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ALTERATIONS OF CLUB CELL ACTIVITY IN EPIDERMIS OF COMMON CARP, *CYPRINUS CARPIO* (ACTINOPTERYGII: CYPRINIFORMES: CYPRINIDAE), DUE TO INFECTION BY *ICHTHYOPHTHIRIUS MULTIFILIIS* (PROTISTA: CILIOPHORA)

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Background. The abundance of club cells in epidermal tissue of fishes in the superorder Ostariophysi is a poorly understood phenomenon. Previous results have suggested that epidermal club cells have a generic role in response to injury and that they display intense phagocytotic activity, having an anti-parasitic function in the host. Earlier works suggested that club cells are usually located in the middle of the epidermis and that they do not communicate with the epidermal surface or do it only when the epidermis has been ruptured by predation. The presently reported study focused on the alterations of club cell activity in carp epidermis induced by ectoparasite, *Ichthyophthirius multifiliis*. We hoped that our observations would help to understand the function(s) of these cells.
Materials and methods. This study was based on 200 four-month old common carp, *Cyprinus carpio* L., with mean body weight of 65 ± 5 g. The fish were experimentally infected with theronts of *Ichthyophthirius multifiliis*. In sequential days post infection, samples of fins and body skin were collected for histological and histochemical examination. The correlation between club cell densities and mucous cell densities was analysed using Pearson correlation analyses.
Results. A local reduction of mucous cells occurred after theront invasion-induced proliferation, and increased club cell density around the parasite during the growth of trophonts. After parasites left the skin due to salt-water treatment, a decrease in the number of club cells was detected. During reinvasion the decrease in parasite activity in areas of club cells proliferation was not noted. It was found that giant mature club cells were opened on the surface.
Conclusion. Club cells have no anti-parasitic function against *I. multifiliis* and these mature cells released their viscous secretion into water. The high density of club cells in the epidermis compensates an overall low density or absence of mucous cells. As it can be hardly concluded that the function of club cells is phagocytic removal of cell debris, an integrated research on mucosal immune mechanisms, as well as studies on epidermal tissue responses on product(s) released by club cells ("alarm substance cells") should be carried out in the future.

Keywords: alarm substance cells, club cell, Ich, *Ichthyophthirius multifiliis*, ostariophysan fishes

INTRODUCTION

The epidermal tissue of fish is often the primary barrier, to pathogens in the environment (Singh and Mittal 1990, Iger et al. 1994, Buchmann et al. 2004, Rakers et al. 2010, Ottesen et al. 2010). The cell-mediated innate mechanisms of epidermal tissues include specialized cells such as macrophages, granulocytes, natural killer cells, and also physical barriers such as mucous layers and skin epithelial tissue lines (Jones 2001, Aoki et al. 2008). The epidermal club cells of many fishes in the superorder Ostariophysi have evolved primarily as part of the immune system (Chivers et al. 2007, Halbgewachs et al. 2009, Ferrari et al. 2010) but their functional mechanisms, with non-specific and/or specific immunity, have not been explored in detail. The evolution of club cells of ostariophysan fish remains enigmatic and controversial as it has been historically linked to chemical alarm signalling (von Frisch 1941, Smith1992, James et al. 2009). However, Carreau-Green et al. (2008) suggested that club cells do not have an alarm function in fathead minnow, *Pimephales promelas* Rafinesque, 1820. Such evidence would support the possibility that club cells of fishes may be maintained by nat-

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ural selection owing to benefits conferred against pathogens (Chivers et al. 2007).

Epithelial cells activated by wounding start to proliferate and to regenerate the epidermal tissue (Whitehead et al. 2005). Previous results have suggested that epidermal club cells have a generic role in response to injury (James et al. 2009). After epidermal damage these cells become differentiated from epithelial cells in common carp (Iger and Abraham 1990) and display intense phagocytotic activity (Iger et al. 1994, Abraham et al. 2001). Their possible role in healing (Al Hassen et al.1985, Wisenden and Stacey 2005), after damage due to parasite attack, has been described (Suzuki and Kaneko 1986, Nakamura et al. 2001) but the mechanism of the immune function of these cells has not been clearly elucidated. Although these ideas have perpetuated in the literature for some time, connecting these functions to a mechanism for maintaining epidermal club cells was not the focus of the original authors (Ferrari et al. 2010).

The host cellular (Cross 1994, Buchmann and Nielsen 1999, Matthews 2005) and molecular (Buchmann et al. 2001, Gonzalez et al. 2007a, b, Whyte 2007, Randelli et al. 2008) responses to *Ichthyophthirius multifiliis* Fouquet, 1876 (Ich) infection in the fish epidermis are well documented. Significant tissue damage is caused by ciliate invasion as a result of histolysis and trauma (Bauer 1958, Ventura and Paperna 1985, Matthews 1994). If club cells have defence functions, then the activation of these cells after invasion should occur. Paradoxically, the proliferation of club cells close to the moving and growing parasite has been described only once, in the channel catfish, *Ictalurus punctatus* (Rafinesque, 1818) (see Chapman et al. 1984). Latest results by Päkk et al. (2011) demonstrate the potential role of club cells in healing process during and after epidermal hyperplasia in koi carp (*Cyprinus carpio*). The sublethal infection pathogenesis combined with clinical signs, histological features of epidermal tissue and remarks in literature refer to possible activation triggers for club cell function in damaged epidermis.

The aim of the presently reported study was to elucidate the alterations in club cells activity and connection between the club and mucous cells due to *I. multifiliis* infection. In this study an epidermal club cells anti-parasite hypothesis (Smith 1992, Magurran et al. 1996, Chivers et al. 2007, Halbgewachs et al. 2009) was tested. If club cells have anti-parasite attributes it was predicted that injury caused by extended exposure to ciliates should result in an increased number of club cells, and a subsequent reduction in parasite activity in the epidermis.

MATERIAL AND METHODS

Experimental animals. Two hundred 4-month old common carp, *Cyprinus carpio* L. (mirror carp) with a mean body weight of 65 ± 5 g were obtained from a fish farm in Ilmatsalu, Estonia. Fish were reared under pathogen-free conditions in the water with temperature of 17–22ºC. Fish were acclimatized for one month in plastic tanks filled with aerated and well filtered tap water at 20ºC under natural photoperiod conditions. Fish were fed *ad libitum* with a commercial koi feed (Danafeed DAN-EX 0333).

Host–parasite interaction. To clarify the function of club cells in epidermis the fish were experimentally infected with *Ichthyophthirius multifiliis* according to the host-parasite interaction model used in several studies (Hines and Spira 1974, Cross 1994, Dickerson and Clark 1998, Wahli and Matthews 1999, Aihua and Buchmann 2001, Matthews 2005, Jørgensen and Buchmann 2007, Ling et al. 2010). Trophonts of the ciliate *Ichthyophthirius multifiliis* grew within the carp epidermis from a diameter of 40 µm to 300–500 µm over the period of 5–10 days, causing extensive damage to the skin. The parasitic stage of Ich can be observed by light microscopy from the time when the parasite invades the fish tissue until it exits the fish to reproduce in the substratum. To study cellular responses associated with recovery from infection, infected carp were treated with marine salt (5 g · L^{-1}). The salt also had an antibacterial effect on the infected fish (Selosse and Rowland 1990, Garcia et al. 2007), which is important for the elimination of secondary bacterial infection (James et al. 2009).

Experiment. Trophonts of *I. multifiliis* were harvested from rainbow trout skin and carp were infected according to the procedure described by Sigh et al. (2004). Test carp were divided into four treatment groups. **Group 1**. A total of 40 infected carp (2000–2500 trophonts per fish) were transferred to one 700-L plastic tank and were treated indefinitely with a low concentration (5 g · L^{-1}) of marine salt (NaCl 97%) solution. **Group 2.** A total of 40 infected carp (2000–2500 trophonts per fish) were transferred into one 700-L plastic tank filled with tap water. **Group 3**. A total of 40 uninfected carp were put into one 700-L plastic tank filled with tap water. **Group 4**. A total of 40 uninfected carp were put into one 700-L plastic tank and treated with a low concentration (5 g · L^{-1}) of marine salt (NaCl 97%) solution.

At the beginning of the study 40 fish were euthanized (as described below). On experimental days 2, 4, 6, and 11, ten fish from each of the four groups were sacrificed. Fish were netted from the tank into 60-L of aquarium water with 0.1 g · L^{-1} of tricaine methanesulphonate (MS-222) buffered with 0.4 g · L^{-1} $NaHCO_3$. After three minutes, all anaesthetized fish were euthanized by cervical dislocation.

Identification of epidermal club cells, mucous cells, and thickness. Samples of fins and skin covering the body were fixed in 10% neutral-buffered formalin, embedded in paraffin, sectioned at 5 µm, and stained with haematoxylin-eosin (H&E) and Periodic-Acid-Schiff reaction (PAS). Sections were examined with a Zeiss Axioplan-2 (Germany) microscope and photographed using a digital camera, AxioCam HRc (Germany). Epidermal club cells around the ciliates were counted in 500-µm perimeter lengths and total depth of fish epidermis from the centre of the ciliates. Epidermal thickness was measured from the basement membrane up to the outer surface of the epithelium (Wisenden and Smith 1997).

Statistical analyses. The results are presented as mean ± standard deviation. To compare the club cell den-

sities, mucous cell densities, and epidermis thicknesses at different days and between groups the Wilcoxon test was perform using the SAS system. The correlation on cells densities between club cells and mucous cell was analysed using Pearson correlation analyses.

Ethics. The research was approved under animal care permit No. 53 by the Commission of the Authorization of Animal Testing Permits of the Estonian Ministry of Agriculture.

RESULTS

Host response.

Day 1. At the beginning of the infection 4.1 ± 1.0 (4.3 ± 1.5 in group 2) mature club cells and mucous cells 8.8 ± 1.1 (8.7 ± 1.1 in group 2) occurred per 1 mm length and at a thickness of 157.8 ± 8.8 µm (157.9 ± 6.5 µm in group 2) of the epidermis (Table 1).

Day 2. Trophonts (max. diameter 77 µm) of *I. multifiliis* were found in the epidermis of the fish in groups 1 and 2. The basal lamina was not damaged. The epidermis beside the trophonts was thickened (186.2 ± 6.3 µm in group 1, and 176.0 ± 13.2 µm in group 2) and hyperplasic and extensive activation of mucous cells (20.0 ± 3.0 in group 1, and 20.9 ± 2.9 in group 2) were noted. The density of club cells was 11.7 ± 2.8 (12.0 ± 2.8 in group 2) per 1 mm length and thickness of epidermis. These densities were significantly different from those counted on day 1 (Wilcoxon test, $P < 0.001$).

Day 4. Trophonts (max. diameter of 212 µm 235 µm in group 2) of *I. multifiliis* were found in the epidermis of fish in groups 1 and 2. The basal lamina was not damaged. The epidermis beside the trophonts was thickened (262.1 ± 17.9 in group 1, and 257.1 ± 28.9 in group 2). Club cells at different developmental stages were noted in the epidermis. The density of club cells was 20.3 ± 4.1 (19.7 ± 4.0 in group 2) and mucous cells 38.5 ± 2.7 (38.1 ± 3.2 in group 2) per 1 mm length and thickness of epidermis. Club cells had no direct contact with the trophonts (Fig. 1).

Day 6. Trophonts (maximum diameter 383 µm (514 µm in group 2) were noted in the epidermis of the fish in groups 1 and 2. In some areas of the epidermis several activated club cell aggregations were found. The density of club cells was 85.0 ± 6.1 (85.8 ± 8.7 in group 2) and mucous cells 7.4 ± 1.5 (6.1 ± 2.5 in group 2) per 1 mm of length and thickness of epidermis. Club cells had no direct contact with parasites. Some club cells on the surface of the epidermis were ruptured (Fig. 2).

Day 11. High invasions of ciliates, and massive or partial necrosis and erosion of the epithelial layers, were seen in the epidermis (thickness 143.2 ± 21.0 µm) of fish in group 2. Three to seven (max 12; 7.5 ± 4.5) trophonts per 1 mm were found from each sample. Club cells at different developmental stages were located near and above trophonts in the region where the epidermal surface was not damaged. In damaged areas only mature club cells (7.7 ± 4.7 in group 2) per 1 mm of length and thickness of epidermis were seen. Club cells had no direct contact with parasites.

Ciliates were absent in the epidermis of group 1. In some areas of the epidermis several activated club cell aggregations occurred with club cells densities of 58.4 ± 6.5 per 1 mm of length and thickness of epidermis were noted. Near the club cells, hyperplasia of new epithelial cells was found. Opening of mature club cells at the surface of the epidermis of infected and uninfected groups was detected.

The salt treatments had no effect on club- and mucous cell density or epidermal thickness of skin of group 4 (control 1) also had any effect filtered tap water on the group 3 (control 2) fish (Table1).

Statistics. Epidermal club cell densities, mucous cell densities and epidermis thicknesses were not significantly different between group 1 and group 2 from day 1 through

Table 1

Club- and mucous cell densities, thicknesses of epidermis of *Cyprinus carpio* and maximum diameter of *Ichthyophthirius multifiliis* at sequential days post infection (mean ± standard deviation)

P	Treatment	Day 1	Day 2	Day 4	Day 6	Day 11
NECC	SW	4.1 ± 1.0	11.7 ± 2.8	20.3 ± 4.1	85.0 ± 6.1	58.4 ± 6.5
	FW	4.3 ± 1.5	12.0 ± 2.8	19.7 ± 4.0	85.8 ± 8.7	7.7 ± 4.7
	c1 SW	4.2 ± 1.5	4.1 ± 1.7	4.3 ± 1.5	4.1 ± 1.5	4.4 ± 1.5
	c2 FW	4.2 ± 1.5	4.2 ± 1.1	4.0 ± 1.1	4.2 ± 1.6	4.3 ± 1.2
NMC	SW	8.8 ± 1.1	20.0 ± 3.0	38.5 ± 2.7	7.4 ± 1.5	0
	FW	8.7 ± 1.1	20.9 ± 2.9	38.1 ± 3.2	6.1 ± 2.5	0
	c1 SW	8.7 ± 1.1	9.1 ± 1.5	8.8 ± 1.1	8.6 ± 1.0	8.6 ± 1.1
	c2 FW	8.7 ± 1.1	8.8 ± 1.5	8.4 ± 1.3	8.6 ± 1.2	8.9 ± 1.5
ET	SW	157.8 ± 8.8	186.2 ± 6.3	262.1 ± 17.9	265.0 ± 15.3	197.1 ± 9.2
	FW	157.9 ± 6.5	176.0 ± 13.2	257.1 ± 28.9	282.4 ± 12.2	143.2 ± 21.0
	c1 SW	157.8 ± 8.7	158.0 ± 8.0	157.9 ± 8.1	160.3 ± 8.9	158.7 ± 7.9
	c2 FW	157.8 ± 8.7	157.9 ± 8.9	157.8 ± 8.5	157.9 ± 8.6	158.0 ± 8.9
CMD	SW	42	77	212	383	—
	FW	42	73	235	514	134 (48*)

P = parameter; NECC = No. of epidermal club cells per 1-mm transect (the transect was 1-mm long, covering the entire thickness of the epidermis); NMC = No. of mucous cells per 1-mm transect; ET Epidermis thickness, [µm] (Epidermal thickness was measured in the proximity of the ciliate parasite.); CMD = Ciliate parasite maximum diameter [µm]; SW = salt water (group 1); FW = fresh water (group 2); c1 = control 1; c2 = control 2; *reinvaded trophonts.

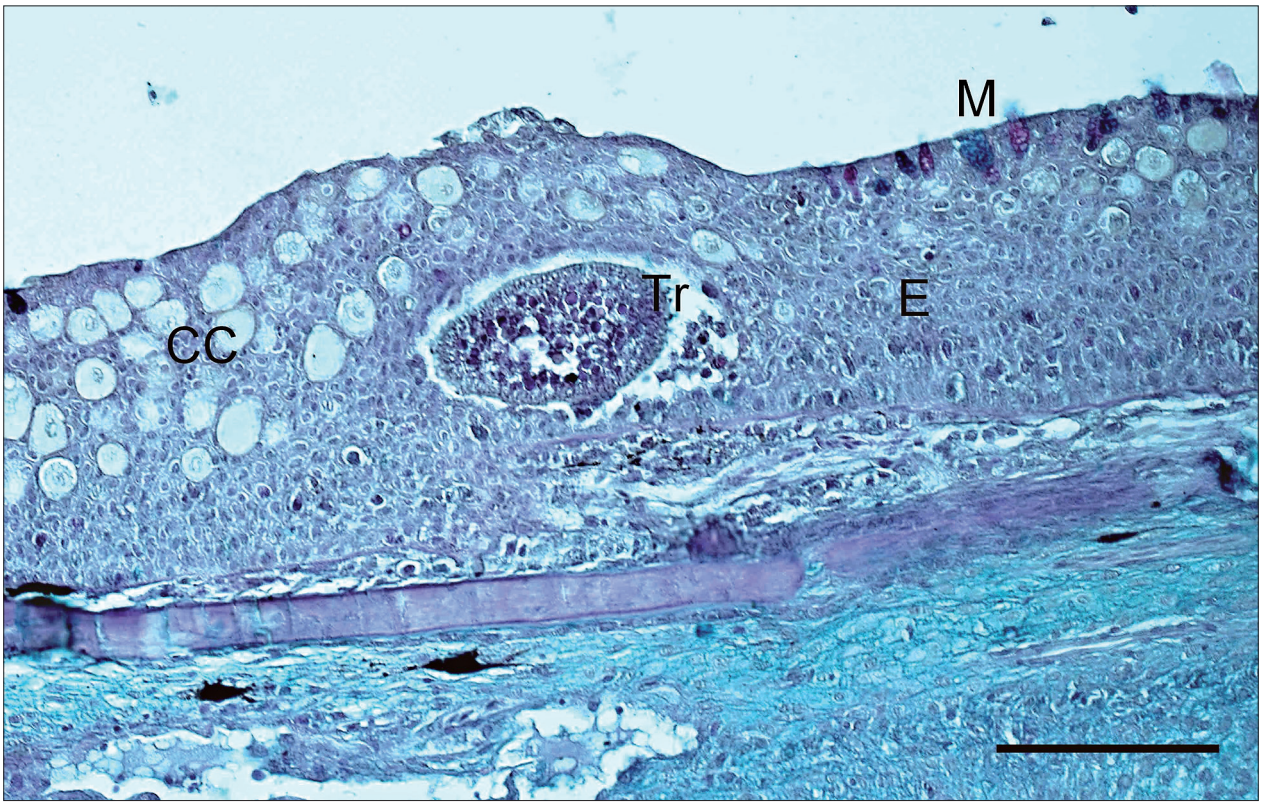

Fig. 1. Skin of common carp, *Cyprinus carpio*, four days after infection; E = epidermis, Tr = trophont of *Ichthyophthirius multifiliis*, CC = club cells, M = mucous cells; PAS staining, scale bar = 150 µm

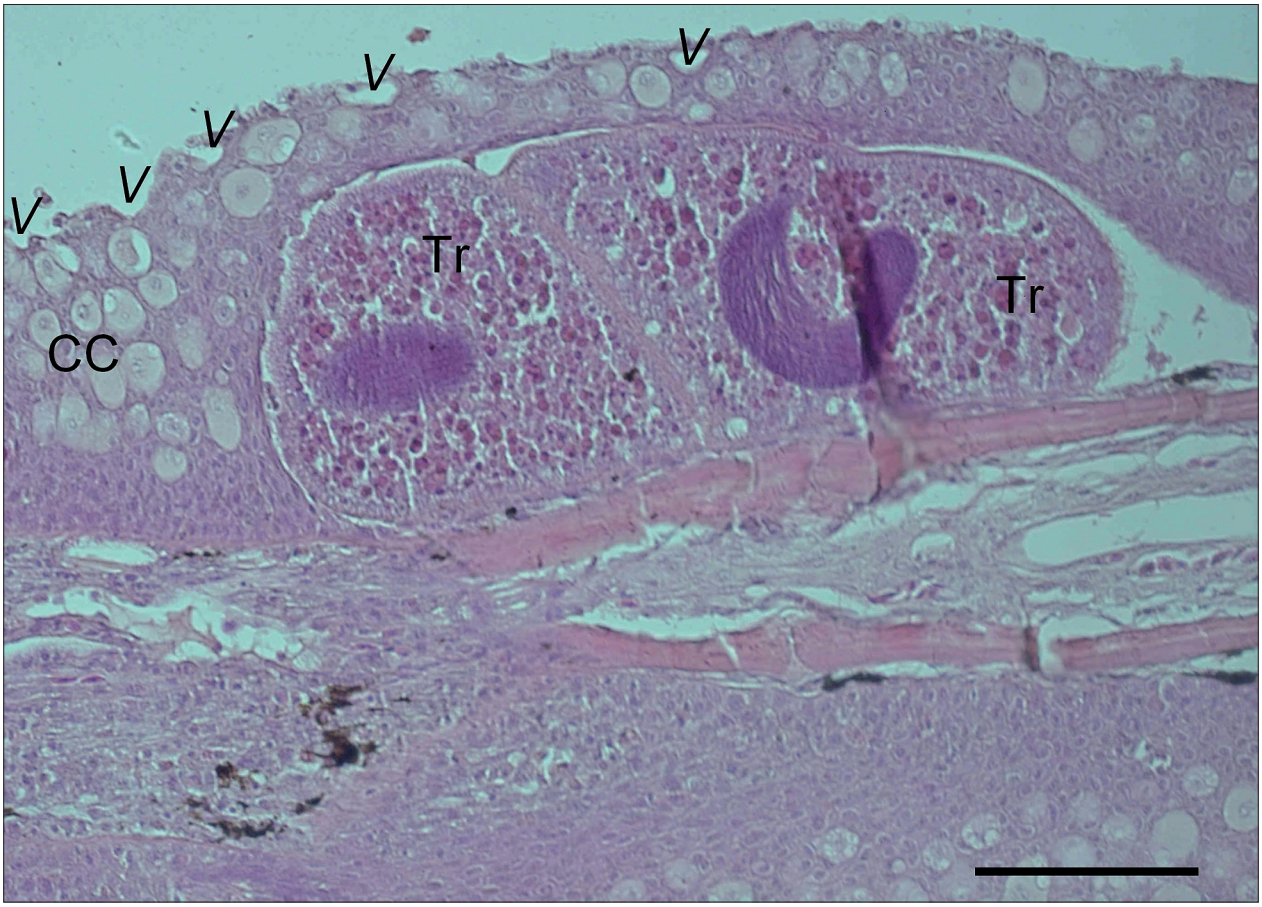

Fig. 2. Skin of *Cyprinus carpio* six days after infection in salt water; E = epidermis, CC = club cells, Tr = trophonts of *Ichthyophthirius multifiliis*, V = opening of club cell; H&E staining; scale bar = 150 µm

day 6 ($P > 0.05$). Significant differences in epidermal club cell densities and epidermis thicknesses between group 1 and group 2 were found at the day 11 after infection ($P < 0.001$).

Epidermal club cell densities, mucous cell densities and epidermis thickness were not significantly different between group 1 (salt water; SW) and group 2 (fresh water; FW) in days 1–6 ($P > 0.05$). Significant differences in epidermal club cell densities and epidermis thicknesses between SW and FW were found on day 11 post infection ($P < 0.001$).

DISCUSSION

In contrary to a previous study (James et al. 2009) this experiment has shown that invasion of *Ichthyophthirius multifiliis* parasites activated epidermal club cells. It certainly needs clarification whether the above inconsistency is related to the fish skin condition, insufficient acclimatisation time, the influence of selective breeding, parasite specific host life cycle, or invasion intensity. These results are in accord with the hypothesis that club cells of common carp are a part of an integrated response to the parasitic damage of host epidermis (Smith 1992, Chivers et al. 2007, Halbgewachs et al. 2009).

We found that epidermal club cells in carp are a component of the epithelial/mucosal barrier, becoming activated after increased mucus production in damaged epithelium caused by invading ciliates. Mucous cell exhaustion with a reduced number of active cells is often seen as a response to injury (Ottesen et al. 2010, Ozerov et al. 2010). Contrarily, club cells respond to parasite injuries with an increase in cell size and density. It is clear that the high density of club cells in the epidermis compensates for an overall low density or absence of mucous cells (Figs. 3, 4).

Club- and mucous cells are integrated into epidermal cell line physical protection mechanisms in the epidermis. The reinvading parasites did not diminish the activity of club cells and previously activated club cells did not diminish the activity of newly invading ciliates in the group 2 fish. The proliferation of club cells indicates that club cells do not provide primary protection against ciliates in naïve fish, nor do they inhibit the growth of the parasite. Even though the "helping" mechanical pressure

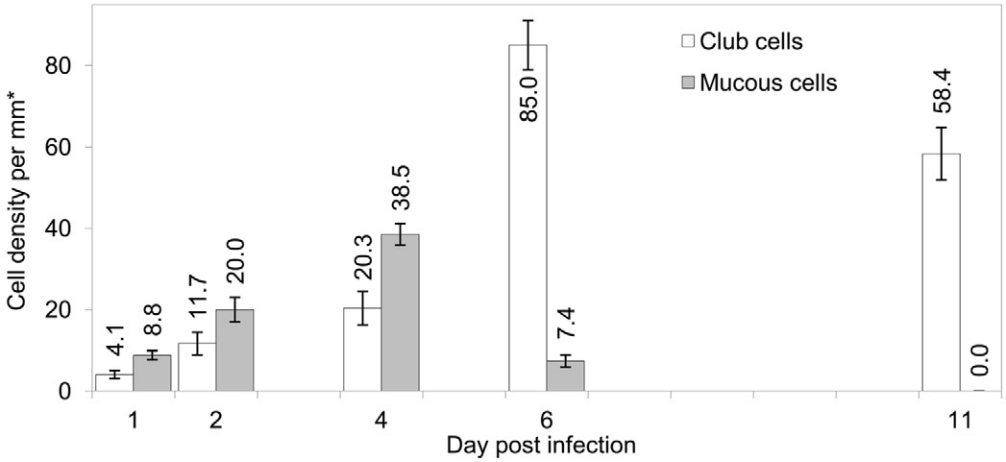

Fig. 3. Densities of club- and mucous cells (mean ± standard deviation) in the epidermis of *Cyprinus carpio* in salt water days 1–11 post infection with *Ichthyophthirius multifiliis*; *determined on 1-mm long transect covering the entire thickness of the epidermis

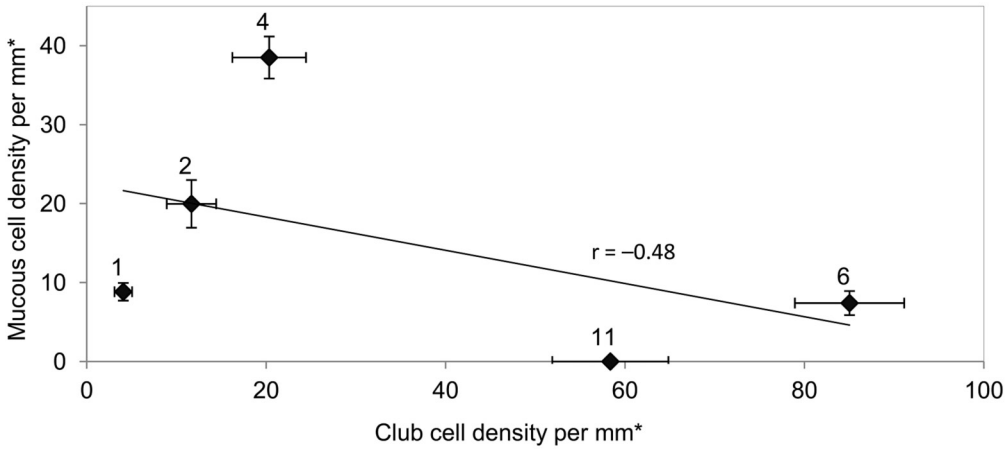

Fig. 4. Correlation on cell densities between club- and mucous cells of *Cyprinus carpio* (mean ± standard deviation) in sequential days post infection with *Ichthyophthirius multifiliis* (corresponding day shown above the marker); *determined on 1-mm long transect covering the entire thickness of the epidermis

of these cells at the beginning of the free-living stage is not excluded (Fig. 2). As pointed out by Selosse and Rowland (1990) salt may act as a general therapeutic agent by promoting mucus production and the healing of damaged skin, and by having beneficial osmoregulatory and anaesthetic effects on the infected and stressed fish. It was found that the growth of ciliates in epidermis was diminished in group 1 maybe by salt water together with a high proliferation of club cells, not because of the activity of club cells alone (see SW day 6 in Table 1).

However, differentiation and activation of club cells associated with mechanical and proteolytic damage by ciliates are like replacements of damaged epithelial cells. At the same time it is hardly concluded that the main function of club cells is phagocytic removal of cell debris, which occurs in skin during tissue damage but see Iger et al. 1994 and Abraham et al. 2001. In contrast, a study by Åsbakk (2001) has shown that Malpighian cells are capable of engulfing foreign material, and thus may function as scavenger cells of Atlantic salmon, *Salmo salar* L.

Earlier works suggested that club cells are usually located in the middle of the epidermis (Iger and Abraham 1990), and that they do not communicate with the epidermal surface (Nakamura et al. 2001, Halačka et al. 2010) or communicate only when the epidermis has been ruptured by predation (Smith 1992). In this study, club cells on the surface of the epidermis released a viscous "secretion" in fishes belonging to groups 1 and 2 from, the fourth to the eleventh experimental days (Figs. 1, 2). The same phenomena were seen in study by Päkk et al. (2011).

There are still no data about the chemical content of club cells (Chivers et al. 2007, Ferrari et al. 2010). The aggregates of mature club cells around the ciliate may be water-specific analogues of pus in terrestrial vertebrates. The contents of these cells may be components of secretory mucosal immunity in the skin of Cypriniformes. Hines and Spira (1974) have hypothesized that passive immunity might be mediated through mucus released from immunized fish into the water. This may occur every time following activation of club cells, because the epithelium of carp skin and mature club cells is indicated by intensive staining and expression of polymeric immunoglobulin receptor (pIgR) around the nuclei (Rombout et al. 2008). These molecular and morphological observations imply that the function of pIgR (a key component of the mucosal immune system that bridges the evolution of innate and adaptive immune defence) may have preceded the emergence of IgA antibodies (Rombout et al. 2008) during evolution. Moreover, club cells may play some role in antibody activity and in passive immunization of naïve fish during/after healing. In this case there may be a role for innate components of the immune system in the development of the olfactory system and the evolution of innate responses to chemical alarm cues released by damaged epithelial tissue.

The genesis of club cells seems to be an evolutionary adaptation for living in muddy/standing waters which contain more potentially harmful substances compared to clean waters. Pathogens are ubiquitous in aquatic habitats and make for a compelling agent of selection for cellular responses in the epidermis (Ferrari et al. 2010). Parasite-host interactions, together with surrounding effects on the organisms, have influenced the evolution of morphological adaptations required for effective defence against pathogens and/or a damaging environment. Cypriniform fishes are mainly prey-species, whose survival depends on healing and protection mechanisms of skin epidermis during post-predation and post-infection recovery. But club cells are not unique only among the Ostariophysi. Fishes in the Percidae (Acanthopterygii) also possess club cells with similar histological properties as club cells in the Ostariophysi (see Smith 1992, Wisenden et al. 2004, Chivers et al. 2007).

It is suggested that club cells do not have an anti-parasite function *per se*. The activation of club cells in carp is induced by skin damage caused by ciliates.

Integrated research on mucosal immune mechanisms, together with studies on the role of evaluation of epidermal barriers in the superorder Ostariophysi, and others, as well as studies on epidermal tissue responses on products released by club cells (alarm substance cells) should be carried out in the future.

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