 'refilled'. In all vertebrates and independent of location (gills, intestinal tract, skin) mucus cells are well equipped with adrenergic

receptors and thus mucus cell responses appear suitable indicators of adrenergic surges as we anticipated would occur with painful stimuli.

We used the novel tank test paradigm to obtain behavioral indicators for the effects of potentially painful stimuli. The effects of fin clipping on swimming activity and freezing behavior of zebrafish in our novel tank gave a first clue on the impairment of habituation to a novel environment, a modulation of a complex behavior which requires cognitive abilities. These results of pain-modulated behavior suggest interpretation of a presumed painful stimulus for which cognitive functions are required. More research should be carried out using such (automated) behavior tests is fish pain research.

3. Nitrogenous waste: compound- and species-specific effects

Water quality is of crucial importance for fish well-being. Threshold levels for water temperature, salinity, pH, oxygen content are well-documented for many species with economical or research interest. The increasing implementation of recirculating aquaculture systems (RAS) enables fish farmers to be less dependent on natural water sources and reduces the impact of this industry on the environment (Piedrahita, 2003; Read and Fernandes, 2003) but does require close monitoring of water quality, in particularly the feeding-associated production of nitrogenous waste. In RAS water is mechanically and biologically filtered to prevent turbidity and accumulation of ammonia, nitrite and nitrate (Van Rijn et al, 2006). Due to high stocking densities in RAS (compared to open farming systems), problems with nitrogenous waste exposure may easily arise. In sub-optimally functioning RAS, fish can be exposed to high and toxic concentrations of any of the nitrogenous substances associated with nitrogen cycling in the system. When starting culture of a new species in RAS, it is quintessential to know optimal conditions of culture, especially regarding formation of nitrogenous waste products. Nitrogenous waste production is particularly problematic under high feeding pressure and/or when the capacity of the bio-filter is exceeded.

RAS systems have high investment and maintenance costs (De Ionno et al, 2006). For the industry to be profitable, fish are farmed at high densities, which is synonymous to a high input of nitrogen from the (potentially uneaten) diet and fish metabolism. High nitrogen input can lead to saturation and malfunctioning of the biofilters of the system, then leading to accumulation of nitrogenous waste. Previous toxicological, acute-exposure studies on nitrogenous waste compounds mostly focused on 96 h LC_{50} values for ammonium, nitrite and nitrate. Such data are important, but not very relevant

for professional fish farmers, which are interested in the concentrations at which growth and welfare of the animals become impaired.

We conducted a series of experiments in which African catfish was exposed to different levels of ammonia, nitrite and nitrate and assessed effects on physiology and growth performance. We chose to investigate realistic concentration ranges, as reported to occur in RAS. Our aim was to be able to discriminate subtle changes on parameters important for the fish farmers, without causing mortalities. In addition, we conducted experiments with a recently introduced species in Dutch aquaculture, the pikeperch. This species is more sensitive to ammonia than catfish, but less sensitive to nitrate. We observed not only different responses between the two species, but also between individuals within the same species, depending on the compound tested. The studies substantiate the need to study species independently and hold a warning to be careful with extrapolations from one to another species.

Ammonia was, as predicted from the literature, the most toxic compound for both species; fish growth became impaired at micromolar concentrations. This toxicity was confirmed by the tight plasma regulation of ammonia by both species. African catfish plasma and branchial physiology together with gill morphology were affected at the highest concentration investigated. These effects were not observed in pikeperch, but the highest concentration used for this species was 49 times lower than for the African catfish. Concentration ranges were selected after literature studies on the species ecology and particularities. African catfish showed gradual morphological changes in the gills in response to increasing ambient ammonia concentrations. Gills harbor a multifunctional epithelium, involved in gas and ion exchange, water balance and waste excretion (Goss et al, 1998; Perry and Gilmour, 2002). In stressful situations, the epithelium may show hypertrophy/hyperplasia as protective mechanism (increase in diffusion distance for toxicants); tissue proliferation impedes gas exchange (which benefits from a thin epithelium) but can be considered a temporal escape/compromise to avoid influx of toxic molecules such as ammonia. African catfish is an air-breathing fish, and although thickening of the branchial epithelium will impair gas exchange in the gills, its air-breathing capacity (via the arboreal tissue associated with the gills arches) may offer an escape.

Chronic exposure to nitrite did not induce high levels of methemoglobin in African catfish as is observed in juvenile pikeperch (Wuertz et al, 2013). Growth of African catfish was only affected at 27 mM, the highest concentration studied. Nitrate did not affect physiological parameters in African catfish, nor pikeperch. Both species were exposed to a similar concentration range. This confirmed the relative low-toxicity of nitrate. It is interesting to

mention that growth was affected at 27 mM nitrate in catfish, but not in juvenile pikeperch at 25.6 mM nitrate. The latter was assumed to be a more sensitive species for ammonia and nitrite (Wuertz et al, 2013). This notion again underlines the importance to study the effect of each component for each species (and at different life stages as well).

African catfish is rather tolerant to constant high concentrations of nitrogenous waste compounds. In practices, fish may experience high concentrations of nitrogenous compounds mostly after the feeding sessions (Jobling, 1981). The peaks in waste products seen are normally buffered by the bio-filters within few hours. As ammonia is oxidized to nitrate (via nitrite), peaks of nitrite and nitrate follow the ammonia peak with a delay. We provided fish farmers safe thresholds for the three compounds below which growth is not affected. We studied the effect of those compounds independently. It is important to realize that in RAS system, fish are likely to be exposed to a combination of those 3 compounds simultaneously. Effects of stressors do add up. The results of these experiments should be seen in the concept of allostasis (McEwen and Wingfield, 2003). An accumulation of stressors may overtax the adaptive capacity of an animal and lead to allostatic overload and poor welfare (Korte et al, 2007). Fish can cope with stressors up to a certain point, where the load can become an overload (too strong) and the effect deleterious for the fish. The accumulation of stressors in RAS and other aquaculture systems may comprise high densities, aggression, feed withdrawal, diseases and pathogens, handling, transportation, noise (Ashley, 2007; Barton et al, 1991; Davidson et al, 2009; Mohapatra et al, 2013; van de Nieuwegiessen, 2009). High concentrations nitrogenous waste compounds are not unrealistic in RAS and exposure to an additional stressor could cause an allostatic overload.

4. Concluding remarks and perspectives

Worldwide aquaculture is a fast growing industry (with fisheries being stabilized, FAO, 2012). Due to increasing societal awareness in various countries, attention has been drawn to fish welfare in aquaculture. This situation is recognized more and more; for instance, several running European research projects aim to address welfare issues (COPEWELL, WELLFISH *etc*.). But fundamental research on stress, pain and welfare in fish is still very limited. In this thesis, we investigated acute and chronic discomfort that may occur in aquaculture. Our results were obtained in laboratory settings. There we could reveal subtle responses that suggest that optimization of fish welfare will benefit aquaculture. We show that improved growth performance is directly

linked to physiological and behavioral parameters indicative of good welfare of the fish.

A next step in studies on pain in fish should focus on early-gene expression such as *C-fos* to directly show involvement of higher brain centers in handling of noxious stimuli. It has been shown recently that local electrical stimuli applied at the base of sedated Atlantic cod (*Gadus morhua*) induced somatory evoked potentials (SEPs) in the central nervous system (Ludvigsen et al, 2013). Additionally, with this new minimally-invasive technique, the authors were able to record quantitative responses from multiple brain regions, which correlated with the stimulus intensity. The behavioral data coupled with fine tuned brain analyses, rendered possible with such techniques could help to determine more accurately the part of the brain involved in pain perception and reaction to painful stimuli in fish.

In our studies on chronic discomfort we investigated the effects of single nitrogenous waste products independently introduced in the water. In aquaculture an accumulation of negative stimuli through combinations of negative stimuli is more realistic to cause an allostatic overload (Schreck, 2000) and result in poor welfare. For example, an increase in nitrogenous waste compounds may occur at low oxygen levels, which boils down to a multiple stressor for the fish. The thresholds that we recommend to protect welfare of the studied species in this thesis are for single compounds only; the simultaneous presence of high concentrations of multiple waste compounds can have more drastic effect as shown for turbot exposed to ammonia at low oxygen levels (Foss et al, 2007). Hypoxia is an important stressor in fish (Bernier et al, 2012) and hypoxic conditions occur all too often in nature and aquaculture alike (Foss et al, 2007; Burt et al, 2012). Some fish species evolved powerful strategies to survive hypoxia (Nilsson and Renshaw, 2004) clearly an issue in the physiology of fishes. Fish that are not hypoxia-tolerant may be predicted to be more vulnerable to nitrogenous waste than those that are.

Acknowledgements

I would like to thank prof. dr. Eric Roubos for constructive comments on the general discussion.

Samenvatting in het Nederlands (Summary in Dutch)

Het hoofddoel van dit promotieonderzoek was het vergroten van kennis omtrent het welzijn van gekweekte vis door het bestuderen van de effecten van toediening van relevante en gedefinieerde uitwendige prikkels op fysiologie en gedrag. In de Algemene Inleiding (**hoofdstuk 1**) van dit proefschrift wordt dit in meer detail verklaard.

In de **hoofdstukken 2-4** worden de effecten beschreven van acute en potentieel pijn-veroorzakende prikkels op verscheidene fysiologische parameters en gedragsparameters. In de staartvin van de karper, *Cyprinus carpio*, zijn zenuwvezels geïdentificeerd die betrokken zijn bij de perceptie van pijn (A-δ en C-vezels) (**hoofdstuk 2**). Wanneer bij Nijl tilapia, *Oreochromis niloticus*, in de staartvin een vinknip werd toegediend ('tail fin clip') werd een uur later een sterke slijmafgifte door de kieuwen gemeten, en zes uur na toediening werd een sterke migratie van branchiale chloridecellen van de epitheelcellen naar de kieuwlamellen waargenomen. Daarnaast werd een verhoogde zwemactiviteit gemeten. Zes uur na de vinknip waren de slijmcellen weer gevuld. Plasmawaarden voor cortisol, glucose en lactaat verschilden niet tussen vissen die een pijnprikkel kregen en vissen die alleen gehanteerd waren.

In het **hoofdstuk 3** worden de effecten bestudeerd van een andere acute, potentieel pijn-opwekkende prikkel toegepast op de vissenstaart van de Mozambique tilapia, *Oreochromis mossambicus* in de vorm van een gestandaardiseerde, zwakke, elektrische schok. Deze prikkel leidde niet tot fysieke schade bij de vis. Gedurende vier uur na de schok namen we een verminderde zwemactiviteit waar. Met uitzondering van glucose veranderden de andere gemeten plasmaparameters niet. In tegenstelling tot vinknip had de elektrische prikkel geen invloed op de afgifte van kieuwslijm of de migratie/proliferatie van brachiale chloridecellen. De verschillen in respons tussen de vinknip en de zwakke elektrische schok moeten daarom worden toegeschreven aan een verschil in de aard van de prikkels zelf en/of de onderzochte vissoorten. Beide prikkels hadden echter wel invloed op het zwemgedrag; een hogere en lagere zwemactiviteit na toediening van respectievelijk de vinknip en het elektrische schokje.

Hoofdstuk 4 behandelt 1) het motivatieconflict in de zebravis tussen respons op een verblijf in een nieuwe omgeving, dat angst kan veroorzaken, en de respons op een vinknip, die mogelijk pijnlijk is; en 2) het effect van de vinknip wanneer de zebravis gewend is aan een nieuwe omgeving. Op dag 1 bij de start van het experiment was er nog geen sprake van gewenning. We veronderstelden dat er op dag 5 wel sprake was van gewenning.

Van gewenning aan een nieuwe omgeving is sprake wanneer vissen hun gedrag veranderen; voor de zebravis is dit een verlaging van de duur en de frequentie van de bewegingsloosheid en grillige bewegingen, en een toename van het zoekgedrag. De verandering in gedragsuitingen wijzen op het verdwijnen van angst en daarmee het optreden van gewenning. Wanneer de zebravis vijf dagen iedere dag in een nieuwe omgeving werd geplaatst en tevens een vinknip kreeg op dag 5, nam alleen de totale zwemafstand duidelijk af. Dit laat zien dat er op dag 5 geen competitie meer bestond tussen de respons op een nieuwe omgeving en de respons op de vinknip; het dier was aan de nieuwe omgeving gewend en laat in gedrag een reactie zien op de toegediende vinknip. De volgende dag (dag 6) was er geen sprake meer van een verschil in de totale zwemafstand voor de zebravis met de vinknip.
Wanneer de vinknip op de eerste dag na overplaatsing naar de nieuwe tank werd gegeven namen we de competitie tussen een respons op een nieuwe omgeving en de respons op de vinknip wel waar; het gedrag van de zebravis werd beïnvloed door de nieuwe omgeving en niet de toegediende vinknip.

Op grond de experimenten in hoofdstuk 2, 3 en 4 concluderen we dat zowel vinknip als een (zwakke) elektrische schok weliswaar op de korte termijn ongerief gaf, maar dat de vis goed in staat was om deze prikkel weer te boven te komen.

In de **hoofdstukken 5-8** wordt de invloed beschreven van stikstofhoudende afvalproducten in het leefmilieu op het welzijn van de Afrikaanse meerval, *Clarias gariepinus,* en de snoekbaars, *Sander lucioperca.* **Hoofdstuk 5** demonstreert dat meervallen die worden blootgesteld aan een ammoniaconcentratie van minstens 90 µM NH_3 te kampen hadden met verminderde voedselopname en een lagere groeisnelheid. Ook de kieuwstructuur veranderde in toenemende mate bij een oplopende ammoniaconcentratie, en bij de hoogste testconcentratie (1084 µM NH_3) trad sterke hypertrofie op van het kieuwepitheel en versmelting van kieuwlamellen. Dit verschijnsel duidde op activering van een verdedigingsmechanisme dat de afstand vergrootte tussen het externe milieu (water) en het interne milieu (bloed), waardoor de passieve influx van ammonia-influx werd beperkt. Het is opmerkelijk dat de vis in staat was om zelfs bij de hoogste externe ammoniaconcentratie het plasma ammonia constant te houden. Ook andere gemeten plasmaparameters werden niet beïnvloed door een stijging van extern ammonia, of lieten slechts matige veranderingen zien bij de hoogste concentratie ammonia in het water. Voedselopname en groeisnelheid waren alleen (sterk) gereduceerd vanaf 90 µM ammonia en hoger. Om het risico van

groeireductie tijdens viskweek te vermijden adviseren wij om een concentratie van 24 μM NH_3 in het water niet te overschrijden.

Hoofdstuk 6 laat zien dat blootstelling van de Afrikaanse meerval aan een toenemende concentratie van extern nitraat gepaard ging met een verhoging van plasmanitraat. Voedselopname en lichaamsgroei werden alleen beïnvloed bij de hoogste nitraat concentratie (27 mM). De hematocrietwaarde van het bloed, bloedplasmaparameters en structuur en werking van de kieuwen bleven onveranderd. Om het risico op groeivertraging van de meerval te vermijden raden we aan om een concentratie van 10 mM extern nitraat niet te overschrijden.

In het **hoofdstuk 7** wordt aangetoond dat blootstelling van de Afrikaanse meerval aan een toenemende concentratie extern nitriet een navenante toename van de plasma nitrietconcentratie tot gevolg had. Daarnaast nam ook de nitraatconcentratie in het plasma toe, wat duidde op een beschermingsmechanisme dat gebaseerd is op interne detoxificatie van omzetting van nitriet naar minder giftig nitraat. Bloedparameters (hematocriet, hemoglobine en methemoglobine), de gemeten plasmaparameters en structuur en functioneren van de kieuwen werden niet door nitrietblootstelling beïnvloed. Wij adviseren dat de externe nitrietconcentratie niet boven de 43 μM moet komen.

In het **hoofdstuk 8** wordt beschreven hoe jonge snoekbaarzen werden blootgesteld aan verschillende externe concentraties van ammonia en nitraat in van elkaar gescheiden experimenten. De plasma ammoniaconcentratie bleek binnen nauwe grenzen te worden gereguleerd, en nam alleen toe bij de hoogste externe concentratie. De plasma concentratie van nitraat, daarentegen, nam lineair toe met de stijgende externe nitraatconcentratie. In beide studies bleven de gemeten fysiologische parameters en de kieuwstructuur onveranderd. Groei werd negatief beïnvloed door ammonia maar niet door nitraat. Daarom adviseren we om een water concentratie van 25,6 mM nitraat (de hoogst bestudeerde waarde) en van 3,4 μM NH_3 niet te overschrijden, opdat het risico op verminderde groei van jonge snoekbaars wordt vermeden.

In de Algemene Discussie (**hoofdstuk 9**) worden alle resultaten bediscussieerd en geïntegreerd in het licht van het hoofddoel van dit promotieonderzoek.

Acknowledgements

I would like to thank prof. dr. Eric Roubos and dr. Hans van de Vis for the preparation of this summary in Dutch.

Résumé en français

La demande en poisson ne cesse d'augmenter, non seulement en tant que source protéique, avec la constante expansion de l'industrie aquacole, mais aussi en tant qu'espèces modèles pour la recherche, animaux domestique ou activités récréatives. En conséquence, des millions de poissons sont manipulés chaque jour, mais pas nécessairement de la manière adéquate. Cela peut s'expliquer par le manque de connaissances sur le bien-être de ces animaux, ainsi que du fait qu'ils évoluent dans un environnement totalement différent du notre, et finalement, leur aspect n'attire pas forcement de l'empathie au premier coup d'œil. L'objectif principal de cette thèse est d'accroitre nos connaissances sur le bien-être des poissons d'élevage à travers l'étude des effets de stimuli externes pertinents et ciblés sur la physiologie et le comportement. Les recherches présentées dans cette thèse traitent de deux différents aspects bien être des poissons: inconfort aigu et chronique. Pour étudier l'inconfort aigu, nous avons sélectionné comme stimuli une section partielle de la nageoire caudale ainsi qu'un choc électrique ciblé, applique dans la même région caudale. Pour étudier l'inconfort chronique, nous avons expose les poissons pendant de longues périodes (plusieurs semaines) a différentes concentrations ambiantes en déchets azotés (ammonium, nitrite et nitrate).

Dans les chapitres 2, 3 et 4 de cette thèse, nous décrivons les effets de stimuli nocifs, potentiellement douloureux, qui sont susceptibles de causer un inconfort aigu chez les poissons. Pour cela, nous avons analysé les structures nerveuses de la nageoire caudale (zone d'application des stimuli), ainsi que des paramètres physiologiques et comportementaux.

Nous avons démontré la présence de fibres nerveuses impliquées dans la perception de la douleur (fibres A-δ et C) dans la nageoire caudale de la carpe commune, *Cyprinus carpio* (**chapitre 2**). Des fibres similaires ont été également mises en évidence dans la nageoire caudale du tilapia du Nil, *Oreochromis niloticus* (**chapitre 3**), ainsi que du poisson-zèbre, *Danio rerio* (**chapitre 4**).

Le tilapia du Nil répond à une ablation partielle de la nageoire caudale par une sécrétion active de mucus au niveau branchial 1 h après avoir reçu le stimulus. De plus une migration lamellaire les cellules a chlorure branchiales et une activité natatoire accrue ont été observées. Ces réactions peuvent être attribués a la procédure d'ablation proprement dite, puisque absentes chez les groupes témoins (non-manipulés et manipulés). Les paramètres plasmatiques (niveaux de cortisol et glucose) ne permettent pas de discerner entre la part de réaction imputable a la procédure d'ablation en elle-même et la part de réaction imputable au stress dût à la manipulation ; cela montre les limites de l'utilisation de ces paramètres dans ce domaine de recherche (**chapitre 2**).

Dans le **chapitre 3**, nous avons utilisé un stimulus nocif, potentiellement douloureux différent, ne s'accompagnant pas de dommages tissulaire; un choc électrique standardisé applique dans la région caudale du tilapia du Mozambique, *Oreochromis mossambicus*. Ce stimulus a été choisi car il peut induire de la douleur sans causer de dommage physique inhérents à d'autres stimulus tels que l'ablation de la partielle de la nageoire caudale. Nous avons observé une diminution de l'activité natatoire jusqu'à une période de 4 h après avoir reçu le stimulus, (3 h pour le groupe témoin seulement manipulé) et un délai différentiel de 15 min avant la reprise du comportement stéréotype 'friction' ont été observée. Le taux de glucose plasmatique a augmenté de manière significative 6 h après avoir reçu le choc; indiquant une augmentation de l'activité adrénergique. Contrairement à l'ablation partielle de la nageoire caudale, aucun effet au niveau branchial (sécrétion de mucus ; migration/prolifération des cellules a chlorure) n'a été observé. Les résultats obtenus démontrent que l'exposition du tilapia du Mozambique à un choc électrique, même léger, affecte son bien-être. De notre point de vue, cette étude supporte les recommandations de l'EFSA sur les conditions d'électro-étourdissement pour les poissons d'élevage; la procédure d'électro-étourdissement doit provoquer la perte de conscience et de sensibilité immédiatement après son application. (EFSA, 2009).

Les réponses différentielles aux 2 stimuli étudiés dans les chapitres **2** et **3** indiquent que les poissons différencient ces stimuli nocifs du stimulus de stress seul inhérents aux procédures de manipulations lors de l'application d'un stimulus nocif.

Les paramètres comportementaux comme l'activité natatoire et la préférence pour les zones d'ombres/illuminées ont été affectées à la fois par l'ablation d'une partie de la nageoire caudale et par l'électrochoc. Dans le **chapitre 4**, nous avons étudié l'effet de l'ablation partielle de la nageoire caudale sur l'habituation du poisson zèbre à un nouvel environnement, en utilisant le test du nouvel aquarium. L'habituation à un nouvel environnement peut être quantifiée par la diminution des comportements liés à l'anxiété ('geler', mouvements erratiques) et l'augmentation de l'exploration du nouvel environnement. Dans notre étude, nous n'avons pas observé de différences concernant ces paramètres comportementaux 'classiques' les jours ou les poissons ont reçu le stimulus (jours 1 et 5 de l'expérience). La distance totale parcourue le premier jour n'a pas été affectée par l'ablation partielle de la nageoire caudale, mais chez les poissons recevant le stimulus le cinquième jour, une fois habitué a la procédure, la distance totale parcourue a diminué de façon drastique. De plus, certains individus ayant reçu le stimulus le cinquième jour

continuent de 'geler' ce jour-ci, alors que les poissons des autres groupes n'affichaient plus ce comportement. L'habituation a été affectée par le stimulus douloureux seulement lorsque celui-ci est appliquée le cinquième jour, alors que la procédure de transfert dans le nouvel aquarium en elle-même cause plus d'anxiété que le stimulus douloureux lorsque celui-ci est appliqué le premier jour. Nous concluons donc que les deux stimuli; l'ablation partielle de la nageoire caudale et l'électrochoc de faible intensité sont des stimuli nocifs qui provoquent un inconfort aigu, mais un inconfort que le poisson peut surmonter.

Dans les chapitres 5, 6, 7 et 8 de cette thèse, nous avons étudié les effets d'une exposition chronique à différentes concentrations ambiantes en déchets azotés sur les paramètres du stress et de la croissance de deux espèces durant les phases de grossissement en système de recirculation (RAS) : le poisson-chat Africain (*Clarias gariepinus*) et le sandre doré juvénile (*Sander lucioperca*).

Dans le **chapitre 5**, les poissons-chats exposés à de fortes concentrations d'ammonium ont montré une sévère diminution de leur apport alimentaire et de leur taux de croissance lorsqu'ils sont exposés à des concentrations supérieures à 90 µM. La morphologie branchiale a été affectée graduellement avec les concertations croissantes ambiantes en ammonium. Une sévère hypertrophie de l'épithélium accompagnée de fusions lamellaires a été observée à la plus forte concentration ambiante testée. Ces modifications ont été interprétées comme mécanismes de défense pour augmenter la distance entre le milieu extérieur riche en composés toxiques et le milieu intérieur du poisson, et ainsi limiter l'afflux passif d'ammonium. Les poissons-chats ont réussi de manière remarquable à maintenir leur taux d'ammonium plasmatique relativement constant et faibles, même à des niveaux ambiant d'ammonium élevés. Les autres paramètres plasmatiques n'ont pas ou peu été affectes; seulement pour la plus forte dose testée. Nous conseillons les professionnels de l'aquaculture de ne pas dépasser une concentration ambiante en ammonium de 24 µM afin de prévenir les risques de prise alimentaire réduite, de croissance détériorée et de détérioration de la morphologie branchiale chez le poisson-chat Africain.

Le **chapitre 6** décrit l'exposition de poisson-chat à différentes concentrations en nitrate ambiant. Nous démontrons que les concentrations en nitrate plasmatique augmentent progressivement, en parallèle avec les concentrations en nitrate ambiantes, pour atteindre le niveau millimolaire (6.6 mM). La prise alimentaire ainsi que le taux de croissance spécifique n'ont été affectés qu'à la concentration ambiante la plus élevée testée (27.0 mM). Les niveaux d'hématocrite sanguin, la physiologie plasmatique ainsi que la

morphologie et physiologie branchiale sont restées inchangées. Nous recommandons de ne pas dépasser une concentration ambiante de 10 mM en nitrate afin de prévenir les risques de prise alimentaire réduite, de croissance détériorée et de détérioration de la morphologie branchiale chez le poisson-chat Africain.

Dans le **chapitre 7**, nous démontrons que les concentrations plasmatique de nitrite augmentent progressivement, parallèlement avec l'augmentation ambiante en nitrite, pour atteindre 32.5 µM à une concentration ambiante en nitrite de 928 µM. De plus, cette hausse est accompagnée d'une augmentation progressive du nitrate plasmatique avec l'augmentation des concentrations de nitrite ambiante, révélant un mécanisme de défense sans-précédant de détoxification interne (conversion du nitrite plasmatique en moins toxique nitrate). Des expériences plus approfondies de ces mécanismes chez les poissons téléostéens semble justifiée. Les niveaux d'hématocrite, d'hémoglobine et méthémoglobine sanguine, la physiologie plasmatique ainsi que la physiologie et morphologie branchiale restent inaffectés, indépendamment des concentrations ambiantes en nitrite étudiées. Nous conseillons de ne pas dépasser une concentration ambiante en nitrite de 43 µM pour prévenir les risques de prise alimentaire réduite, de croissance détériorée et de détérioration de la morphologie branchiale chez le poisson-chat Africain.

Dans le **chapitre 8**, nous avons exposé des sandres dorés juvéniles à différentes concentrations ambiantes en ammonium et nitrate. Les concentrations plasmatiques en ammonium ont été strictement régulées jusqu'à une concentration ambiante de 11.2 µM, mais à la concentration maximale étudiée, les concentrations plasmatiques ont atteint le niveau millimolaire (1.4 µM). Les concentrations plasmatiques en nitrate ont augmenté de manière linéaire jusqu'à 6 mM, atteints à la plus forte concentration en nitrate étudiée. Pour les deux études, les taux d'hématocrite sanguin, la physiologie plasmatique ainsi que la physiologie et morphologie branchiale n'ont pas été affectés. La croissance a été affectée par une concentration en ammonium de 11.2 µM, mais n'a pas été affectée pour aucune concentration en nitrate de la gamme étudiée. Nous recommandons de ne pas excéder une concentration ambiante de 25.6 mM NO_3^- (la concentration maximale étudiée) et 3.4 µM NH_4^+ pour prévenir les risques de croissance détériorée chez sandre doré juvénile.

Compte tenu des différentes tolérances des deux espèces aux différents déchets azotés étudiés, nous préconisons que la sensibilité/tolérance envers ces composants déchets azotés doit être étudiée pour toutes les espèces d'intérêt aquacole, en tenant compte de leur stade de développement.

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Acknowledgements

The research described in this thesis was carried out in the frame of two projects, both supported by the Dutch Ministry of Economic Affairs: Project KB08 009001 ASG, and Project BO-07-011-028.

This PhD journey started long before the official contract was signed, in 2009. While I was trekking with my dear friends from Mayotte, I received a phone call from Mireille Charmantier that announced me that I was accepted in the last year of her bachelor program. She renewed her trust in me by accepting me the following year in the her master program. When I told her my wish to go abroad for my first internship, she sent me to Nijmegen, where they had together with her husband, Guy Charmantier, a long-lasting fructuous collaboration. I was glad to be part of it myself with my yearly lectures in the 'Ecology' course. I would like to thank Mr. and Mrs. Charmantier for their trust and kindness that was at the origin of this journey.

I met Gert during his lecture in Montpellier, at the end of it, I shyly approached him and introduced myself; 'I am supposed to be your student'. What was supposed to be a 3 month stay in the Netherlands lasted more than 6 years. After completing the internship, Gert agreed that I perform the second year of Master. Meanwhile, he was writing together with Hans van de Vis the current PhD project that they trusted me to work on. Wout, freshly appointed junior researcher at IMARES was my daily supervisor.

I am very grateful to Gert for the trust you had in me from the beginning. You gave me a lot of freedom with designing and arranging the experiments, my working schedule and my holidays. I could always count on your support, kindness and generosity. You were a very good supervisor and head of department as you are hard working, but also understands that there are other things in life and allowed us to do all the activities with the departments. Most of all, I enjoyed our yearly teaching trips to Montpellier.

Wout, thank you for my daily-weekly supervision. It was a pleasure to be your first-supervised PhD. I enjoyed the work we did together. Thank you for your trust and the freedom you gave me with organizing my schedule and my experiments. I always liked the side-experiments you got me involved in, it was nice to travel around and try different things.

Hans, we first met when we were trying to set-up an experiment with eels in Madagascar. The project aborted quickly, independently of our will, but I was the start of our collaboration. I am grateful for your advices and suggestions, as well as for your material and human support from IMARES.

Daisy, you helped me in so many occasions, thank you very much for your help and your kindness. It was always a pleasure to drop by in your office for some chatting.

Tom, I am sorry for having brought back eels to our department, I learnt my lesson and latter on focused on easier species! Thank you for your help with the fish as well as for your precious advices.

I am grateful for the students that helped me with the different projects: Femke, you were my first official, good job with the mucus cell counting! Aurélie, merci aussi pour le comptage des cellules a mucus aussi ainsi que du visionnage des heures de vidéos. Margot was a bit luckier because she could use Ethovision, but first she had to master the program, which she did much faster (and better) than me. Gaétan, merci pour les assays et le Saint-nectaire ! Yanick, bon travail avec les poissons-chats et les dimanches pour les séances de volley-basket. Bonne adaptation au climat hollandais depuis La Nouvelle-Calédonie via Montpellier. Although not in this thesis, Erik Ducker, my first non-official student that I was 'supervising' during my first internship, we discovered the work in the lab together. And finally Tirsa that helped me during my last experiment with the 'stinkende meerval'.

I would like to thank my PhD colleagues and students from the department of Organismal Animal Physiology, also known as 'Beer Physiology'. I spent a nice time with you guys, thank you for the help in the lab as well as the outside the lab activities.

Jeroen 'Postbode', it was fun to be cubical-mates, discussing about serious thing and also showing each-others some silly YouTube videos. The Ing. Remy Manuel, specialized in cortisol assays, catfish with option on facebook and foosball. Nacho, my Movember and Zombies! mate I liked our collaboration with the seriola project, I hope I can visit Cadiz in the future, I assume you will be employed there forever, or that you will come back here one more last time for the last time Paco de Cadiz, the other foreigner of the department, even when you got the position at the NCMLS you couldn't abandon our department. Maartje and Erik we started (almost) together, and finished (almost) together as well, it was nice to growth up together during the PhD, thank you for your help, advices and knowledge with the zebrafish work and the general introduction. Marnix, thank you for organizing activities as well as for your help in the laboratory, especially with the tiny zebrafish, and your help with finalizing this thesis. Yvette, the other (part-time) cubical mate and coin collector (only for cool-people). Stefan 'Bokito', one day neither Jeroen nor me were there and we found latter our cubical totally changed, with you in it. Next day we rearranged our desks the same way, Jeroen K, the unofficial member of the department, Juriaan which family kept on growing during my stay in The Netherland, as well as, Kasper, Clara, Martha, Edwin and the other persons that were once in this department.

I am grateful to the staff of the department and the other collaborating cells for their held and advices: Peter K., I admire your scientific spirit and dedication, Ron, thank you for your kindness and your help with the fish during

week-end shifts. Jan, Tony, Liesebeth, Walter (R.I.P), Jelle, Geert-Jan and Wim A. thank you for your assistance.

Wout and Hans got me involved into different interesting projects, thanks again for that, and thank you the collaborators and co-authors that were involved in them. First of all, Edward, the two first catfish projects that are in this thesis are your ideas. Thank you for involving me in them and your trust. Ainhoa, it was always very nice to go to Yerseke once in a while to sample the seriola (they also taste good!). I guess we tested everything parameters in the end so they can be cultured in Europe! Thank you as well to the other co-authors and collaborators that I had the occasion to work with; Ruud, thank you for the nice discussions on behavior and Paris, thanks for being part of the committee and I am sorry I misspelled your name in one our papers… Dirk, Pepijn, Henk, Angelo, Femke, Tiedo and Gavin, and to the people that helped me with their advices and articles: Lynne Sneddon, Bruce Lynn, Lauriane Michel, Mark Westerink, Allan Kalueff and Jonathan Cachat. Divna and Liliane, Хвала вам на љубазности.

Thank you to our (ex) joint-lab of cellular physiology for the nice event spent together; Tom R., it is like you were part of the Beer Physiology department, Rick, Linda, William, Claudia, Flore, Debbie, Frouwke, Bruce, Tamas, Gea, Feico. Peter C., thank you for your help and your good mood, your enthusiasm and your singing in the lab. People downstairs in the ecology department are lucky to have you now. Eric, thank you very much for your help with finalizing this manuscript.

Lu we had nice time at the lab but also outside with the wonderful diners, bowling games, Xbox-kinect performances with Leo, sharing experiences about travelling, food and life. I was happy to be at your side during your defense. Welcome to Summer!

Maria and Flore, the Erasmus time is long ago now, but it was nice and still seems like yesterday. Saro, my big brother, it was always nice to see you in those (too little) occasions, for some crazy dancing and other fun activities. ‘Fais-chier la vache, biloute!’

Anny and Johannes, it was worth it to leave in Den Bosch to be neighbors for almost 2 years, so easy to jump on the floor/knock on the ceiling to announce that the diner was ready or that it was time to play games. Nice trips to Terschelling and France.

Steve and Chin Chin, it was always nice to come to Amsterdam/Amstelveen or to have you in Nijmegen for a cozy week-end with nice food, chats and board games.

Vitoria than you for starting the Friday’s (latter became Sunday’s) basketball and introducing me to the CNS people (and Co.). We had a lot of fun together with the basketball, the impro-club Ya-γ-moto, board games,

barbecues, parties ect. Kriss, Ioannis and Maria, Declan ('everybody knows Declan!'), Marius, thank you for the wonderful trips to Austria, although I lost y knee in the second), Matthias (where are you now !?!), Daphne, Giovanni, Tom, Flora, Paul, Steve and Vicky, Rocio and Freek, Daniel, Santosh, Ming, Suzanne, Lauriane, Lie, Tang, Jiankang,… Ashley, the volleyball, tennis and pubquiz master.

Michalis and Svetlana, thank you for the lovely evenings, your sweetness and generosity (and Michalis sweatiness on the basketball court), all the laughs we had together and the trips we made together (RHCP, Austria, Fantasialand, the CNS sailing week-end, numerous games nights and civilization V week-ends, and of course Greece in 2012 with Tix and Catalina, and Skiathos in 2013; those holidays with the 6 of us were simply fantastic).

Christian and Carmen, we had lovely time being neighbors, thank you for the parties, the diners and games and the sports. Constantin-Codrut (You can claim I don't know how to pronounce it, but at least I got the spelling right!) my dear room-mate we had fun with a lot of things, too bad that Skyrim was only playable by one player, but we had borderlands 1 with co-op mode!

Cheng Huaaaaaaaaaa, thank you for your enthusiasm and your energy on the stage. It was always nice to hear the door bell ringing and do improvised activities. Thank you for introducing me to Carcassonne, and the games we still played online.

Malte, the king of impro, on the stage and outside, it was always to do unplanned activities, do and talk about crazy things, watch western movies and play some music together. What nice surprises that you still ring the bell and join our rehearsals when you surprise-visit Nijmegen. What's wrong, this picture?

Bronagh, Jeff, Toru and Nietz, my band-mates, thank you for the time we rocked together with instruments and elsewhere, for making me pick the bass guitar again. And don't forget; it's a small world after all! We had fun! Jeff, thank you for your patience while (trying to) teach me music and making me discover good artists, Nietz my rhythmic-section, gym (until I dropped out…), basketball partner and NBA-fan mate. Just too bad that you chose the Spurs over the Mavericks…

Aux amis de LBO et BGAE, revus depuis lors de leurs traditionnelles montées aux Pays Bas ou de mes descentes lors des non-moins traditionnelles lectures d'Ecology. Merci de vos visites, et merci pour mes provisions de produits gastronomiques et culturels (BD's): David et Olivier, Jeremy, Lola et Olivier (merci pour l'aide apportée pour mon résumé en français), Melanie, Anne-Claire, Romain, Lucille et Manu, l'autre expat' de doctorat. Aurore R, le temps de la prepa est loin et révolu, merci de ton soutient durant cette période et de ton amitié depuis.

Aux amis de Mayotte, éparpillés ici et là mais qui sont venu nous rendre visite ou que nous avons visités. Loin des yeux mais pas loin du cœur. Je suis désolé pour les traditionnelles randos que je n'ai pas pu joindre. Etienne, mon pote aventurier, quand j'y repense on aurait du se voir plus souvent lors de ton sejour sur Amsterdam, Anne, Flo et Flo, Daye, Cécile, Marine, Doudou et Juliette, Clément et Julie, Yatrika et François-Nicolas, Malik.

The Rabdal team, thank you for welcoming me in. Tuesdays were holy-days and tournaments here and there were fun: Lucky Skywalker that quickly nicknamed me 'Chocka', Eric and Erik, Sip, Johnny, Naima, Roberto, Martini, Bart, Joris, Floor, Anneke, Elles, Bianca, Joke, Philip, Sietze, Anchel, Antoine, Jochem, and Pim. It was fun to be a trainer: Alright, 2 balls, 2 lines, Spelen!!!

Thank you to the people that were around and helped me during the surgery and rehabilitation period, some already mentioned, Leo and Michalis for the rides to the clinic, Dr Donk and his crew at ViaSana, Patricia, Anne and Jos my physiotherapists that followed me and helped me for months, and my dear friend, ex-neighbor and unofficial chiropractor Jacob with his previous advices and program.

Thanks to the people that helped me out and made me work in very different areas than science while finishing this thesis; Lu and Leo, Andrew, Henry, Adil, Radbal and the Donder's friends.

Thank you to Emil and his team that always helped me to fix my bike!

Many thanks to Kiyoko for the cool design of the cover of this thesis; when I opened the picture, I was really like 'Wahoo, so cool!!!'.

Merci a ma famille qui certainement ne pourra pas lire tout le contenu de cette thèse ☺, mais elle a malgré tout été rendue possible grâce a eux. Maman et papa pour soutient, leur support moral et financier qui m'ont permis de poursuivre mes études aux Pays Bas, et pendant ma convalescence. A ma petite sœur Rebecca, notamment la semaine passée ensemble ici a été super, puis les 3 semaines de 'stage' et bougnagnas durant ma convalescence, avec le concerts des Kaisers Orchestra. Papy qui lui aussi est passionné par les poissons, mamy Vivianne, tonton Gérard, tatie Claudia, Christel et Quentin, Justine, et a la mémoire de papy Charlie et mamy Jeanne. Papé et mamé, Maribel, Manu et les enfants, Françoise, Patrick, Melo, merci pour Châtaigne!

I am also grateful to all the fish that were sacrificed for the completion of this thesis. I hope that at least with it I could bring some more scientific evidences that these animals deserve consideration.

Last but not least, Mimi, thank you for everything, for loving me for what I am and being at my side from the beginning of this thesis and for long after, now along with T-2. Gaou.

Publication list

Schram E#, Roques JAC#, Abbink W, Spanings T, De Vries P, Bierman S, Van de Vis H, Flik G (2010) The impact of elevated water ammonia concentration on physiology, growth and feed intake of African catfish (*Clarias gariepinus*). Aquaculture 306:108-115.

Roques JAC#, Abbink W#, Geurds F, van de Vis H, Flik G (2010) Tailfin clipping, a painful procedure: Studies on Nile tilapia and common carp. Physiol Behav 101:533-540.

Roques JAC, Abbink W, Chereau G, Fourneyron A, Spanings T, Burggraaf D, van den Bos R, van de Vis H, Flik G (2012) Physiological and behavioral responses to an electrical stimulus in Mozambique tilapia (*Oreochromis mossambicus*). Fish Physiol Biochem 38:1019-1028.

Schram E#, Roques JAC#, Abbink W, Yokohama Y, Spanings T, De Vries P, Bierman S, Van de Vis H, Flik G (2012) The impact of elevated water nitrate concentration on physiology, growth and feed intake of African catfish *Clarias gariepinus* (Burchell 1822). Aquac Res, *in press*.

Roques JAC#, Schram E#, Spanings T, van Schaik T, Abbink W, Boerrigter J, de Vries P, Van de Vis H, Flik G. The impact of elevated water nitrite concentration on physiology, growth and feed intake of African catfish (*Clarias gariepinus*). Aquac Res, *in press*.

Schram E#, Roques JAC#, van Kuijk T, Abbink W, van de Heul J, de Vries P, Bierman S, van de Vis H, Flik G. The impact of elevated water ammonia and nitrate concentrations on physiology, growth and feed intake of pikeperch (*Sander Lucioperca*). Aquaculture, *in press*.

Roques JAC, Bekhuis M, Abbink W, van de Vis H, Flik G. Effects of a tailfin clip on habituation to novelty in zebrafish (*Danio rerio*): a pilot. Anim Welfare*, submitted.*

Abbink W, Blanco Garcia A, Roques JAC, Partridge GJ, Kloet K, Schneider O (2012) The effect of temperature and pH on the growth and physiological response of juvenile yellowtail kingfish *Seriola lalandi* in recirculating aquaculture systems. Aquaculture 330-333:130-135.

Manuel R, Boerrigter J, Roques J, van der Heul J, van den Bos R, Flik G, van de Vis H. Stress in African catfish (*Clarias gariepinus*) following overland transportation. Fish Physiol Biochem, *in press*.

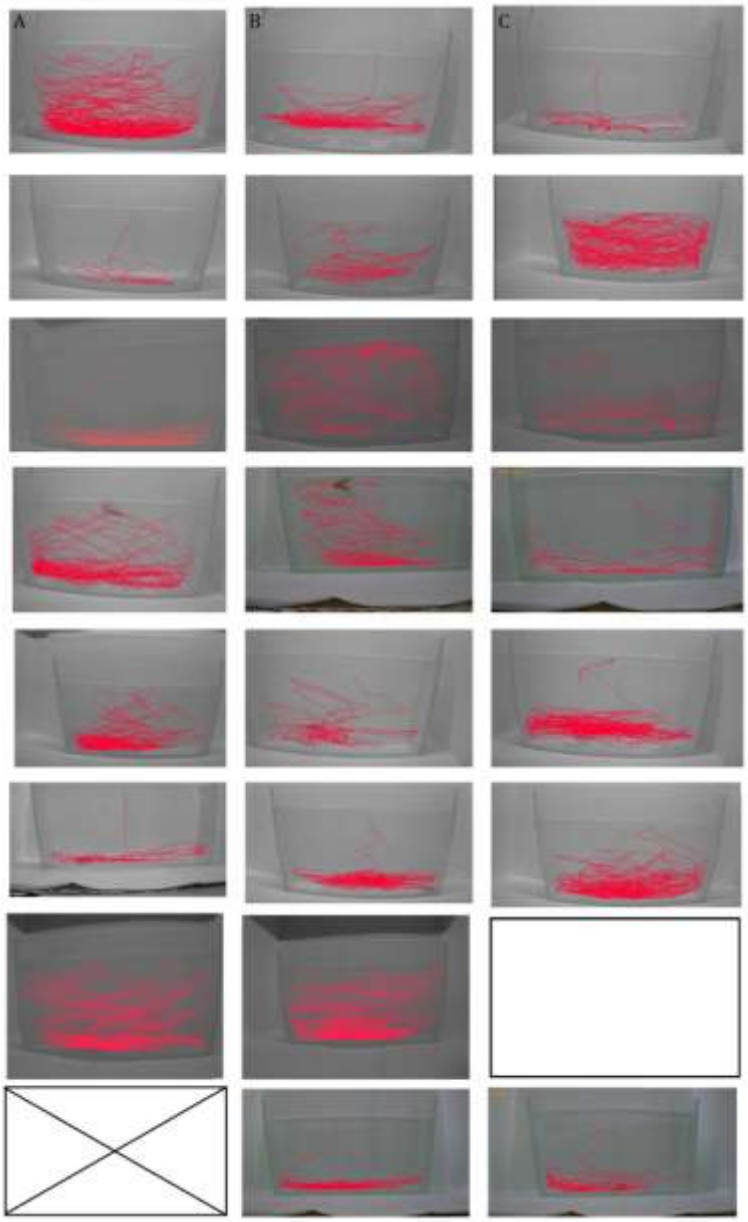
Boerrigter J, Manuel R, van den Bos R, Roques JAC, Spanings T, Flik G, van de Vis H. Recovery from transportation by road of farmed European eel (*Anguilla Anguilla*). Aquac Res, *in press.*

Blanco Garcia A, Abbink W, Roques JAC, Partridge JG, Kloet K. The effect of salinity on the growth and physiological response of juvenile yellowtail kingfish (*Seriola lalandi*) in recirculating aquaculture systems. Aquac Res, s*ubmitted*.

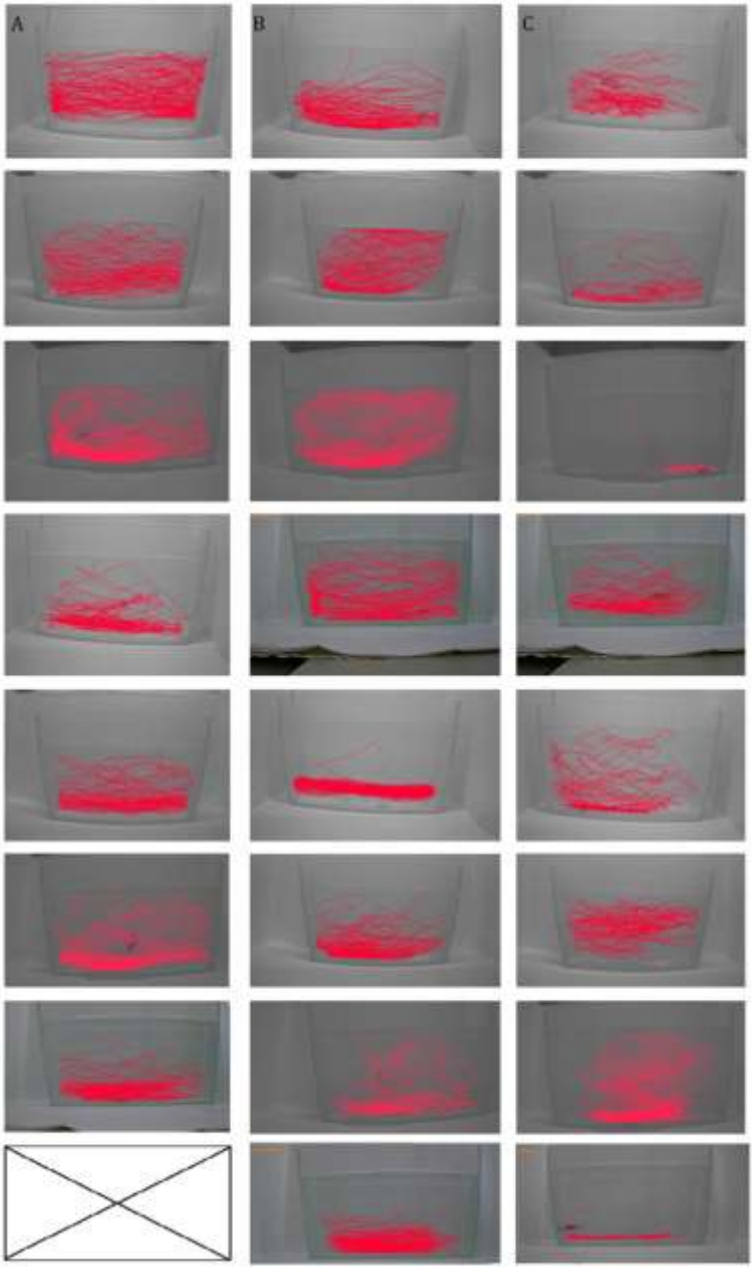
Curriculum vitae

Jonathan Armand Charles Roques was born on August 1, 1985 in Montpellier, France. In 2003 he finished his secondary education at the Lycée of Mamoudzou (Mayotte) and proceeded to study biology at the Preparatory classes of Lycée Joffre (Montpellier). He completed a bachelor degree at the University of Montpellier II in 2006 where he began a master program latter on the same year. In March 2007 he moved to The Netherland for what was supposed to be a three months internship, at the Department of Animal Physiology of the Radboud University Nijmegen under the supervision of professor Gert Flik. The following year he completed is master education in the same department. After obtaining the MSc degree, he started his PhD research leading to this thesis, in February 2009, under the supervision of Gert Flik, Hans van de Vis and Wout Abbink (both from IMARES Wageningen UR).

Appendices



Appendix 1. Swimming profile of the experimental fish on day 1. 1A. control (N=7), 1B. FC D1 (receiving the fin clip) (N=8), 1C. FC D5 (N=7. Only 7 profiles are displayed because of a technical camera problem we were not able to analyze the profile of one fish on that day).



Appendix 2. Swimming profile of the experimental fish on day 5. 2A. control (N=7), 2B. FC D1 (N=8), 2C. FC D5 (receiving the fin clip) (N=8).