Differential responses of Nile tilapia (*Oreochromis niloticus*) to fin clip wounding and related stress: perspectives

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Summary

The debate around fish welfare is intensifying in The Netherlands. As a result, more research is carried out to enhance knowledge on fish welfare in aquaculture. Detailed information is lacking on how production procedures causing discomfort to the fish may affect welfare. That fish must perceive adversive stimuli follows from the fact that nociceptive mechanisms similar to those in mammals are present in fish. However, whether and how nociceptive stimuli are perceived or interpreted by a fish is a far more difficult question that requires significantly more effort from fundamental research, both neurophysiological and behavioural studies, than now available. The study presented in this report aimed to define selected readout for the acute response to a supposedly painful stimulus: a standardised tailfin clip.

In a pilot study, common carp was clipped, and mixed nerves were identified in the clip. Ultra structural analysis (TEM) indicates the presence of Aα-, Aβ-, Aδ- and C-type axons typical for nerves transmitting nociceptive signals in higher vertebrates. An experimental design was chosen to discriminate in Nile tilapia (*Oreochromis niloticus*) responses to the painful stimulus and the acute stress that is unavoidably associated with the handling required to make a clip. A series of key parameters for further studies could be defined. The responses seen in 'classical' stress parameters such as plasma cortisol, glucose and lactate levels did not allow discrimination between the clip and the handling stress. However, several other readouts indicated a differential, stronger response to the painful stimulus compared to the handling stress alone: I) swimming activity was more increased and clipped fish spent more time in the light (in a tank where half the volume is dark and the other half illuminated; II) in the gills a fast (observed 1 h after the clip), transient (response no longer observed 6 h after the clip) increase in Na^+/K^+-ATPase activity assayed *in vitro*; III) a kinetically similar response was observed in the mucus cell population in the gills: a decreased number of stainable mucus cells 1 h after the clip, indicative of a temporary increased mucus secretion as response to the clip.

In conclusion, we succeeded to demonstrate differential, stronger responses to a presumed painful stimulus than to the handling stress *per se* associated with the administration of the pain stimulus. These parameters will be the focus of future research within this welfare project.

1. Introduction

Dit onderzoek heeft plaatsgevonden in het kader van het Kennisbasisonderzoeksprogramma Diergezond en Dierenwelzijn (KB8).

In humans, awareness of pain, fear and stress depends on functions controlled and executed by the highly developed hippocampus, amygdale, frontal lobe and neocortex of the cerebrum. In fish, the telencephalon, that 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 and Rose, 2002). One of the endeavours of research on welfare in fish is to identify whether these early vertebrates have some form of consciousness and thus may experience pain and fear. There is ample evidence to conclude that fish experience stress and successfully mount behavioural and neuroendocrinological responses to cope with stress (Wendelaar Bonga, 1997).

Reviews by Braithwaite and Huntingford (2004) and Chandroo and coworkers (2004) present convincing evidence that fish, despite their less developed telencephalon, have significant intelligence (= learning ability = cognitive ability). For some species (trout, cod, salmon) researchers have recently advanced the first evidence that fish have the capacity to perceive painful stimuli and should have nervous substrate to experience fear and to suffer (Sneddon, 2002; Nilsson et al., 2008). However, it has to be emphasised that it is unlikely that fish, as well as other animals, except maybe higher primates, have the capacity to suffer as humans do (Braithwaite and Huntingford, 2004). As far is known to date, the cognitive abilities of fish are limited to (simple) concurrent discrimination learning (perch can discriminate four, trout up to six independent objects; the ability is directly linked to brain size; Rensch, 1967); the ability to learn class concepts (*e.g.* categorise objects as chairs or birds) is one step up in the hierarchy of learning abilities, restricted to the best of our knowledge to some birds and mammals (Thomas, 1996). Although the limited concurrent discrimination learning mentioned would suggest that fish are not very likely to show concept learning, it is worthwhile to stimulate research in this direction as the strict experimental criteria to establish such learning has rarely been met (even in studies on mammals). Moreover, the number of species of fish is overwhelming (60% of vertebrate species are fish), increasing the possibility to find a species which should not be excluded in advance. One could wonder for instance, how African catfish manage their pack-hunting of tilapia (Merron, 1993), a highly complex social behaviour better known from large predators such as wolves, hyena dogs and lions. In the latter, such activity may be assumed to require concept learning.

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 the translation harmfulness to feeling of pain. Therefore, fish should possess both a nociceptive system and some cognitive capacities to experience pain in the human sense. Indeed, a very limited literature supports that fish detect harmful stimuli, respond to nociceptive stimuli and may conceptualise pain (Sneddon 2002, Braithwaite and Huntingford 2004; Chandroo et al. 2004).

Next to the feeling of pain, fear and stress are motivational affective states relevant to fish welfare. In their seminal reviews Braithwaite and Huntingford (2004), and Chandroo and colleagues (2004) conclude that these affective states may well be attributed to fish. Recently, Nilsson and coworkers (2008) have demonstrated explicit memory in Atlantic cod and salmon and therefore it seems more than reasonable to adhere that fish indeed have capacities to be aware of pain.

Studies that deal with the welfare of fish are limited to only a few species, while fishes may come in up to 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. The current literature suggests that fish deserve a better moral consideration than they have received so far.

In studies on mammals, various methods have been developed to assess and attempt to quantify or scale pain and the awareness of pain: physiological parameters, facial expressions, substance-P levels, behavioural activity and EEG measurements. However, next to none of such methods have been applied to demonstrate or quantify painful stimuli in fishes. A complicating factor in pain research on fish 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 (additive or synergistically) with the response to the pain stimulus. It is difficult to distinguish between stress responses and pain responses as these responses share a larger part of the stress physiology.

Goal

In this study, behavioural and an array of stress endocrinological responses of the Nile tilapia (*Oreochromis niloticus)* to a pain stimulus were investigated. In a pilot with common carp, the neuro-anatomy of fins was investigated at the ultra structural levels to determine whether and which nerve fibres obeying classification as mammalian pain fibres are present. Swimming behaviour was monitored and the fish were observed for their preference to reside in the light or the dark section of the aquarium thus designed. The stress parameters plasma cortisol, glucose and lactate, and skin colour were measured. Parameters for osmoregulatory performance including Na^+/K^+-ATPase enzymic activity and chloride cell abundance in gills and plasma activities 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 response inherent to the application of a fin clip as 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. This study is the first within the KB8 project that aims to develop (non-invasive) methods to measure pain and chronic discomfort in farmed animals including fish.

Nerve fibres are categorized as Aα, Aβ, Aδ and C fibres. The A-fibres are myelinated for fast conduction of action potentials and these fibres account for the majority in nerves conducting pain stimuli (Sneddon, 2002).

C-fibres lack a myelin sheet (are simply isolated by glia) and therefore slowly conduct action potentials and account for up to 4% of the axons in nerves conducting pain stimuli.

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

2. Material & Methods

Experimental fish

Female Nile tilapia (*Oreochromis niloticus*), weighing around 200g, were obtained from a fish farm, and after transport to the laboratory acclimatised for two weeks in the aquarium facilities of the Radboud University Nijmegen. The fish were kept in 140 l flow-through tanks with nine 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=0-0,5 mg/l; NO_3=12.5mg/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 monitored continuously, the light regime was 12h light :12h dark. The study was approved by the Animal Experimental Committee of Lelystad (Protocol: 2008139).

Experimental set-up

Fishes were caught with a net and restrained manually by one experimentor, while another clipped the caudoventral corner of the tailfin (Fig. 1) with a sharp, sterile pair of dissection scissors; next, the fish were returned to their tank. In the control stress-treatment, fishes were handled the same way but not given the clip.

Figure 1. *The fin clip as given to Nile tilapia. The fish on the left is a control. The red spot resulted from puncture of the caudal vessels for blood sampling.*

Seven groups of nine fish each were used (Table 1). The blank group was sampled one day prior the treatments of the other, eight experimental groups. Clipped and control groups were sacrificed at 1, 6 and 24 hrs after the clip procedure. Fish were not fed 24h before sampling. To investigate whether an extra stressor on top of the treatment stress resulted in a stronger response to the pain or stress, two groups were exposed to sub-optimal water quality by closing the water-flow and refreshment to the tanks after the treatment. As a result, the water quality deteriorated significantly (NO_2=1.0 mg/l; NO_3=25-50 mg/l; NH_4=2.5 mg/l; O_2=6.0 mg/l) and became turbid. However, this treatment did not result in discernable (by the parameters analysed in this study) extra stress nor did it affect the response to the pain stimulus. Therefore, these results are not included in this report. Data are available on request.

Table 1. *A total of seven groups of fish were evaluated: a blank, untreated group, three pain groups and three stress groups. The groups that received the fin-clip were designated to have received a combined pain and stress stimulus, and referred to as 'pain groups'.*

Group	Treatment	Time (h)
control	control	0
1h pain	pain	1
1h stress	stress	1
6h pain	pain	6
6h stress	stress	6
24h pain	pain	24
24h stress	stress	24

Sampling

The fish were rapidly caught using a net and deeply anaesthetized with 1:1000 2-phenoxyethanol (Sigma-Aldrich) within 2 minutes. Blood was sampled by puncture of the caudal vessels with a heparinised needle and syringe and immediately centrifuged at 4°C and 13000rpm for 10min 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:7.3) for later determination of Na^+/K^+-ATPase enzymatic activity or fixated in Bouin's fixative (15 volumes saturated picric acid: 5 volumes formaldehyde: 1 volume glacial acetic acid) for histological analyses of the mucus cells and chloride cells.

Nerve bundles

Tail fin clips of common carp (*Cyprinus carpio*) were fixated with 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 of 70 – 90 nm were cut with an ultratome and mounted on square mesh nickel grids. On-grid sections were post-

stained for two minutes with uranylacetate and with lead citrate for two minutes and rinsed thrice with double distilled water (routine procedure for electron microscopy at the Department of Animal Physiology, Radboud University Nijmegen). Nerve fibre types in cross sections were categorised based on diameter and the presence of myelin to distinguish Aα-, Aβ-, Aδ- and C-fibres (Sneddon, 2002).

Parameters measured

Skin colour

Skin colour was assessed proceeding from the L*a*b-colour model procedure, where L (Luminance) is the light intensity component from 0 (black) to 100 (white) and a and b are two colour components. The a-component ranges from -127 (green) to +127 (red) and the b-component ranges from -127 (blue) to +127 (yellow). The skin colour was measured with a Konica Minolta chromameter, type CR-400/410.

The L*a*b-values were measured at three standardised positions on the right side of the fish (Fig. 2). The choice of the three points as most suitable was based on a pilot experiment (W Abbink, unpublished results).

- Position 1: below the first spine of the dorsal fin.
- Position 2: halfway the lateral line.
- Position 3: at the base of the most distal ray of the anal fin.

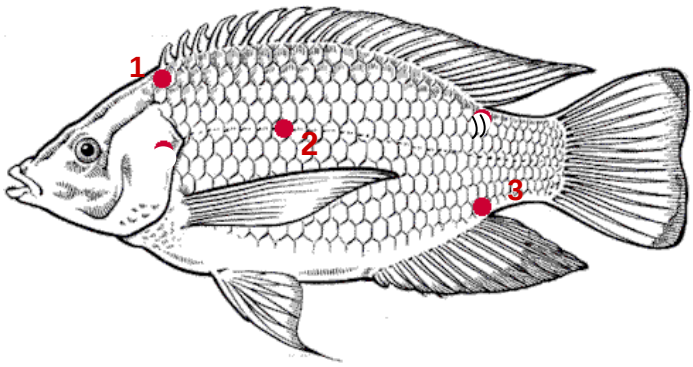

***Figure 2**. The three selected points were the skin colour was measured.*

Dark-light preference and swimming activity

Tanks were covered with black plastic to make 50% of the volume dark and 50% illuminated. The preference for residence 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.

Blood plasma

Plasma was analysed for cortisol according to a protocol obtained from the Department of Animal Physiology of the Radboud University Nijmegen (Metz et al., 2005). Concentrations of Na^+, K^+, Ca^{2+}, pH, glucose and lactate in plasma were measured using Stat Profile pHOx plus analyser (Nova Biomedical, Waltham, MA, USA).

Gills

Gill samples were fixated in Bouin's fixative for 24h, dehydrated with a series of alcohols and embedded in paraffin. The samples were cut at 7µm using a microtome and sections stained for the presence of mucus cells and chloride cells as follows. Mucus in mucus cells was stained with alcian blue according to a protocol used at the Department of Animal Physiology from the Radboud University Nijmegen. The mucus cells density was estimated by counting the alcian blue positive cells in a designated representative cross-section stretching along 400 lamellae of the sampled gill arch. Following stress stimuli, mucus cells may expel their mucus content and thus is mucus cell frequency scored in test animals relative to controls a measure for mucus secretion. Mucus cell frequency was assessed for each fish twice by two independent persons. Mucus cells were scored on both the leading and trailing edge of the gill filament to avoid potential topological bias. Statistical analysis provided evidence 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 report.

The chloride cells in the gills were stained through Na^+/K^+-ATPase immunohistochemistry. Chloride cells (mitochondria rich cells) are central in fish osmoregulation, and through a link between bicarbonate and sodium exchange, also in aspects of gas exchange in fish (Perry, 1997). Chloride cells normally predominate on the trailing edge of the filament (where the water flow exits the gill) and adjacent interlamellar space of the gill filamental epithelium (Van der Heijden et al., 1997). 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 of the enzyme in a gill homogenate according to the protocol described in 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 capacity of the pump.

Statistics

Data are expressed as means ± 1 standard deviation (SD). Differences among groups were assessed by one-way ANOVA followed by Bonferroni post-hoc analysis to assess level of significance. For all analyses, $p<0.05$ was taken as fiducial limit.

3. Results

Nerve bundles.

In carp tail fin clips several nerves were found, both within the lepidotrichia segment and between these dermal bone structures of the fin rays. The nerves were symmetrically distributed (Figs. 3 & 4). Morphometric analyses revealed four categories of neuritis, three types of myelinated A-fibres and one type of unmyelinated C-fibres (Fig. 4). Neurites in five nerves were analysed for diameter to score them as C and A-δ, A-βand A-α type (Table 2). The type distributions in the nerves were tested for homogeneity (χ^2 test of homogeneity of proportions, $p > 0.05$).

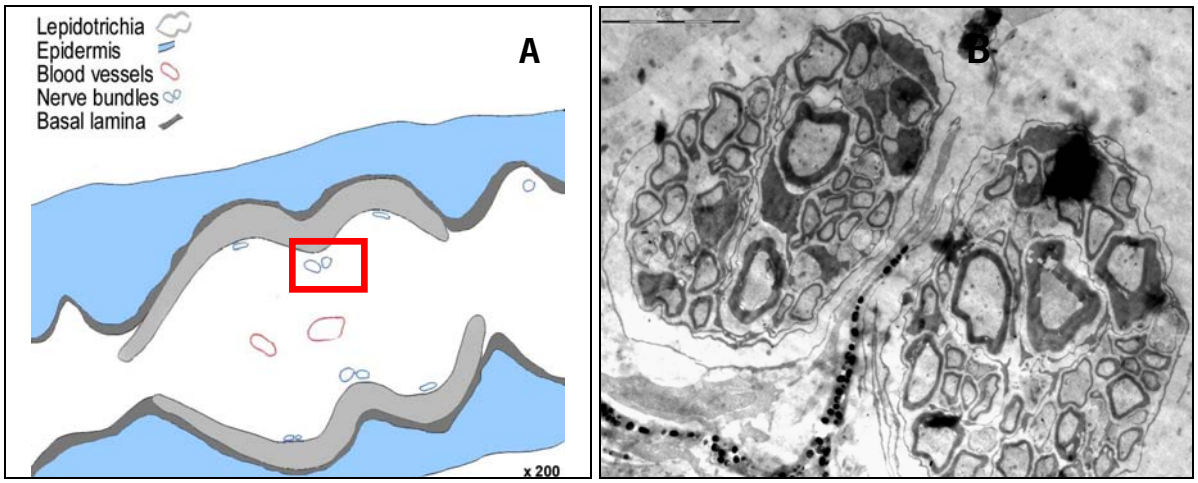

***Figure 3A**. Nerves in tail fin of common carp (Cyprinus carpio), the red box is detailed in **Figure 3B**, and shows a transverse section of the interior of the lepidotrichia segment of the tailray showing two nerves (EM, Scale bar = 5 µm).*

***Table 2.** Neurite type frequency (in %) of five independent nerve sections in a tail of a common carp, following the classification as published for rainbow trout trigeminal nerve (Sneddon, 2002). No statistical differences between average frequencies were found among the five nerve cross sections analysed (p=0.9). Classification of the neurite types is based on diameter (Lynn,1994).*

Fibre type	Bundle 1	Bundle 2	Bundle 3	Bundle 4	Bundle 5	Average	Trigeminal nerve (trout, average)
C and A-δ	46.7	38.7	33.3	26.8	47.8	**38.7**	**37**
A-β	40.0	48.4	56.9	57.1	41.3	**48.7**	**53**
A-α	13.3	12.9	9.8	16.1	10.9	**12.6**	**9**

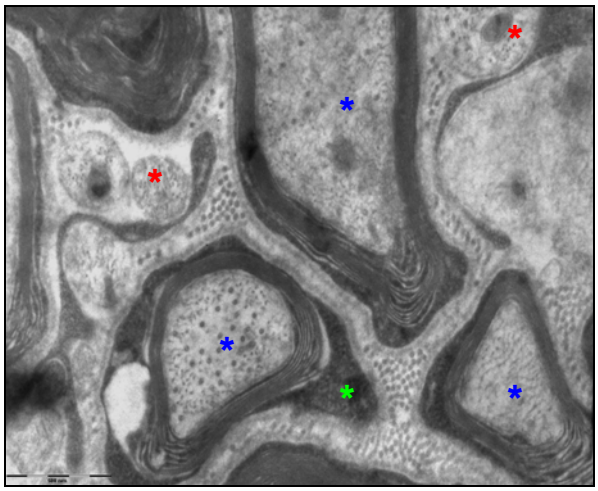

***Figure 4**. Nerve fibres in tail fin of common carp (TEM, scale bar = 500 nm). Both C-fibres (red asterisk) and three categories of A-fibres (blue asterisks) are present within the nerve. (Green asterisk: Schwann cell producing the myelin sheets around A-fibres). Black spots in the neurite neuroplasm represent microtubules.*

Skin colour

The colour measurements did not reveal any changes related to the clip or handling. The colour of the skin of the tilapia changed rapidly and visibly during the handling and anaesthesia, making this assay inappropriate to score and discriminate effects of the pain-stimulus and the handling stress.

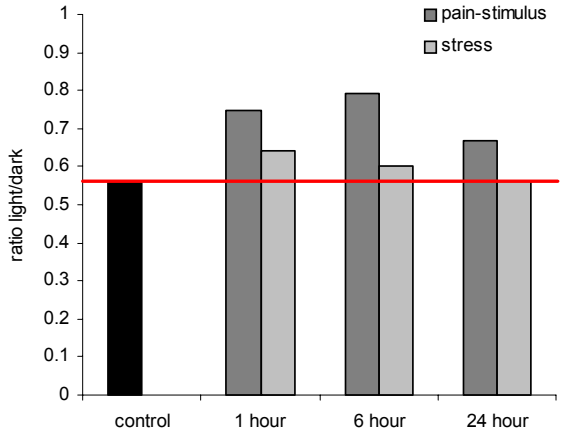

***Figure 5.** Dark/light preference. Compared to control, untreated fish, a pain stimulus induces a larger shift in preference than the handling stress alone. This effects lasts for at least six hours.*

Dark-light preference and swimming activity

The fish in the control situation showed a slight preference for the darker side of the tank (Fig. 5). Following a pain stimulus, the fish showed increased swimming activity that resulted in a more random movement of the fish through the tank (data not shown). The response was strongest in the groups 1h and 6h after the clip and had essentially faded after 24h. In the stress groups, the effect on swimming activity was mild (at 1h after handling) and had faded at 6h and 24h following the stress.

Stress and plasma analyses

Plasma concentrations of cortisol and glucose (Fig. 6 and Table 3) showed the predictable changes imposed by stress, but 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.

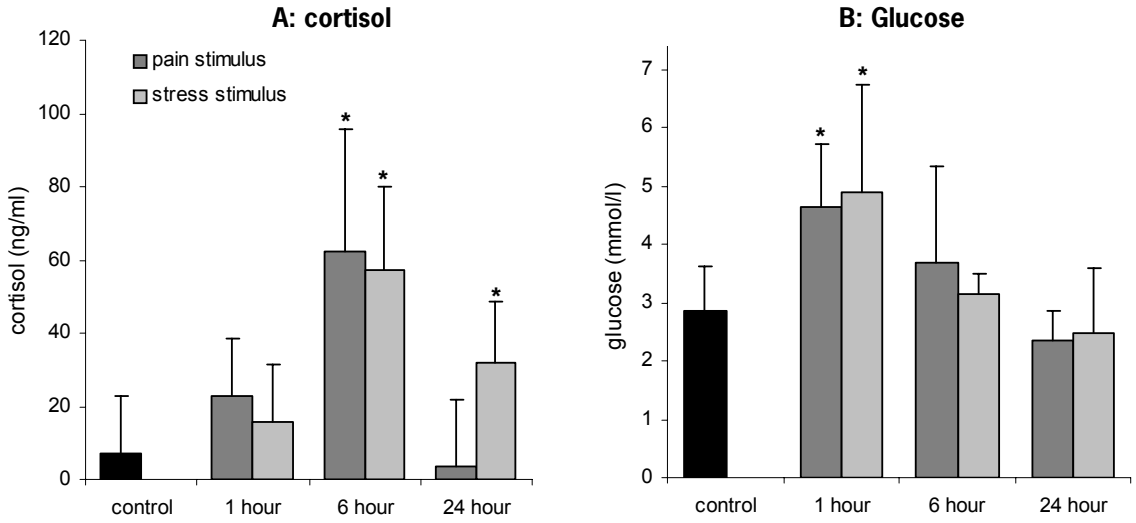

Figure 6. *Plasma cortisol, glucose and lactate. following handling stress an tail fin clipping and in untreated fish.*

Osmoregulation related parameters

The levels of plasma Na^+, K^+ and Ca^{2+} were not affected by any treatment (Table 3). Plasma pH (Table 3) was not affected by the clip or handling stress; this was taken to indicate that the fish cope well with the treatments and were not overly stressed; indeed, this tilapia species is well known as a truly sturdy fish.

The Na^+/K^+-ATPase enzymic activity transiently increased specifically in the group sample 1 h after the fin clip (Fig. 7 and Table 3). Further no differences in Na^+/K^+-ATPase activity were found among the groups tested

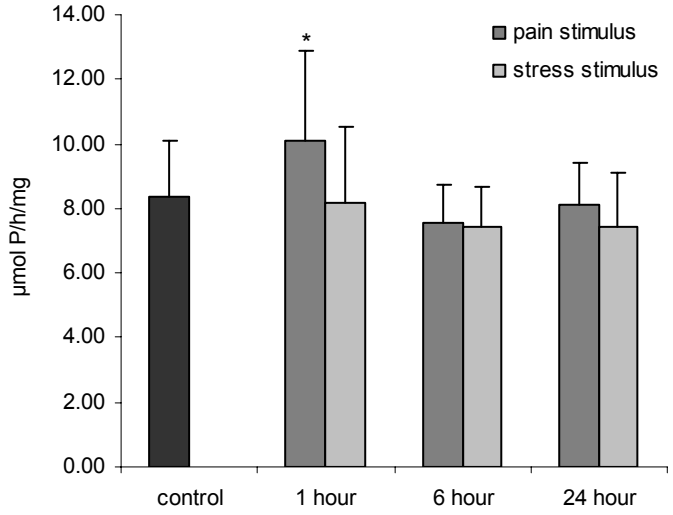

Figure 7. *Na^+/K^+-ATPase activity increased one hour after the fin-clip. In stress group or in any of the other test groups, no differences compared to the controls were found.*

Table 3. *Plasma parameters and Na^+/K^+-ATPase activity (in µmol P_i/h per mg). Asterisks indicate significant differences compared to untreated controls.*

Group	Cortisol (ng/ml)	Glucose (mM)	Lactate (mM)	pH	Na^+ (mM)	K^+ (mM)	Ca^{2+} (mM)	Na^+/K^+-ATPase
Controls	7.20±15.71	2.87±0.75	2.56±1.40	7.72±0.11	161.4±3.0	3.76±0.52	1.15±0.18	8.33±1.74
Pain 1h	22.60±15.72	**4.63±1.10***	2.41±1.30	7.81±0.09	157.9±1.9	3.52±0.30	1.09±0.15	**10.08±2.83***
Stress 1h	15.49±12.73	**4.89±1.85***	2.72±2.42	7.83±0.06	158.5±3.4	3.41±0.32	1.10±0.14	8.19±2.31
Pain 6h	**62.09±33.82***	3.68±1.67	1.41±0.59	7.68±0.04	158.1±5.2	3.97±0.48	1.25±0.23	7.55±1.19
Stress 6h	**57.01±23.01***	3.14±0.34	**0.65±1.31***	7.72±0.03	158.7±3.7	3.76±0.35	1.22±0.14	7.41±1.25
Pain 24h	3.65±5.01	2.34±0.43	1.69±0.64	7.81±0.04	157.5±1.7	3.62±0.33	0.99±0.10	8.14±1.29
Stress 24h	**32.01±21.01***	2.49±0.43	1.39±0.51	7.81±0.03	157.9±1.5	3.47±0.44	1.05±0.12	7.44±1.68

Both the clip and handling stress induced migration of chloride cells to lamellar regions. This migration was observed as of 6h post treatment and lasted at least for 24hrs (Fig. 8). The cells migrated from the central filamental epithelium up the lamellae, way up to the tips. No difference between the clip and handling stress groups were found. Interestingly, our Na^+/K^+-ATPase activity analysis revealed only a transient rise in Na^+/K^+-ATPase capacity in the fish that received a clip. From this we should conclude first, that a fast (phosphorylation?) event is at the basis of this phenomenon and second, that the cell migration observed concerns migration of residing cells, and is not a result of cell hyperplasia.

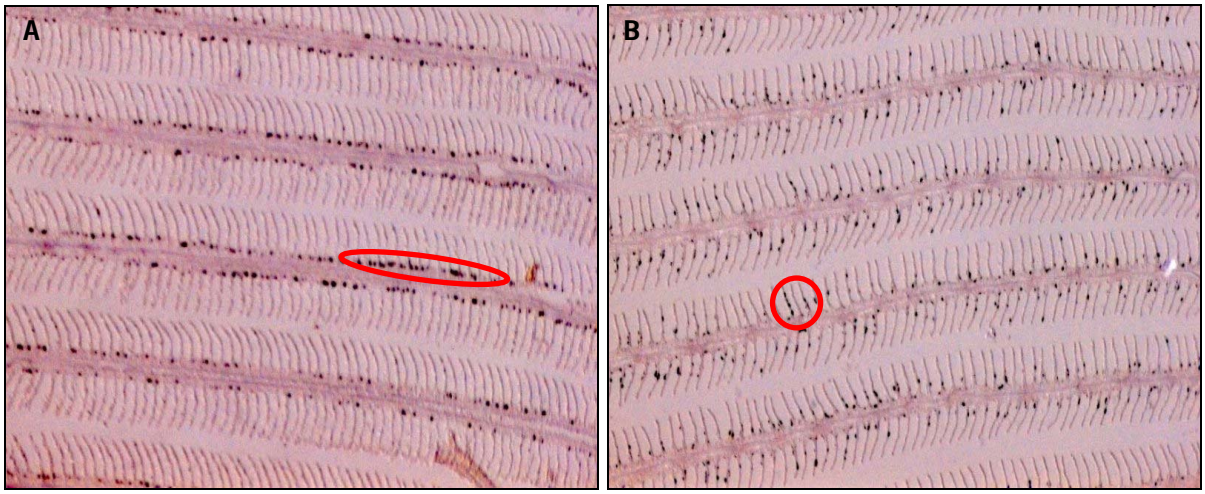

Figure 8. *Chloride cells, seen as dark dots with examples encircled, are situated in the filamental epithelium at the base of the lamellae. Control fish (**A**). In the 6h and 24h post treatment groups, chloride cells had migrated towards the apices of the lamella (**B**). This phenomenon was observed in both the clipped and handled fish. Magnification 100x.*

Mucus cells

The mucus cells in the control group are seen as blue dots between the lamella on the filaments, at a similar location as the chloride cells (Fig. 9A). In response to the tail fin clip, 1h after the clip (Fig. 9B), the frequency of mucus-containing cells had drastically decreased. This we take as a measure for release of mucus. This response was not observed in any of the other groups. At 6h and 24h after the clip (Figs. 9C and E), mucus cells had recovered to control status. In the 1h-, 6h- and 24h-stress groups, no difference in mucus cell frequency was found compared to the controls.

Figure 10 summarises the quantification of mucus cell frequencies in controls and all experimental groups. The lower incidence of alcian blue positive cells (mucus cells) in the group 1h after the clip is a highly significant factor two lower compared to controls.

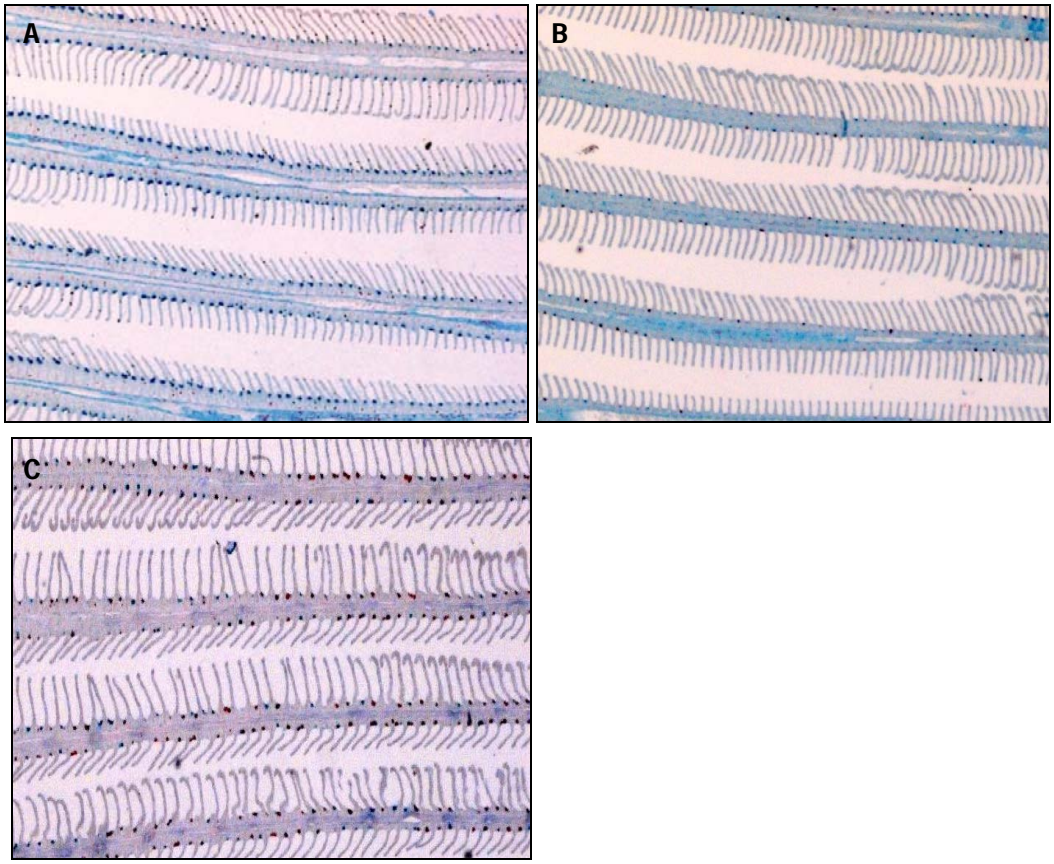

Figure 9 A. *Mucus cells containing mucus and stained with alcian blue show up as blue dots between the lamella in the filamental epithelium.* ***B****. In the group analysed 1 h after the tail fin clip, mucus was secreted from the cells and the number of visible mucus cells decreased.* ***C.*** *At 6h and 24h 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. Magnification 100x.*

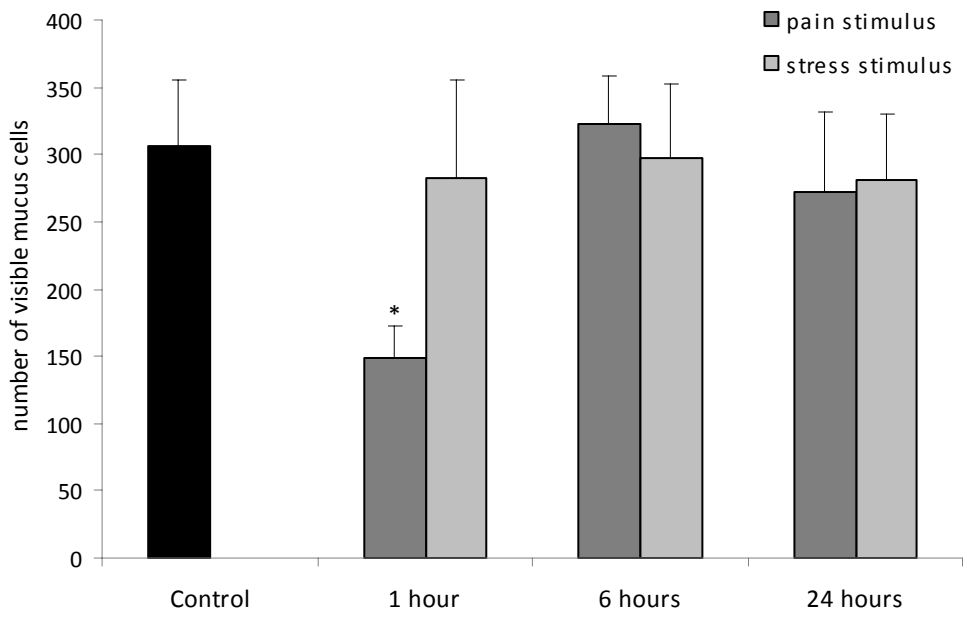

***Figure 10**. Quantification of the mucus cells frequency in gills. A significant decrease in mucus-filled mucus cells in the gill filaments in the 1h-pain group. In the accompanying stress group, this decrease was not observed. Different letters stand for significant differences.*

4. Discussion and perspectives

This study investigated acute physiological and behavioural 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 tail fin). In carp, in similar clips, the presence of nerves could be demonstrated that fulfil all requirements to be designated as nerves that can rely pain stimuli. In the tilapia, three parameters were found that show a differential response to the pain stimulus compared to the accompanying stress response. Fish that receive a pain stimulus show more swimming activity and less preference for the darker part of the tank compared to controls. This was found both 1h and 6h after the fin clip was given and indicates that the clip experience, which is likely harmful, is either memorised or still experienced as painful for several hours.

The Na^+/K^+-ATPase activity, reflecting sodium pump capacity in the gills, increased transiently in the fish that received the pain stimulus. This effect was only seen 1h after the fin clip.

A similar transient response was seen in the mucus cells in the gills: strong mucus secretion in the branchial epithelium was seen in fish 1 h after the clip.

Nerves in fin clips

Several nerves were found between and within the fin rays where the fin was clipped. Four different types of neurites were identified in the nerves on the basis of their diameter (Sneddon, 2002). C-fibres and A-δ fibres are involved in pain perception. In mammals, the unmyelinated C-fibres mediate slow dull pain signals and the myelinated A-δ fibres mediate acute pain (Erlanger and Gasser, 1937; Sneddon, 2002). The presence of these two types of fibres in the clipped tissue, combined with the behavioural and physiological parameters, support strongly that this teleostean fish discriminates a nociceptive stimulus from handling stress. This conclusion is in accordance with recent literature (Munro and Dodd, 1983; Sneddon, 2003; Chandroo et al., 2004; Braithwaite and Huntingford, 2004; Huntingford et. al., 2006; Reilly et al., 2008).

The presence of the nerves with remarkably similar make-up as seen in mammalian (and trout) nerves that carry pain signals, highlights that a fin-clip may be a well chosen stimulus to study acute pain responses in teleostean fishes. The well-taken stress response that goes with this procedure indicates that the fish are well able to recover from this harsh invasive procedure.

The relative abundances of C-fibres and A-δ fibres among the neurites we scored in cross-sectioned nerves are rather similar to those reported for the trigeminal nerve of rainbow trout by Sneddon (2002). In trout, a low 4% C-fibres (compared to 10% in carp) contrasts with the percentage in terrestrial vertebrates where it can reach 50% (Young, 1977). According to Sneddon (2002), the difference in proportion can be attributed to the water-to-land transition and the subsequent adaptation to terrestrial life that requires a more sophisticated alarm system to cope with the complex environment. The difference between trout and carp could relate to the phylogenetic distance between these fish (trout being an older species) or maybe the very different life styles (fast swimming predator vs. bottom-dwelling omnivore).

The presence of nerves in the carp tail fin with characteristics of pain nerves in other species warrant similar analyses in the two target species for this project, Nile tilapia and Dover sole. In these, nerve tissue should be analysed at vulnerable sites such as the fins, opercula, mouth and lips and skin. Also, the search for neurites penetrating between skin epithelium as seen in mammals should be pursued.

Skin colour

Tilapia change the colour of their skin very rapidly in response to (environmental) conditions, for instance during reproduction, aggression or stress (Van Eys and Peters, 1981; Arends et al., 2000). This is mainly a neural response, although hormonally regulated mechanisms are also involved in skin colour changes, albeit on the somewhat longer term than the acute changes seen after stress. The pituitary α-MSH plays an important role in this process (Arends et al., 2000), in particular in the melanin production. MSH not only stimulates the production of melanin in the skin melanophores, it may also induce spreading of pigment in the melanophores, with darkening of the skin as a result. Several studies have revealed an increase in the α-MSH level and expression of α-MSH mRNA in different fish species at various stressful conditions (Wendelaar Bonga, 1997). Colour change actions of MSH are mediated via the melanocortin receptor 1, a receptor controlled by MSH and the natural antagonist agouti-related protein. MSH is pleiotropic and among its other functions are control of cortisol-producing cells and lipolytic acions in the liver. Chronic stress in fish is always associated with elevated plasma MSH levels, but not necessarily with skin darkening.

In the current study, the skin colour measurement did not discriminate groups. The effect of the sampling procedure induced responses that masked possible effects of the pain stimulus application. This procedure was therefore considered unsuitable for assessment of responses to pain or stress in this fish.

Dark-light preference and swimming activity

The fish that received the pain stimulus showed an increased swimming activity in the 1h-and 6h- pain group that was not observed in stress-only groups. In an earlier study, it was shown that pain influences the behaviour of fish lastingly (several hours) as we observed here and that behavioural studies can be used to study pain perception in fish (Sneddon, 2003a and b).

Behavioural studies will be intensified in the course of this project. Future experiments will focus on interactions between fish when a designated part of a group is given the fin clip; this may surface a role for pheromone-like alarm signals. In addition, the effects of tank enrichment on the behavioural responses after a painful stimulus will be addressed.

Stress response

The cortisol level increased in response to the treatments, but no effect between treatments was found. Basal cortisol levels are usually around 10 – 20 ng/ml, depending among others on species, sex and life stage. In the present study, cortisol levels increased up to 60 ng/ml 6h post treatment, generally referred to as a mild stress response. A rapid increase to a level above 100 ng/l is generally referred to as a more severe stress response. A peak in cortisol level occurred around 3 hours after the stressor, after which a gradual decline set in.

When fish experience chronic stress, the plasma cortisol level should remain elevated compared to the control concentration (Wendelaar Bonga, 1997). No such observation was made which leads us to conclude that the treatments given in this study were relatively mild even though the clipping is invasive and may have damaged pain nerves.

Biswas et al. (2004) measured basal cortisol levels of 14 – 36 ng/ml in Nile tilapia and a rapidly increased level of 182 ng/ml after an acute light stressor. After 8 hours, the cortisol level had returned to basal. Monteiro et al. (2005) measured a basal level of 28 – 34 ng/ml and highly increased levels of 163-278 ng/ml in copper-exposed fish. These results highlight that the mild increase of the present study represents a mild stressor compared to such highly stressful treatments as copper exposure.

In addition, glucose and lactate levels showed a comparable result, with mildly increased glucose levels compared to the controls, but no differences between the pain and stress groups. Lactate levels slightly decreased 6h and 24h post treatment, but no difference between the stress and pain groups was observed.

Monteiro et al. (2005) found resting levels of 2.38 – 2.90 mg/dl (1.32 – 1.61 mmol/l) in Nile tilapia and increased levels of 4.29 – 5.46 mg/dl (2.38 – 3.03 mmol/l) in copper exposed fish. These values are in the same range as the glucose level that was measured in the present study.

Since these parameters are considered to be basal stress parameters and are relatively easy to measure, these parameters will be measured in future experiments, although no differential response was found between the pain and stress groups.

Osmoregulation

Na^+/K^+-ATPase activity had increased specifically in the 1h-pain group. In the accompanying stress group, as well as the 6h and 24h post treatment groups, no differences were observed. Ionic concentrations of Na^+, K^+ and Ca^{2+} in the plasma were rather constant after the pain stimulus and handling. This once again supports the relative mildness of the stressor applied and indicate no major loss of control over permeability to water and ions, as is often seen in (severely) stressed fish (due to stress-related, catecholamine-induced epithelial lifting and dysfunction of the gills).

The chloride cells are responsible for the majority of the in Na^+/K^+-ATPase activity. 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 24h post treatment groups, whereas at 1h post treatment, no increased migration was observed. 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.

Although we did not assay catecholamine levels, the pain stimulus must have evoked an adrenergic response that in the gills could easily increase permeability to water and ions (Wendelaar Bonga, 1997). The rapid transient rise in Na^+/K^+-ATPase activity observed in the most severely stressed fish (those receiving the fin clip) may have counteracted imminent loss of ions. It is unlikely that newly synthesized enzyme explains the increased activity. Rather phosphorylation and increased activity would well explain our observations. Catecholamines are known to exert such effects on this enzyme. At 6h post treatment, the activity had decreased to basal, indicating that the gills had recovered from the primary adrenergic response.

The increase in activity and the migration of the chloride cells are a combined adaptive osmoregulatory response to the pain stimulus and the likely endocrine changes occurring in the fish. The rapid increase in Na^+/K^+-ATPase activity can be seen as a emergency response, 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 pain stimulus.

Mucus

In the 1h-pain group, an increased mucous secretion was observed compared to the controls. In the 6h-pain group, the cells had recovered and were re-filled with mucus, suggesting the observed effect is an acute reaction to the pain stimulus 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 pain stimulus 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, analysing 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 1h after a pain stimulus and the mucus on the tail section that received the pain stimulus deserve attention.

General conclusions

This study is the first within the KB8 project to develop (non-invasive) readout to assess pain and discomfort in fish. This experiment aimed to confirm involvement of pre-selected parameters in the response to a 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 pain stimulus 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 was rightly chosen as a pain stimulus. The differential response to the pain stimulus 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, this choice is not exclusive; additional parameters related to pain, such as substance-P, endorphins or EEG-measurements, for future experiments are indicated.

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

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Justification

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Approved: Dr. J.W. van de Vis
Researcher

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