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

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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, three 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.

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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 [1]. 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 [2,3]. One of the endeavours 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 behavioural and neuroendocrinological responses to cope with stress [4].

Reviews by Braithwaite and Huntingford [5] and Chandroo et al., [6] 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 [7–9]. 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 [5]. 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 [5–7,10–12].

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 [5], and Chandroo et al., [6] conclude that these affective states may well be attributed to fish. Recently, Nilsson et al., [8] 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,

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may impinge on welfare-related aspects such as aquaculture-related stress physiology [13]. 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 [14].

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 [15]. 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 behavioural 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, behavioural 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 fibres classified in mammals and rainbow trout as pain fibres. 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 fibres are categorized according to their diameter, conduction velocity and degree of myelinisation as A-α, A-β, A-γ, A-δ B- and C-fibres [16]. The A-fibres are myelinated for fast conduction of action potentials. The A-δ fibres are involved in the transmission of well-localized acute pain, while C-fibres 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 [7,13,17]. Fibres conducting in the velocity range of A-δ and C-fibres were identified in the trigeminal nerve of the rainbow trout and characterized as nociceptive fibres by Sneddon [7]. A-δ fibres (25%) were predominant over C-fibres (4%), displaying a different pattern compared with other vertebrates, where C-fibres can comprise from 50% (cat, human) up to 65% (frog) of the total fibre type [18].This difference in fibres composition is attributed to the water-to-land transition in vertebrate evolution [7].

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 fibre types in cross sections were categorized based on diameter and the presence of myelin to distinguish Aα-, Aβ-, A-δ and C-fibres [7,17] (Table 1).

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 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 ($NO_2^- = 0.5$ 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 nine fish were used (Table 2). Two control groups were sampled one day prior the treatments of the six 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 versus 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

Table 1
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 [7]. No statistical differences between average frequencies were found among the five nerve cross sections analysed (chi-square test, $p = 0.9$). Classification of the neurite types is based on diameter [17].

Fibre type	Bundle 1 (Hypodermis)	Bundle 2 (Hypodermis)	Bundle 3 (Lepidotrichia)	Bundle 4 (Lepidotrichia)	Bundle 5 (Lepidotrichia)	Average (SD)	Trigeminal nerve (Trout, average)
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

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 analysed for cortisol as described in detail elsewhere [19]. Activities of Na^+, K^+, Ca^{2+}, pH, and concentrations of glucose and lactate in plasma were measured using Stat Profile pHOx plus analyser (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 [20,21]. 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 [22] and, therefore, sections of the trailing edge were observed for chloride cell distribution. Under stressful conditions chloride cells may migrate into the lamellar epithelium [23]; 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 [21]. 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 finrays. The nerves were symmetrically distributed (Figs. 1 and 2). Morphometric analyses revealed four categories of neurites, three types of myelinated A-fibres and one type of unmyelinated C-fibres (Fig. 2). Neurites in five nerves were analysed 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

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).

Group	Cortisol (nM)	Glucose (mM)	Lactate (mM)	pH	Na^+ (mM)	K^+ (mM)	Ca^{2+} (mM)	Na^+/K^+-ATPase activity (μmol P_i/h per mg protein)	Osmolality (mOsmol/kg)
H (Kruskal–Wallis)	$H(6,71) = 44.60$	$H(6,71) = 41.07$	$H(6,70) = 24.22$	$H(6,71) = 30.94$	$H(6,71) = 15.31$	$H(6,71) = 11.12$	$H(6,71) = 15.22$	$H(6,71) = 8.32$	$H(6,70) = 13.43$
	$p < 0.01$	$p < 0.01$	$p < 0.01$	$p < 0.01$	$p = 0.018$	$p = 0.085$	$p = 0.019$	$p = 0.22$	$p = 0.037$
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}	7.92 (1.53)	321.8 (9.5)
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}	10.01 (2.83)	314.3 (5.3)
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}	8.20 (2.31)	316.4 (9.9)
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}	7.55 (1.19)	314.9 (11.8)
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}	7.42 (1.25)	311.3 (8.1)
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}	8.14 (1.29)	312.3 (6.3)
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}	7.44 (1.68)	312.1 (5.3)

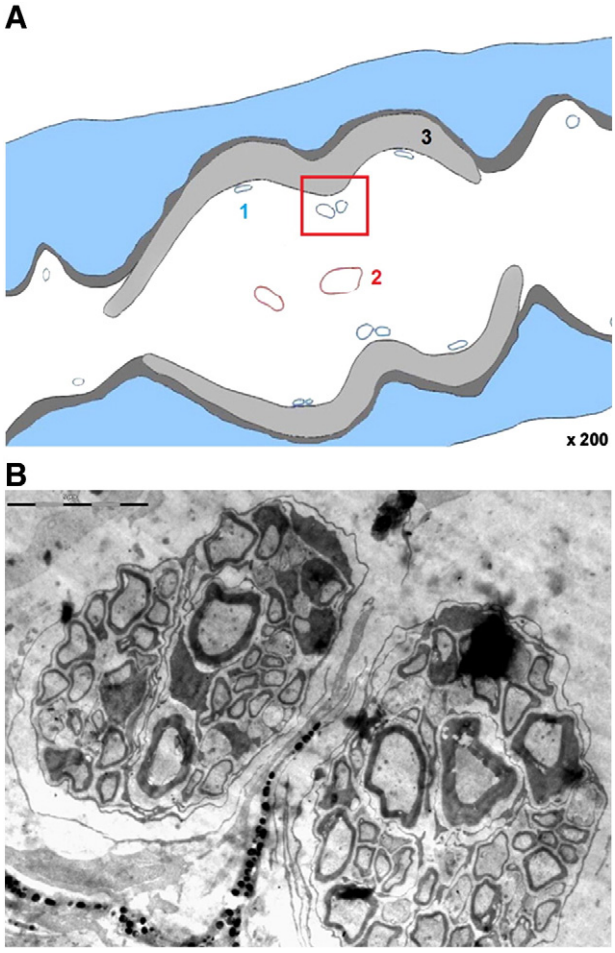

Fig. 1. A. nerves in tailfin of common carp (x 200), the red box is detailed in B, and shows a transverse section of the interior of the lepidotrichia segment of the tailray showing two nerves (EM, Scale bar = 5 μm). 1: nerve bundle, 2: blood vessel, 3: lepidotrichial hemisegment.

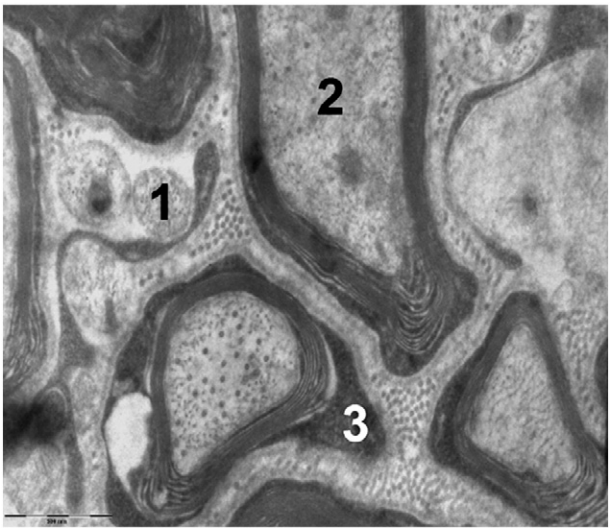

Fig. 2. Nerve fibres in tailfin of common carp (TEM, scale bar = 500 nm). Both C-fibres [1] and three categories of A-fibres [2] are present within the nerve. [3] Schwann cell producing the myelin sheets around A-fibres. Black spots in the neurite neuroplasm represent microtubules.

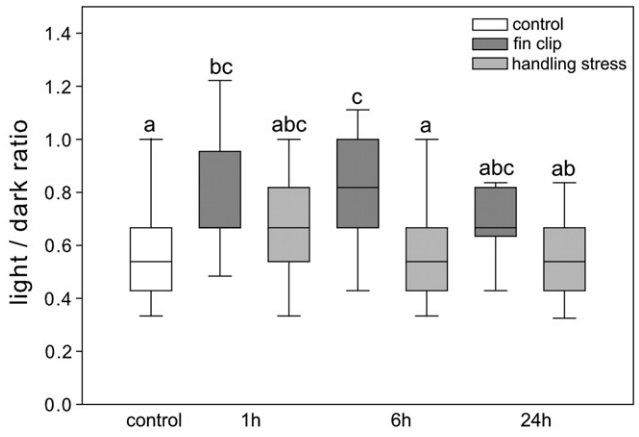

Fig. 3. Dark/light preference of Nile tilapia, in function of treatment. Compared to control, untreated fish, a fin clip induces a larger shift in preference than the handling stress alone. This effect lasts for at least 6 h. Different letters stand for significant differences at $p = 0.05$ (Post-hoc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

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.

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 [24].

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

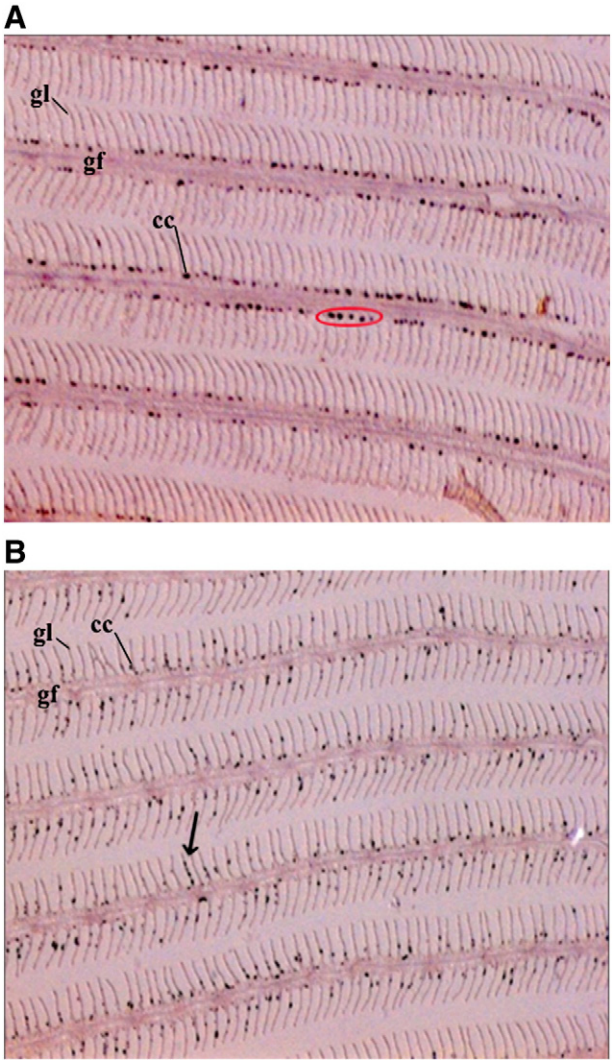

Fig. 4. Chloride cells (cc) of Nile tilapia, seen as dark dots with examples encircled, are situated in the filamental epithelium at the base of the lamellae (gl). Control fish A. In the 6 h and 24 h post treatment groups, chloride cells had migrated towards the apices of the lamella (arrow) B. This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

stress, no difference in mucus cell frequency was found compared to the controls (Fig. 6).

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

4. Discussion

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

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 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* [25].

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 [7,17]. 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 [7,16,17]. The presence of these two types of fibres in the clipped

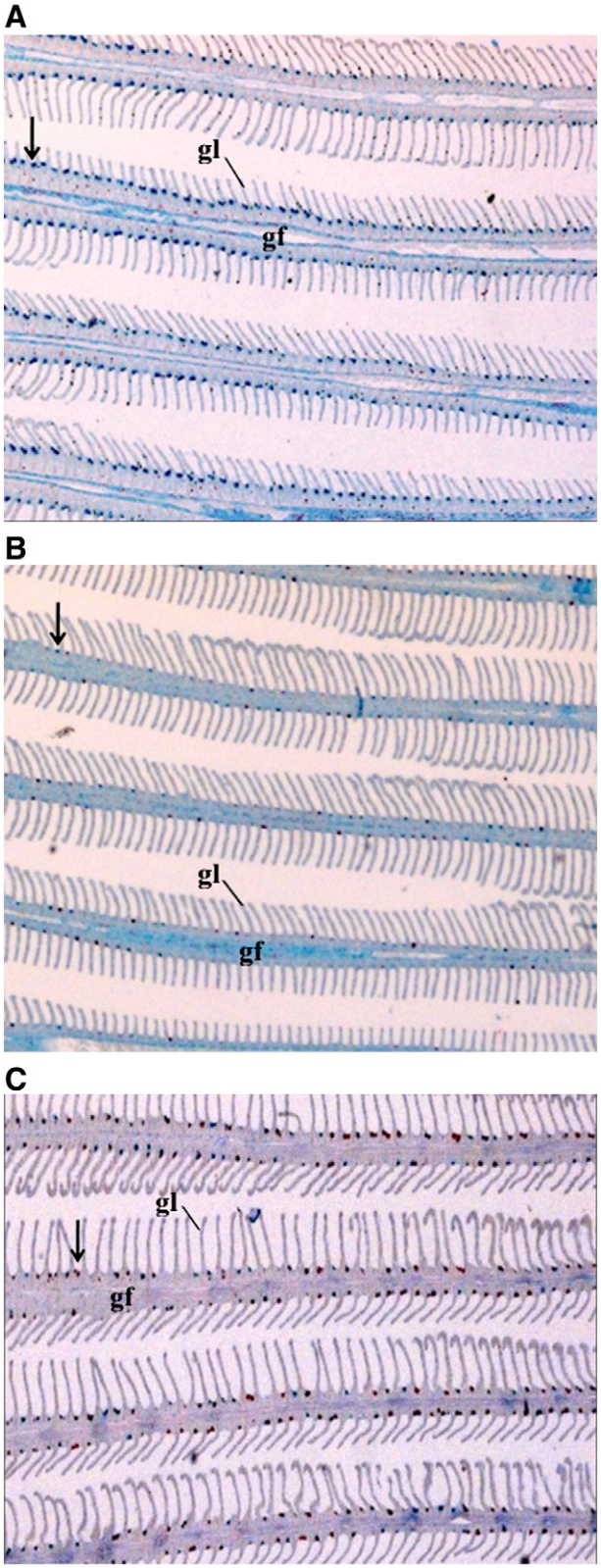

Fig. 5. A. mucus cells (arrow) of Nile tilapia containing mucus and stained with alcian blue show up as blue dots between the lamella (gl) in the filamental epithelium. B. in the group analysed 1 h after the tailfin clip, mucus was secreted from the cells and the number of visible mucus cells decreased. 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.

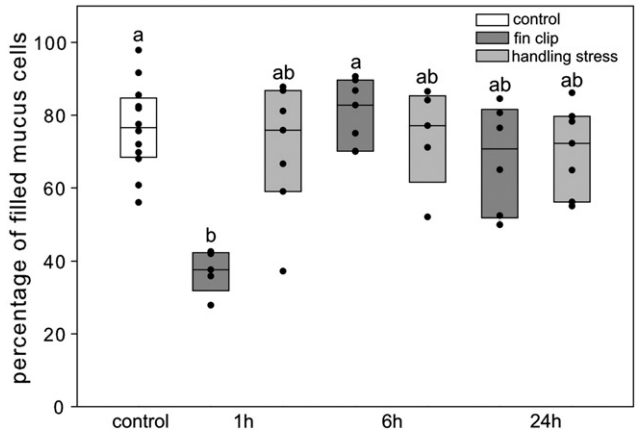

Fig. 6. Quantification of the mucus cells frequency in gills of Nile tilapia, in function of treatment. A significant decrease in mucus-filled mucus cells in the gill filaments in the 1 h after fin clip group. In the accompanying stress group, this decrease was not observed. Different letters stand for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

tissue, combined with the behavioural and physiological parameters, support strongly that Nile tilapia discriminate nociceptive stimuli from handling stress, a conclusion in accordance with recent literature [5,6,10,26–28].

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-fibres and A-δ fibres among the neurites we scored in cross-sectioned nerves are similar to those reported for the trigeminal nerve of rainbow trout [7]. In trout, the low 4% C-fibres clearly contrasts with the percentage in terrestrial vertebrates where this type of neurite may represent 50% [18] This low C-fibres percentage was also found in our study where it was estimated to ±5% of the total amount of fibres.

The presence of C-type fibres in fish provides a further substrate for the discussion on pain perception in these animals. The 6 h duration of behavioural response in our tests suggests that signals comparable to those transducing lasting dull pain in mammals are carried by C-fibres [7,17].

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 analyse 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 [29].

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 fulfil 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, behavioural studies are instrumental to study pain perception in fish [10,11].

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; 4). 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 [4]. When fish experience chronic stress, plasma cortisol level should remain elevated compared to controls [4], 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 [30].

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 [31]. Copper exposure may induce even higher cortisol levels (over 600 nM; 35). 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 behavioural 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 et al. [32] 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 [4].

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 [4]. 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 [33]. 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 [34]. Mucus has a very high water content captured by glycosaminoglycans and glycoproteins [35]. In addition, mucus contains substances, such as lysozyme, IgM's, calmodulin and pheromones (reviewed in [36]). 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 [36].

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, 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 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 behavioural response, enzymic osmoregulatory activity and the mucus cell response and these will be the focus for future experiments.

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