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Club cells active role in epidermal regeneration after skin hyperplasia of koi carp *Cyprinus carpio*

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Abstract. Carp pox is a flat epidermal hyperplasia affecting common carp (*Cyprinus carpio* Linnaeus, 1758) and its ornamental form koi. The seasonal nature of this disease has been noted by many researchers. The aim of the study was to elucidate processes involved in epidermal hyperplasia regeneration of koi carp which occurs during a steady temperature increase. In the study 14 immature male koi carps, divided into 7 affected and 7 healthy fish, with the body weight of 130–450g were observed. The koi carps were raised in cages with the water temperature of 9ºC which was raised up to 18.5 ºC. Fish health condition and alterations of cellular structure during epidermal regeneration were studied by the means of visual observation, routine histology and histochemistry, morphometric analysis during 78 days. Erosion and desquamation, beginning from the line of new epidermis (newly differentiated mucous cell line), was noted at the final phase of regression of epidermal hyperplasia. Our investigations showed clear interrelation between epidermal regeneration and the number of club cells.
Key words: carp pox, club cells, epidermal hyperplasia, koi carp.

Introduction. In contrast to higher vertebrates, the outermost epithelia of fish skin is metabolically very active living tissue (Noga 2000a) which is regularly remodelled, retaining balance between proliferation and differentiation (Rakers et al 2010) but the mechanisms controlling growth, differentiation, and maintenance of the fish epidermis are poorly known (Webb et al 2008). Epidermis of Cyprinids consists of about 90–140 µm thick stratified layer, composed mainly of epithelial cells, mucous cells and club cells (Iger & Abraham 1990), migrating leucocytes, macrophages and lymphocytes (Ferguson 2006; Genten et al 2009). The epithelial cells extensive hyperplasia (pathological proliferation of the epithelial cells), called historically fish pox or carp pox or candle wax disease has been studied in ornamental varieties and common carp (*Cyprinus carpio* Linnaeus, 1758) and in other fish species (Hoole et al 2001; Kortet et al 2002; Dixon 2008). Epidermal hyperplasia is a common phenomenon in fish and is caused by a wide variety of agents (Noga 2000b; Korkea-Aho et al 2008). These lesions are discrete expansive growths of altered epidermis that tend to obliterate the other mucous cells and club cells by displacement or necrosis (Ferguson 2006). In Nordic climate, the disease in fish occurs during colder winter period where temperatures fall below 14^{o}C, but in the summer (in warm water) the lesions reduce both in number and severity (Morita & Sano 1990; Lu et al 2009) during the 2 months (McAllister et al 1985). Healing and regeneration of tissues involves complex processes of physiological factors, immune components (Medzhitov 2008) and transformations in epidermal cellular structure. However, in warm water the infiltrates perhaps activate the regeneration processes in epidermis (Morita & Sano 1990) but information about alterations in these regressed "hyperplastic plaques" was absent. At the same time as pointed out by Hoole et al (2001), the normal epidermis usually regenerates under the lesion.

The objective of this case study is to examine interrelation between epidermal regeneration and the alterations of cellular structure at final phase of hyperplasia healing in warm water.

Material and Methods

In autumn, when water temperature decreased to 9°C, the specific pox symptoms were detected on fins and body of koi carps breed in cages in a pond in South Estonia. Stocking density of fish in the period was 1 kg /m^3. The pox lesions were palpable and visible, rosy in colour, gel-like in consistency (Fig. 1). The large waxy coatings covered body and both sides of the caudal fin. A number of 14 affected (7 fish) and unaffected (7 control fish) immature male koi carps (sex ratio was determinated after euthanasia by autopsy) with the body weight of 130–450 g where put into a 700 litre plastic tank filled with tap water. During 13 days of acclimatization in laboratory conditions water temperature was raised up to 18.5 ± 1.3°C (imitating the ponds warmth regime situations in spring).

During study the fish where fed *ad libitum* with commercial koi feed (Danafeed DAN-EX 0333). Water was continuously changed (about 25 % per week), aerated and well filtrated.

Scarceness of experimental material made us to design our study in the way which allows observing the final phase of regression (lesions reduce during the 2 months; McAllister et al 1985). To study the cellular structure of caudal fins skin comparatively in unaffected and affected (U & A) fish, samples for light microscopy were taken at days 1 (2U+2A fish), 62 (3U+3A fish) and 78 (2U+2A fish). For sampling, fish were netted from the tank into 60 L of aquarium water with 0.1 gL^{-1} of tricaine methanesulphonate (MS-222) buffered with 0.4 gL^{-1} $NaHCO_3$. After 2 min, the fish became anaesthetized and were then euthanized by decapitation. During the study were taken 6 samples per fish (3 from damaged areas and 3 from not-damaged areas of affected fish and 6 from unaffected fish for longitudinally sections). Longitudinally sections illustrate better the all alterations in hyperplastic areas from proximal to distal.

Routine histology. For light microscopy, samples were fixed in 10% neutral buffered formalin, dehydrated in ethanol, embedded carefully in paraffin. Sections with 5 µm in thickness were stained with haematoxylin and eosin (H&E) and/or periodic acid-Schiff (PAS). Sections were examined with a Zeiss Axioplan 2 (Germany) microscope and photographed using a digital camera AxioCam HRc (Germany).

Morphometric analysis. Morphometric analysis of the epidermis was performed on H&E and PAS-stained sections using the computer program Image J 1.42. Epidermal thickness was measured from the basement membrane to the outer surface of the epithelium (Wisenden & Smith 1997).

Results

During the skin regeneration in warm water the lesions continuously decreased and changed from rosy to milky white in colour and changed partially transparent (capillary's radiating trough the plaques) (Fig. 2). No bacterial or ectoparasite damages of skin were detected during study.

Microscopic data of cellular types of epidermis of infected, healed and unaffected fish were referenced in Table 1.

Day-1. At the beginning of the experiment the epidermal hyperplasias were located in different areas on both sides of caudal fins. They were pink, 7–39 mm in diameter and raised for 0.36–1.4 mm from skin surface. Extensive hyperplasia of epithelial cells and absence of club and mucous cells was observed in epidermis tissue. The epithelial cells on the epidermal surface were flattened. Significant inflammatory infiltrates were absent (Fig. 3).

Figure 1. Epidermal hyperplasia in caudal fin of koi carp *Cyprinus carpio* (first day of study).

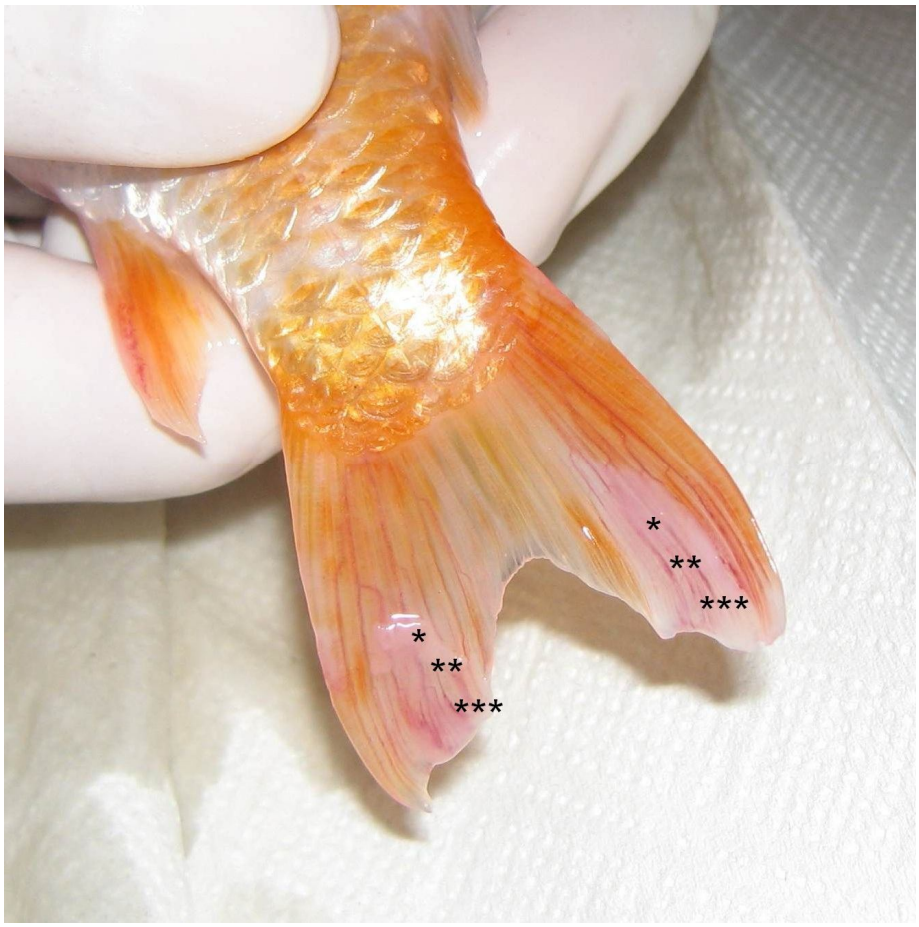

Figure 2. Regressed epidermal hyperplasia in warm water in caudal fin of koi carp *Cyprinus carpio* 62 days after rising water temperature (* Part of hyperplastic areas; ** Part of areas after desquamation of surface; *** Part of regenerating areas).

Table 1

Microscopic data of cellular types of epidermis (per 1 mm of length and total thickness) and epidermal thickness in normal, affected and healed koi carps caudal fins

Number of cells	Normal epidermis (control group)	Hyper-plastic epidermis (day 1)	Regene-rating epidermis (*) (day 62)	Regene-rating epidermis (**) (day 62)	Regene-rating epidermis (***) (day 62)	Healed epidermis (day 78)
Club cells (per mm of length and total depth)	8 ± 3	-	825 ± 330	118 ± 8	9 ± 3	9 ± 2
Mucous cells (per mm of length and total depth)	29 ± 3	-	28 ± 6	63 ± 5	29 ± 6	29 ± 4
Epidermal thickness (µm)	96 ± 6	850 ± 550	770 ± 440	98 ± 9	94 ± 9	88 ± 16

(*) – Part of hyperplastic areas
(**) – Part of post-hyperplastic areas after desquamation of surface
(***) - Part of regenerating areas

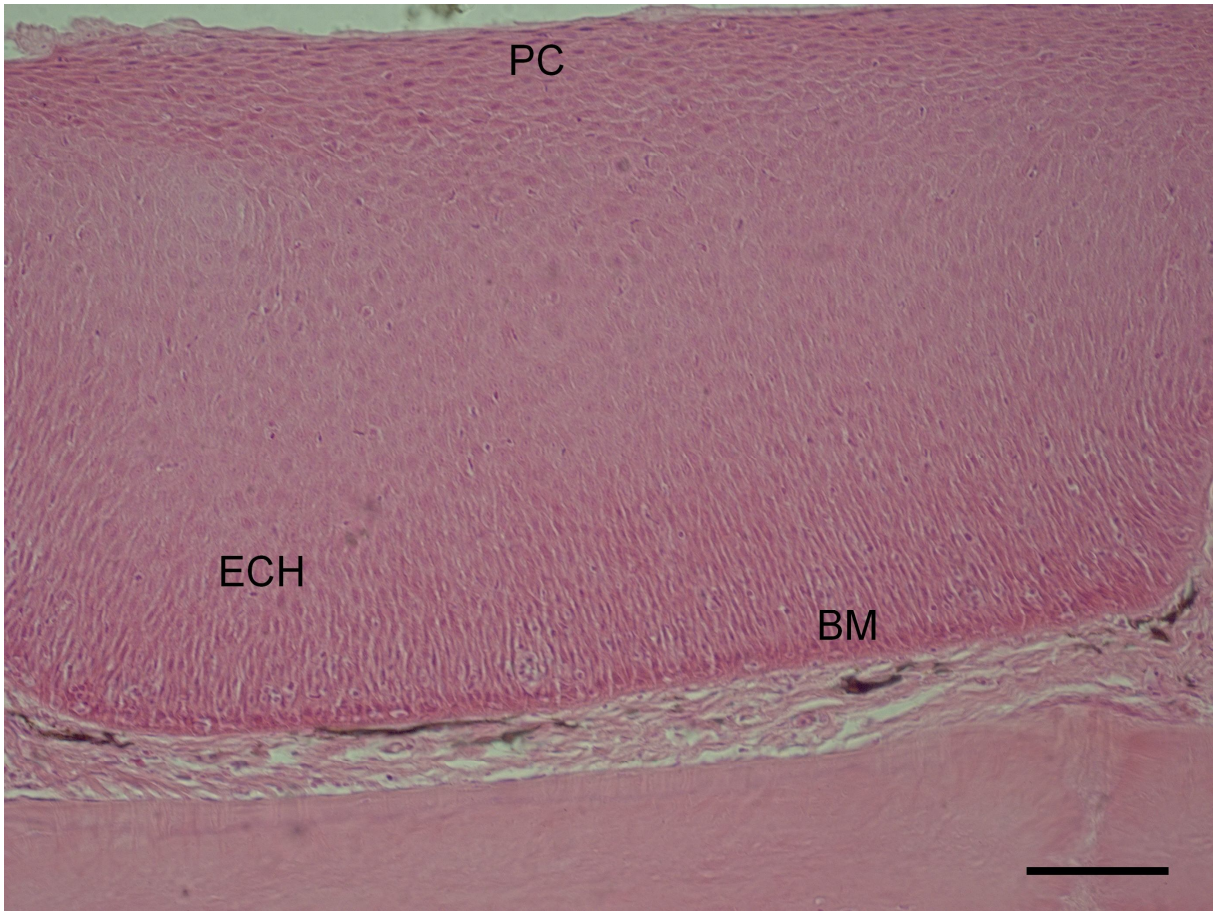

Figure 3. Extensive hyperplasia of epithelial cells (EC) in caudal fin of koi carp *Cyprinus carpio*. Note the absence of club cells and mucous cells. Epithelial cells are smaller in size and tightly packed (PC). The basal layer (BM) forms pegs and is slightly scalloping. PC- epithelial cells. First day of study. Bar = 50 µm (H&E).

Day-62. Microscopical examination of three affected fish biopsies from epidermal growths (thickness 770 ± 440 µm) area showed proliferation of eosinophilic large pale club cells. Club cells (max. diameter 38µm) have pink cytoplasm that is frequently scalloped at the edges and have central nuclei.

Mature club cells density was extremely high on both sides of the fins skin in all affected fish and varied in different locations of regressing hyperplasias (825 ± 330 club cells were present per 1 mm of length and total thickness of sectioned epidermis) (Fig. 4). Loss of epithelial integrity and evacuation of decomposed club cells from top layer and sloughing of surface was seen in distal parts of hyperplasias. Desquamation of hyperplastic areas was observed in line at the distance of 105 (± 5) µm from basal layer. Mature mucous cells were present only inside of lesions at distance 32 (± 4) –110 (± 13) µm from basal layer (32µm in middle and 110µm in distal areas of lesions tissue).

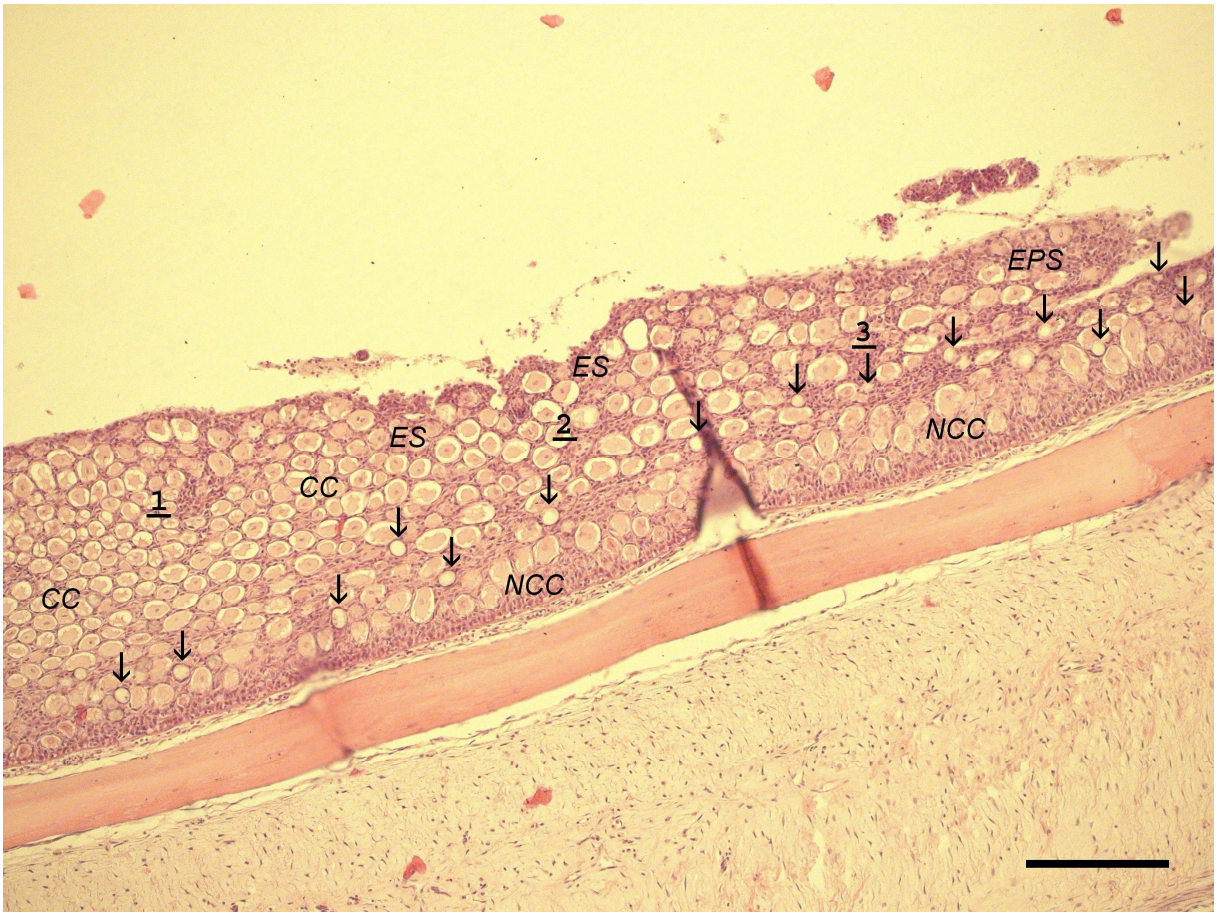

Figure 4. Epidermal hyperplasia medial area of caudal fin of koi carp *Cyprinus carpio* 62 days after rising water temperature. Loss of epithelial integrity (ES) and eventual desquamation (EPS): 1. - section of hyperplasia with high proliferation of eosinophilic enlarged club cells (CC) and undamaged surface; 2. – section of CC hyperplasia with erosion of surface (ES) and the evacuation of club cells; 3. - section of CC hyperplasia with erosion and peeling of surface (EPS) beginning from the line of newly differentiated mature mucous cells (arrows) and new club cells (NCC). Bar = 100 µm (H&E).

In epidermis areas after desquamation of surface a large number of mucous cells in outer part and high secretory activity of superficial layer epithelial cells was observed. Severe new mucous cell differentiation was seen beneath of club cells proliferation layer up the basement membrane. At the mean 118(± 8) club cells as well as 63(± 5) mucous cells counted per 1 mm of length and total thickness of sectioned epidermis (Fig.5).

In epidermal tissue from earlier peeled areas to apex of fins, normal density of epithelial cells could be detected (mucous 21(± 6) and club cells 9(± 3) per 1 mm of length and total thickness).

Day-78. Examination of affected two kois 78 days after start of the rising temperature study showed absence of neoplasm on both sides of the caudal fins. Epidermal thickness was measured as 88 (± 16) µm-s, 18(± 4) mucous cells and 9 (± 2) mature club cells were noted per 1mm of length and total thickness.

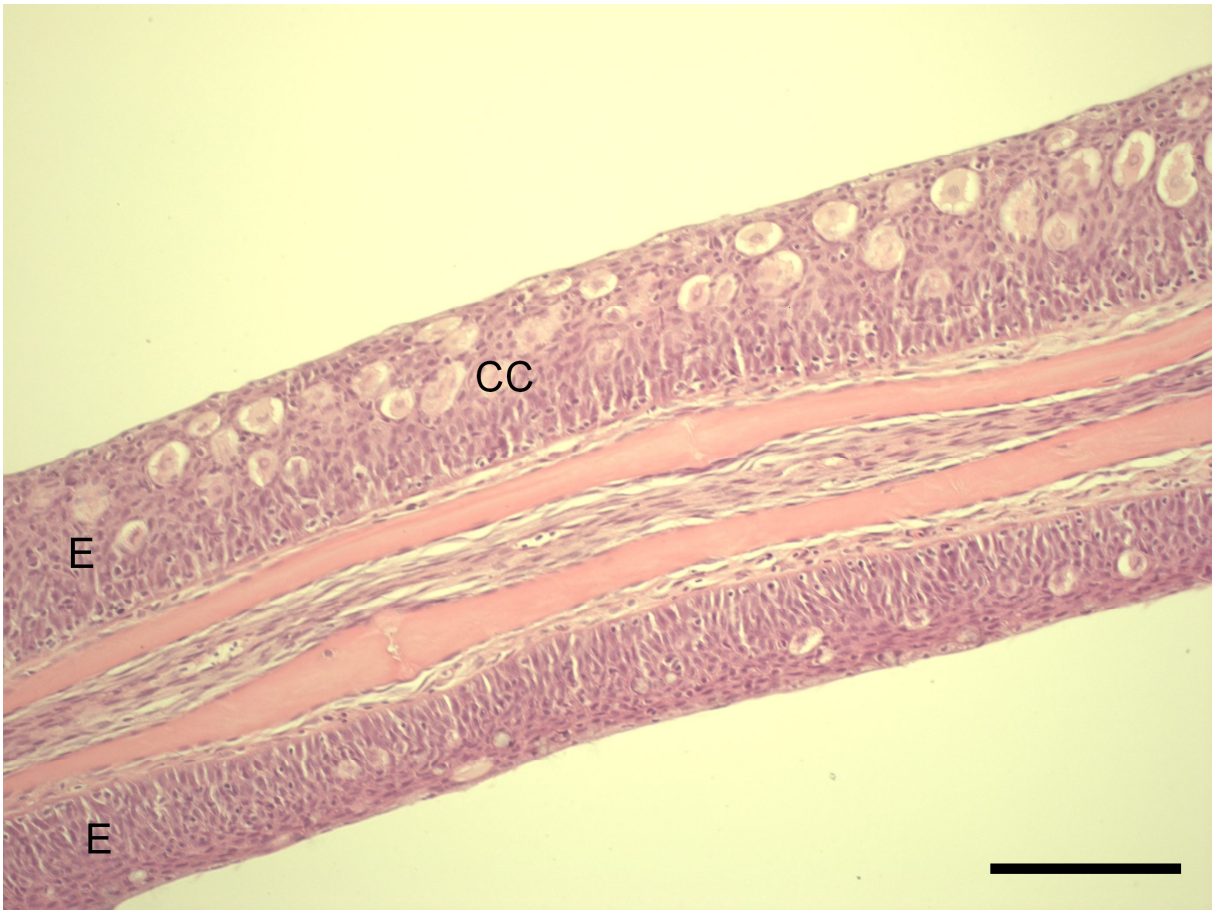

Figure 5. Epidermal hyperplasia area after desquamation of surface of caudal fin of koi carp *Cyprinus carpio* 62 days after rising water temperature. E- Epidermis. CC- Club cells. Bar = 100µm (H&E).

Control group. In sections of caudal fins of the samples of fish of control group (unaffected fish) or in undamaged areas of affected fish the epidermal outer layer contains stratified squamous epithelial cells and mucous cells, with underlying layers usually consisting of cubical epithelial, active mucous and mature club cells (Table 1). The long time treatment had no effect on body condition, club and mucous cell density or epidermal thickness of skin in unaffected fish during study.

Discussion

The final phase regression of epidermal hyperplasia takes place through proliferation of club cells and it is followed by desquamation of cells from growths surface (Fig. 4).

Fluctuating number of club cells is may caused by different epidermal thickness above and between fin rays. However by our understanding the difference in normal epidermal thickness (96 ± 6µm) does not very influence the general high number (825 ± 330) of club cells in longitudinally sectioned hyperplastic (thickness 770 ± 440µm) lesions. Anyway the activation of club cells in regressed hyperplastic tissue is important information because the function of these cells has been historically correlated to chemical alarm signalling as "alarm substance cells" (James et al 2009; Genten et al 2009).

The high proliferation of club cells in hyperplastic epidermis and desquamation of these cells containing areas from upper part of "new" but not stabile epidermis (from line of newly differentiated mucous cells) noticed in our studies may indicate that the club cells play any role in healing damaged epithelial tissue and the club and mucous cells have integrated into function of re-establishing the normal structure of epidermis. In our cases, severe mucous cell hyperplastic area as described by Ottesen et al (2010) in Atlantic halibut (*Hippoglossus hippoglossus* Linnaeus, 1758) was not noted in koi carp but

high cell turnover and mucous cells secretory activity were identified after desquamation in epidermis. Mucous cell exhaustion with reduced numbers of active cells is often seen as a response to injuries (Buchmann et al 2004; Ozerov et al 2010). Still, high numbers of active mucous cells after desquamation in our samples indicate to the active protection against environmental factors by the healed epithelium. At the same time the club cells proliferation under mucous cells line indicate clearly that the high density of club cells compensates an overall low density of mucous cells inside epidermis as an adaptation for an effective healing and/or protective mechanisms.

Conclusions. Our results demonstrate the potential role of club cells in healing process during and after hyperplasia in koi carp but for better understanding of club and mucous cells integrated functions further studies should conduct more extensive sampling from all parts of the freshwater Cypriniformes fish.

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