Eesti Maaülikool
Estonian University of Life Sciences

ALTERATIONS OF EPIDERMAL CELLS` FUNCTIONAL ACTIVITY IN FISH DUE TO INFECTION

NAKKUSTE POOLT PÕHJUSTATUD EPIDERMISE RAKKUDE TALITUSLIKU AKTIIVSUSE MUUTUSED KALADEL

PRIIT PÄKK

A Thesis
for applying for the degree of Doctor of Philosophy in Hydrobiology

Väitekiri
filosoofiadoktori kraadi taotlemiseks hüdrobioloogia erialal

Tartu 2012

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The English in the current thesis was revised by David Arney and the Estonian by Jaagup Alaots.

European Union
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To my wife Margit.

CONTENTS

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original articles, referred to in the text by the Roman numerals I to III:

I.   **Päkk P.,** Hussar P., Järveots T., Paaver T. (2011). Club cells active role in epidermal regeneration after skin hyperplasia of koi carp *Cyprinus carpio*. *AACL Bioflux*, **4** (4), 455–462.

II.   **Päkk, P.,** Hussar, P., Paaver, T. (2011). Alterations of club cells activity in epidermis of common carp, *Cyprinus carpio*, due to infection by *Ichthyophthirius multifiliis*. *Acta Ichthyologica et Piscatoria*, **41** (3), 185–192. DOI: 10.3750/AIP2011.41.3.06

III.   Ozerov M.Y., Lumme J., **Päkk P.,** Ristamäki P., Zietara M.S., Barskaya Y.,   Lebedeva D., Saadre E., Gross R., Primmer C.R., Vasemägi A. (2010). High *Gyrodactylus salaris* infection rate in triploid Atlantic salmon *Salmo salar*. *Diseases of Aquatic Organisms*, **91** (2), 129–136. DOI: 10.3354/dao02242

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Priit Päkk`s contribution to each article (%):

Contribution	I	II	III
Original idea and structure of papers	100	100	5
Data collection	100	100	30
Data analysis, statistics	80	80	10
Writing	90	80	5

ABBREVIATIONS

ACC   alarm substance cells
BM   basement membrane
CC   club cells
CyHV-1   *Cyprinide herpesvirus* 1
D   dermis
E   epidermis
ECC   epidermal club cells
ECH   epithelial cells hyperplasia
epid-s   epidermis
EPS   erosion and peeling of surface
ES   erosion of surface
H&E   haematoxylin and eosin
ICH   ichthyophthiriosis
IgA   immunoglobulin A
IL-1   interleukin-1
M   muscle layer
MC   mucous cells
Me   melanophore
MS-222   tricaine methanesulphonate
NCC   new club cells
PAS   Periodic-Acid-Schiff reaction
PC   epithelial cells
pIgR   polymeric immunoglobulin receptor
SAS   Wilcoxon test system
Sc   dermal scale
Tr   trophont of *Ichthyophthirius multifiliis*
U&A   unaffected and affected fish
UV   ultraviolet radiation

1. INTRODUCTION

The mucosal surface of the skin, gills and intestinal tract is a very delicate and fragile barrier between the fish and its environment (Ottesen *et al.,* 2010). Fish skin is a multi-purpose tissue that serves numerous vital functions including chemical and physical protection, sensory activity, behavioural purposes or hormone metabolism. Further, it is an important first-line defence system against pathogens, as fish are continuously exposed to multiple microbial challenges in their aquatic habitat (Rakers *et al.,* 2010). Pathogens are ubiquitous in aquatic habitats, as anywhere, and make for a compelling agent of selection for cellular responses in the skin (Ferrari *et al.,* 2010). Because of the intimate contact of fish with the environment, cutaneous diseases are relatively more common in fish than in terrestrial vertebrates and are one of the primary disease conditions presented to the aquatic animal practitioner.

The epidermis of fish comprises only living cells, in contrast to that of terrestrial vertebrates which are covered by an outer layer of dead cells (Saadatfar, 2010). The epidermis of fish is regularly remodeled, retaining a strict balance between proliferation and differentiation. To participate in tissue regeneration and repair, stem cells need to be multipotent and, in the case of fish epidermal stem cells, be able to differentiate into epithelial cells, mucous cells, club cells or sensory cells (Rakers *et al.,* 2010). Activated by wounding, they start to proliferate and to regenerate the tissue (Whitehead *et al.,* 2005).

In fish, the epidermis represents an initial site for complex immune responses against waterborne pathogens (Whitear, 1986; Kearn, 1999). Immunological components, such as lymphocytes, macrophages, and several types of granulocytes found in fish skin, have been shown to reduce the infectivity, growth, and reproduction of various parasites at the site of infection (Shephard, 1994). Another cellular component of fish epidermis that has been linked to host immunity comprises the club cells of fishes in the superorder *Ostariophysi* (Chivers *et al.,* 2007; Halbgewachs *et al.,* 2009; James *et al.,* 2009).

Communication is also an early function that persists, since animals have used the integument as a canvas for message displays (Wu *et al.,* 2004). However, injured fish release a variety of chemicals, some of which may

be food cues in some contexts and alarm cues from injured conspecifics, which are mediated by the olfactory system (Maniak *et al.,* 2000; Døving *et al.,* 2005; Derby *et al.,* 2008). For almost 70 years, researchers have documented that receivers of alarm cues often show adaptive changes in their behaviour, morphology, and life history upon exposure to the cues (Wisenden and Chivers, 2006; Ferrari *et al.,* 2007; Chivers *et al.,* 2008; Halbgewachs *et al.,* 2009), epidermal club cells are the main source of a specific alarm substance acting as a pheromone, as postulated by Pfeiffer (1978). However, the development of club cells has remained poorly understood (Smith, 1997; Henderson *et al.,* 1997; Chivers *et al.,* 2007; Ferrari *et al.,* 2010) and understanding of the evolution of these cells in an alarm context has been difficult and controversial.

Tissue damage in fish occurs due to many events such as handling in aquaculture and physical trauma. Pathology, usually a combination of clinical signs and histopathological features of tissues and organs, is a key diagnostic discipline and is heavily reliant on the experience of the pathologist and the availability of reference materials and other experts for consultation. Epidermal damage not only provides access to infectious agents, but it also produces an osmotic stress that can be life threatening (Noga, 2000a). Healing and regeneration of tissue damage imply complex processes involving both physiological factors and immunological components (Medzhitov, 2008). Epidermis regeneration has a vital function as a defence against the external environment (Quilhac and Sire, 1999; Böckelmann, 2010). In lower vertebrates such as fish, the responses to tissue injury and the following regeneration of tissue on a molecular and cellular basis are poorly understood. However, a few studies have been made on the cyprinid species zebrafish *Danio rerio* and the common carp *Cyprinus carpio* (Lee *et al.,* 2009; Gonzalez *et al.,* 2007). In spite of numerous histological and histochemical studies, some of the functions of the epidermal club and mucous cells in the healing of damaged epidermis remain hypothetical to date. The data obtained from special studies identify the need for additional study regarding epidermal barriers and defence against pathogen attacks to better understand the functions, and functional interactions, of fish epidermal cells important for investigative dermatologists (see review by Rakers *et al.,* 2010).

The aim of this thesis was to elucidate the alterations in fish epidermal cells' activity and the connection between them due to infection. In this study an epidermal 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 the ciliate *Ichthyophthirius multifiliis* should result in an increased number of club and mucous cells, and a subsequent reduction in parasite activity in the epidermis. The objectives of this study were also to examine relationships between epidermal regeneration and the alterations of cellular structure in the final phase of epithelial cell hyperplasia (*carp pox*) healing in warm water, and the response of mucous cells to invasion by the external parasite *Gyrodactylus salaris*.

2. REVIEW OF THE LITERATURE

The aquatic milieu is the oldest of all environments, leaving aquatic life forms a long evolutionary opportunity to innovate and develop novel molecular / developmental mechanisms in their integument and appendages that allow vertebrate animals to live in different ecological environments. The surviving species on this planet provide only a small glimpse into the full spectrum of cutaneous phenotypes that must have been generated throughout evolution (Rakers *et al.*, 2010).

2.1. Evolution of fish skin

The last common ancestor of fish and mammals is probably more than 350 million year old if one considers this to be the lungfish, and more than 420 million years in the case of the zebrafish. Despite this enormous temporal divide in evolution, fish skin shows architectural similarities with that of mammals, even though it follows specific construction principles that are perfectly adapted to aquatic life: similar to the epidermis of terrestrial vertebrates such as mice or man, the fish epidermis is a multilayered tissue that is separated from the dermis by a distinct basement membrane (Henrickson and Matoltsy, 1968). It is, however, generally a „mucous" and not a „keratinized" system (Figure 1). This evolutionary achievement is linked with important features of fish skin: a stratified mucogenic epidermis and alpha-keratogenic potential. In striking contrast to mammals, a cornified cellular envelope is restricted to specific body regions (e.g. the barbel) or found only in some teleosts such as, e.g. *Periophthalmus* or the *Syngnathidae* (sea horses), which have some „cornified" adaptations. This is because the epidermis of fish usually does not contain proteins connected with interkeratin matrices and corneous cell envelope formations (filaggrin, loricrin). These intermediate filaments are the basis for the stabilization of mammalian epidermal cells, forming a largely water-impermeable apical layer. Instead, fish epidermis is covered by a layer of mucus, a slimy layer of glycoproteins that is heavily enriched with antimicrobial factors, including antibodies, complement, lysozyme, C-reactive protein, lectins, proteases, transferrin and polypeptide antibiotics (Noga, 2000a).
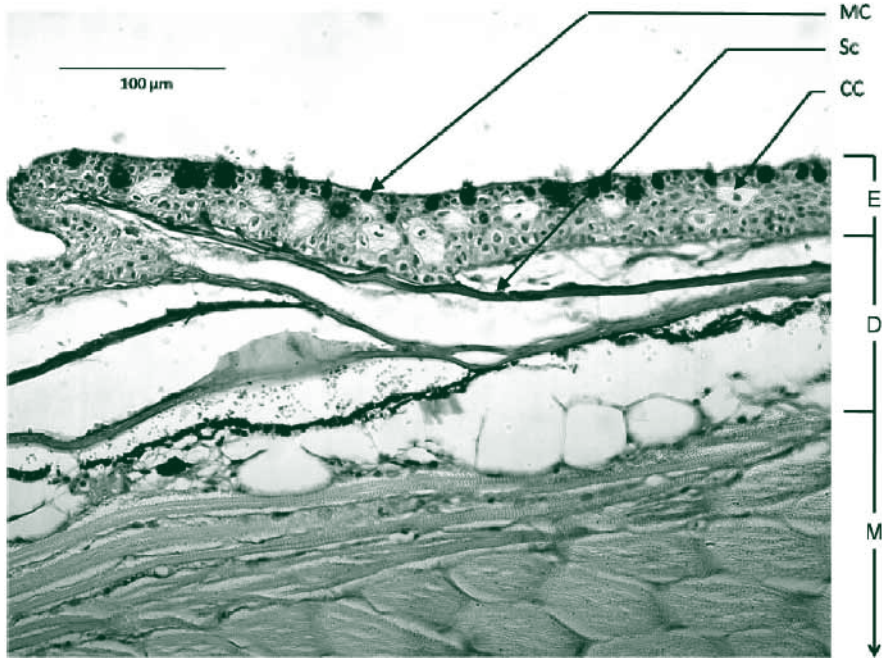
Figure 1. A cross section of fathead minnow skin showing the epidermal (E), dermal (D), and muscle (M) layers. Club cells (CC), mucous cells (MC), and dermal scales (Sc) are indicated with arrows. This section was stained with periodic acid-Schiff`s reagent and then counterstained with haematoxylin. Club cells are PAS negative appearwhite with dark central nuclei, while mucous cells are generally PAS positive and appear dark. Photo by R. Pollock (2011).

2.2. Skin construction/functional principles

The skin of all fish species, as that of any other vertebrate, consists of three basic layers: an outer, the epidermis, and inner, the dermis and hypodermis. The entire outer surface of a fish, including the body and fins, is completely covered by the epidermis. Beneath its protective mucus, which is also very important against hydrodynamic drag, cell-junctions of the stratified squamous superficial epithelial cells, also called filament cells or Malpighian cells, provide the only epithelial coherence. Investigating the functions of tight junctions in fish skin could be directly relevant for investigative dermatologists, because the role of tight junctions, which are expressed not only by keratinocytes but also by epidermal Langerhans cells, is of interest in human epidermal biology (Proksch *et al.,* 2008). In fish, this boundary is separated by mucus-secreting cells. All of these cells are part of the *stratum superficiale*; the outermost of the three epidermal

strata followed medially by the *stratum spinosum* with differentiated cells and proximally by the *stratum basale* with basal cells and the basement membrane. The thickness of these epidermal structures is often bound to ecological factors, to seasonal variances or to variances between males and females. Undifferentiated epidermal progenitor cells emerging from the basal layer are induced to proliferate and differentiate in the *stratum spinosum* when needed, and are subsequently recruited to the outermost epidermal layer. Depending upon the fish species, fish age, location on the body, epidermis thickness and number of epidermal layers, various specialized cells, including goblet cells, sensory cells, club cells and chloride cells, may be present in the epidermis (Whitear, 1986). Fish epidermis is separated from the underlying dermis by a layer of filamentous proteins, which form the basement membrane.

The adjacent dermis is composed of the *stratum laxum* and the *stratum compactum* and, in striking contrast to mammalian skin, is separated from the hypodermal adipogenic tissue by yet another endothelial layer, called the dermal endothelium (Whitear *et al.*,1986). The thin *stratum laxum* of fish dermis consists of loosely arranged connective tissue, complemented with blood vessels and nerve fibres. Dermal cells are mostly fibroblasts, interspersed with different chromatophores. Scale-building cells, the scleroblasts of fish skin, are arranged in the scale pockets. The most frequent scale type in teleosts is the elasmoid scale, which consists of a plate of collagenous tissue, with superficial mineralization, surrounded by scleroblasts and fibroblasts (Whitear, 1986). On the lower side, the scale pocket is lined by modified fibrocytes with desmosomes and caveolae. Bundles of collagen fibres anchor the scale in its pocket. The posterior edge of the scale is more or less covered by the epidermis, depending on the species and their biology. Regions of derma-epidermal interactions in fish skin may be present as has been reported for reptilians (Alibard, 2004). Here, morphoregulatory molecules are exchanged, and may then have a significant influence on the structural composition of the epidermis and dermis. In mammals, small derma-epidermal connected regions migrate into the dermis and form dermal papillae and then hairs (Alibard, 2004; Rakers *et al.,* 2010).

2.3. Fish epidermis

The composition of the three aforementioned epidermal strata is invariably the same in all teleost fishes (Figure 2). The *stratum superficiale* acts as a seal between the animal and its surroundings. Its surface is ornamented with species-specific microridges (Sire and Akimenko, 2004), which are raised, actin-rich structures, that serve to maintain the mucous layer on the surface of the fish (Webb and Kimelman, 2005). The cells of the *stratum superficiale* are stabilized by microfilaments such as 70A a-keratin. While this type of keratin is abundantly found in all fish epitheliocytes, ß-keratin is absent. In almost all fish species, the epidermis does not have a dead, keratinized surface as in terrestrial vertebrates, but consists entirely of living cells (Meyer *et al.,* 2007). As in mammals, fish skin is metabolically very active. Immediately following an injury, cells from the margins close the wound by secreting mucus, which transports lymphocytes into the damaged area (Quilhac and Sire, 1999).

The mucus is produced by mucous cells of the *stratum spinosum* (Figure 1). Their structure is similar to that of mammalian goblet cells (Harris and Hunt, 1975). Mucous cells die when they release their glycoconjugates; hence there is a continuous turnover in the outer layers of the epidermis. Alongside the mucous cells, club cells and sensory cells are embedded in this layer.

Club cells exhibit enormous metabolic reaction capacities to meet various external influences (Meyer *et al.,* 2007). Chemical substances that are dissolved in water can be detected by chemosensory systems, which allow the recognition of mates, conspecifics, predators or prey; they mediate migration and homing and support reproductive and feeding activities. The products of club cells are not secreted; these cells store proteins and acidophilic substances. They are often species-specific and are also known as „*Schreckstoffzellen"* taking into account the experimentally supported hypothesis that when the skin is damaged by a predator, these alarm pheromones are released into the water and warn conspecifics of a predator's presence (Derby and Sorensen, 2008; Mathis, 2009).
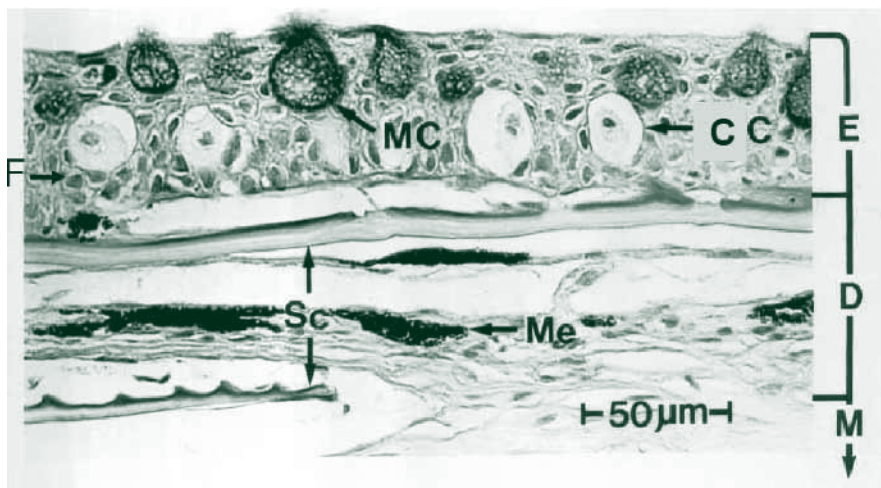
Figure 2. A cross section of fathead minnow skin showing the epidermal (E), dermal (D), and muscle layers. An epithelial (filament) cells, club cells (CC), mucous cells (MC), melanophore (Me), and dermal scale (Sc) are indicated with arrows. PAS. Photo by D.M. Hugie (1990).

However, most epithelial cells in the *stratum basale* remain non-differentiated; this is why this layer may be seen as a stem cell reservoir. Epithelial cells are not continuously peeled off, like the outer epidermal cells of mammals, but only replaced by cells from the *stratum spinosum* upon cell death or injury. Thus, although the fish epidermis does not appear to be constantly renewed, homeostatic mechanisms must be in place to ensure the maintenance of this tissue (Webb and Kimelman, 2005).

2.3.1 Epithelial cells

The surface of undamaged teleost epidermis appears as a barren field of epithelial (Malpighian) cell surfaces (Crouse-Eisnor *et al.,* 1985; Iger et al., 1988). In the literature, other names commonly used for Malpighian cells include keratocytes and filamentous or filament-containing cells (Bullock and Roberts, 1974). Migrating epithelial cells cover wounds rapidly to provide a barrier against opportunistic micro-organisms (Phromsuthirak, 1977; Bullock *et al.,* 1978). Indeed, it is normal in both freshwater and seawater fish for the superficially located epithelial cells to slough off into the water and be replaced by new cells from deeper within the epidermis (Iger *et al.,* 1988; Wendelaar Bonga and van der Meij, 1989; Iger and

Abraham, 1990). This turnover of cells tends to increase with increasing content of bacteria, parasites and other potentially harmful substances in the water (Iger *et al.,* 1988).

Such observations indicate clearly that epithelial cells have a role in the removal of foreign material from fish skin. Åsbakk (2001) shows that these cells are capable of engulfing foreign material and thus may function as scavenger cells, of Atlantic salmon (*Salmo salar*). By exerting this function, the cells may clean the wound surface to pave the way for the undisturbed inward migration of other malpighian cells to cover the wound. By doing so, the cells at the leading migrating front would remove bacteria, and this activity would result subsequently in the rounding up and disintegration or detachment of the cells. Indeed, it is a typical finding in many studies of skin disorders of fish and fish exposed to exogenous stressors (e.g. micro-organisms or heavy metals such as copper, cadmium or lead), that superficial epidermal cells tend to round up. Often this is accompanied by a concomitant loss of intercellular contact, leading eventually to sloughing off of the cells or whole parts of the epidermis (Roberts and Bullock, 1976; Johansson and Svensson, 1982; Iger *et al.,* 1988; Iida *et al.,* 1991; Urawa, 1992; Iger *et al.,* 1994).

2.3.2. Club („*Alarm substance*") cells

Club cells, known as alarm substance cells, are present mainly in *Cypriniformes* (Whitear, 1986). The club cells are large eosinophilic, round, fragile cells positioned near the surface of the epidermis (Figure 2). They lack ducts to the exterior and they do not communicate with the epidermis surface (Wisenden, 2000). Although the function of club cells has never been definitively established, it is considered that the club cells are multifunctional (Zaccone *et al.,* 2001). Two main functions of these cells can be identified: (1) immune activity against ubiquitous environmental challenges and skin penetrating pathogens or parasites (Chivers *et al.,* 2007; James *et al.,* 2009) and (2) behavioural protection, i.e. the alarm substances of club cells are released into the water following their rupture after injury, e.g. by predators (Frisch, 1941; Pfeiffer, 1977; Wisenden *et al.,* 2004; Pollock *et al.,* 2005).

Epidermal club cells that contain the chemical alarm cue known as „*Schrekstoff*'" are a defining characteristic of the ostariophysi (Pfeiffer, 1977). It is now well established that damaged skin serves as a mechanism for risk assessment by fish (Chivers and Smith, 1998). These cells pose a metabolic cost (Wisenden and Smith, 1997), although the mechanism by which individuals that invests in these cells gain fitness benefits is not clear because the cells release their contents only when ruptured by a predator. The lack of obvious fitness benefits to justify metabolic investment in epidermal club cells poses a challenge for evolutionary theory (Williams, 1992). One idea that neatly resolves the evolutionary enigma is the possibility that epidermal club cells contain anti-pathogenic or anti-parasitic agents (Smith, 1992; Magurran *et al.,* 1996). Recently, Chivers *et al.,* (2007) provided several lines of empirical evidence in support of the anti-parasite hypothesis.

Regarding the persistence of epidermal club cells over evolutionary time a recent result suggests that club cells and their contents are not required for skin damage to elicit alarm behaviour (Carreau-Green *et al.,* 2008). This result further supports the idea that club cells are not specialized for the production and release of a pheromone (alarm cue). Rather, these data suggest that club cells serve other function(s) which provide direct benefits to the individual that produces them. This result, if corroborated by future study, would firmly place the „warning substances of minnows" squarely in the realm of chemical information, and not as the enigmatic anomaly it has posed to evolutionary theory for nearly half a century (Williams, 1992; Ferrari *et al.,* 2010).

Recent studies have associated the epidermal club cells with immunological and anti-parasite functions (Chivers *et al.,* 2007; Halbgewachs *et al.,* 2009). It is suggested that the club cells display intense phagocytotic and apoptotic activity. A large number of leucocytes which have penetrated from the dermal region are phagocytosed by the club cells and are continuously expelled from the tissue at the epidermal surface. These cells have a very high proliferation rate; control of the cellular turnover of filament cells and the elimination of leucocytes may represent functions for club cells (Abraham *et al.,* 2001).

2.3.2.1. Club cells antipathogen hypothesis

The antipathogen hypothesis maintains that epidermal club cells play a role in deterring bacterial infection, parasite penetration, and promote general wound healing (Ferrari *et al.,* 2010). Although these ideas have been in the literature for some time, connecting these functions to a mechanism for maintaining epidermal club cells was not described, nor was it the focus, of the original authors (Ferrari *et al.,* 2010). Recent data explicitly testing this hypothesis show that epidermal club cells in percids are more abundant on the dorsal surface than on the lateral and ventral regions, suggesting a link between these cells and exposure to UV radiation (Chivers *et al.,* 2007). Experimental exposure to trematode cercariae and to zoospores of the pathogen fungus *Saprolegnia ferax* and *S. parasitica* stimulate the production of club cells in fathead minnows (Chivers *et al.,* 2007). These cells primarily serve an immune function (Halbgewachs *et al.,* 2009) and have only secondarily acquired a role in mediating predator-prey interactions through selection acting on receivers to exploit chemical information in the environment (Ferrari *et al.,* 2010).

One intriguing possibility for the contrasting results obtained in two studies by James *et al.,* (2009) is that club cell proliferation, and perhaps other forms of epidermal immunity, may be most detectable in hosts exposed to novel pathogens and parasites. Supportive evidence for this hypothesis would include the identification of mechanisms used by specialist parasites such as *Ornithodiplostomum* sp. that migrate through the epidermis without causing direct damage to club cells. Migrating schistostomules of *Schistosoma* spp. navigate between cells in the epidermis of experimentally exposed mice on their way to blood vessels located within the dermis (McKerrow and Slater, 2002). One extension of the novelty hypothesis is that strong and consistent club cell responses may only exist for those parasites or pathogens that remain for extended periods in the epidermis of fish, such as some pathogenic water moulds (e.g. *Saprolegnia*) or those metacercariae that cause „black spot" in the epidermis of their intermediate hosts (Chivers *et al.,* 2007). The data obtained by James *et al.,* (2009) add support to the hypothesis that epidermal club cells of the ostariophysi are part of a complex, generalized response system that confronts pathogens and parasites such as cercariae (especially dead

ones in the case of *Teleorchis*, fungal hyphae *Saprolegnia*), or bacteria in the case of secondary bacterial infection after epidermal damage from mechanical abrasion or exposure to ultraviolet light. Thus, club cells in both ostariophysian and perciformes (perch and darter) fish likely serve this function (Chivers *et al.,* 2007). Taken together, the results by James *et al.,* (2009) are inconsistent with an anti-parasite function for club cells and instead suggest a generic role in response to injury.

2.3.3. Mucous cells

Fishes and other aquatic vertebrates are covered with a mucous epidermis over their entire body surface. Virtually all fish are covered with an integumental mucus secretion that is involved in many aspects of their biology (Daniel, 1981 a, b). Mucous, or goblet, cells open into small pores between the outer epidermal cells (Figure 2). Mucous cells are mainly visible in the apical half of the epidermis; they are PAS positive, indicating the presence of mucopolysaccharide.

Skin mucus has several functions: reducing friction (Rosen and Cornford, 1971), reducing mechanical damage (Pinky *et al.,* 2008), osmoregulation (Van Oosten, 1957), gas exchange (Park *et al.,*2006) and a natural defence against invading microorganisms (Ingram, 1980). Continual replacement of mucus by secretions from goblet (mucous) cells may prevent colonization by pathogenic microbes on the body surface.

Mucous cells usually originate in the middle layer of the epidermis where they develop and mature. Mature mucous cells may have their base on the basement membrane (Bullock and Roberts, 1974). Mucous cells of various types are found in fish (Pinky *et al.,* 2008). Mittal *et al.,* (2002) reported that the major constituents of the surface secretions in fish, in general, consisted of glycoproteins of heterogeneous molecular structure. These glycoproteins were assumed to play a wide variety of roles in relation to the ecophysiological conditions inhabited by fishes. The density of mucous cells varies greatly by fish species, regions on the body and stage of growth (Bullock and Roberts, 1974; Yamamoto *et al.,* 2011).

The study by Buchmann *et al.,* (2004) confirmed that intact rainbow trout epidermis contains several hundred mucous cells mm^2 and this indicates that these cells represent an important part of the structural make-up of the epidermal surface.

2.4. Diseases affecting skin epidermis

The normal structure and function of the piscine integument reflects the adaptation of the organism to the physical, chemical, and biological properties of the aquatic environment, and the natural history of the organism. Because of the intimate contact of fish with the environment, cutaneous disease is relatively more common in fish than in terrestrial vertebrates and is one of the primary disease conditions presented to the aquatic animal veterinarian. Many of the most common fish diseases affect the skin, and skin damage may occur either as a direct or an indirect response to different stressors (Noga, 2000b) and irritants. However, cutaneous lesions are generally nonspecific and may be indicative of disease that is restricted to the integument or a manifestation of systemic disease (Groff, 2001).

A covering epithelial layer forms much more rapidly in fish than in warm-blooded animals, but after the wound is covered further recovery in fish is extremely slow (Van Oosten, 1957). In aquaculture, general husbandry (e.g. transport, handling, grading), together with unfavourable water quality, may create acute or chronic stress that can affect skin health (Noga, 2000a). Stress may induce a cellular response, inducing increased levels of cellular degeneration, inflammation, apoptosis and necrosis, and increased mucus production (Nolan *et al.,* 2000) and may lead to erosion and ulceration (Udomkusonsri *et al.,* 2004).

2.4.1. Carp pox

Extensive hyperplasia of epithelial cells (pathological proliferation of the epithelial cells), historically called „fish pox" or „carp pox" or „candle wax disease" has been studied in common and ornamental varieties of carp (*Cyprinus carpio*) and in other fish species (Hoole *et al.*, 2001; Kortet *et al.*, 2002; Dixon, 2008). Epidermal hyperplasia (sometimes called plaques or papillomas) 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). They may occur on any part of the body, but are often located on the fins. In the Nordic climate, the disease in fish occurs during the colder winter period when temperatures fall below 14 °C, but in the summer (in warmer water) the lesions are reduced both in number and in severity (Morita and Sano, 1990; Lu *et al.,* 2009) within a two month period (McAllister *et al.,* 1985). Healing and regeneration of tissues involves complex processes of physiological factors, immunological components (Medzhitov, 2008) and transformations in epidermal cellular structure. However, in warm water the peripheral blood leucocytes may be involved in hyperplasia regression process (Morita and Sano, 1990) but information about alterations in these regressed „hyperplastic plaques" is absent. At the same time, as pointed out by Hoole *et al.*, (2001), the normal epidermis usually regenerates under the lesion. In general, hyperplasia of fish skin may have multifactoral aetiology, including pathogens and pollutants (Roberts and Bullock, 1976). Carp pox has never been reported in Estonia (J. Kasesalu personal communication).

2.4.2. Ichthyophthiriosis (ICH)

The freshwater ciliate *Ichthyophthirius multifiliis* was described by Fouquet (1876) in France, where the parasite caused problems due to its rapid multiplication in trout ponds during the warmer season.

The life cycle of the parasite is divided into three distinct stages. The trophont resides and feeds in the epidermis of the host where it can grow to a diameter of up to approximately 1 mm. The mature trophont escapes from the epidermis into the freshwater surroundings, where some of the parasites settle and develop into encysted tomonts. In this tomontocyst stage numerous daughter cells (tomites) are produced. The number of tomites resulting from one tomont varies from between 50 to a few thousand (Aihua and Buchmann, 2001). These tomites escape the cyst as so-called theronts (length 20–60 μm) ready to infect the fish epithelium. A number of studies have shown that all of these life cycle stages are extremely temperature dependent. Thus, the time required for the development and release of theronts from a trophont

can be as long as nine days at 5 °C but is reduced drastically to 18 h at 25 °C (Matthews, 2005).

It is known that the theront penetrates the host epithelia on gills and skin. Evidence has been presented suggesting that the theront enters the host by moving in between two epithelial cells to the underlying epidermal layers. As this is exactly where mucous cells open to the fish surface it has been proposed that the invasive stages gain access to the epidermis by invading mucous cells (Buchmann *et al.,* 1999). This is also supported by the observation that theronts are attracted by host mucus and mucous cell-rich skin regions. The theronts are not attracted to the fish epidermis from longer distances (Wahli *et al.,* 1991) but respond chemotactically to serum components in mucus following a random encounter with the host. Theront behaviour upon contact with the epithelium is well known (Geisslinger, 1987), and consists of a fast rotation around its central axis. It is likely that this is the mode of penetration into the mucous cell opening. The trophont does not penetrate below the basal lamina (Ventura and Paperna, 1985) and is covered by an intact epithelial layer which ruptures when the trophont escapes (Ewing and Kocan, 1987).

Low to moderate primary infection, and secondary reinfestations, do not induce significant histopathological changes in the integument. The proliferative response of the integumentary epithelium appears only following subsequent infections. Repeated heavy reinfections induce massive cellular necrosis, and in very heavy infections extensive lysis of the epithelial tissue (Ventura and Paperna, 1985).

2.4.3. Gyrodactylosis

Gyrodactylosis is a parasitic disease of salmonid fishes caused by the viviparous ectoparasite the monogenean flatworm *Gyrodactylus salaris*, which belongs to the *G. wageneri* species-group, of the subgenus *Limnonephrotus* (family *Gyrodactylidae*). Due to „hyperviviparity" (also known as the „Russian doll" style of reproduction), combined with a rapid generation time, it reproduces quickly, and in a matter of weeks a single worm can produce thousands of progeny (Bakke *et al.,* 2007; Buchmann, 2008). Importantly, Atlantic salmon populations' exhibit marked differences in susceptibility to *G. salaris* infection, with populations

from the Atlantic and the White Sea coasts exhibiting higher infection rates and increased mortality, while salmon populations from the Baltic basin are more resistant to the parasite (Bakke *et al.,* 1992; Rintamäki-Kinnunen and Valtonen, 1996; Dalgaard *et al.,* 2003; Kuusela *et al.,* 2009). The most severe gyrodactylosis outbreaks have been observed in Norway, where epidemics of this parasite have devastated salmon stocks in more than 46 rivers over the last 25 years (Johnsen and Jensen, 1991; Johnsen *et al.,* 1999; Bakke *et al.,* 2007; Kuusela *et al.,* 2007, 2009; Buchmann, 2008). Despite the fact that Baltic salmon are generally believed to be more resistant to *G. salaris*, the susceptibility level varies among populations. For example, salmon populations from the Luleälven and Indalsälven rivers in Sweden (Bakke *et al.,* 2002, 2004; Dalgaard *et al.,* 2003) have been shown to be more susceptible to *G. salaris* infection than salmon populations from the Neva river in Russia and the Tornio/Torne river along the border of Finland and Sweden (Bakke *et al.,* 1990, 1992; Anttila *et al.,* 2008). Moreover, the severity of infection also depends on the particular parasite strain (Lindenstrøm *et al.,* 2003; Jørgensen *et al.,* 2007; Kuusela *et al.,* 2007; Zietara et al., 2010). For example, outbreaks associated with rare clones of *G. salaris* have been recorded on Baltic salmon in fish farms (e.g. Rintamäki-Kinnunen and Valtonen 1996; Kuusela *et al.,* 2007). In a few cases it has been demonstrated that the farm parasites were species hybrids, such as *G. pomeraniae* × *G. lavareti* on rainbow trout (Kuusela *et al.,* 2008), or unusual backcross combinations of *G. salaris* in Denmark (Lindenstrøm *et al.,* 2003), Poland, and Macedonia (Zietara *et al.,* 2010).

The presence of a dense layer of mucous cells beneath the epithelial cells in rainbow trout has prompted suggestions that this cell type could be involved in fish immunity to ectoparasite infections (Pickering, 1974; Buchmann and Bresciani, 1998). This hypothesis is related to the fact that mucous and goblet cells from mammalian mucosal surfaces are known to participate in the host response against intestinal nematodes (Castro and Harari, 1982; Ishikawa *et al.,* 1994). The presence in mucous scrapings from fish of several biologically active substances such as immunoglobulin, peptides (Buchmann and Bresciani, 1998), lysozyme and proteases (Yano, 1996), varying carbohydrates (Buchmann and Bresciani, 1998) and lectins (Yano, 1996) add further evidence for the suggestion that mucus affects the survival of invading monogeneans. The release of mucous cell contents due to IL-1 production by injured epithelial cells could connect these two cells type with monogenean invasion.

G. salaries damages fish not only by consuming the mucous and epithelial cells of the host but also by piercing the fish epidermis with its hooklets, compromising the osmoregulatory function of the fish, and leaving it vulnerable to fungal and bacterial infections (Ozerov *et al.,* 2010).

In addition, depressions in the epithelium, presumably caused by the opisthaptor, are found at the site where these gyrodactylids feed. Secretions from the parasite are likely to affect the epithelium. As monogeneans regurgitate intestinal contents (Smyth and Halton, 1983), enzymes (esterase, aminopeptides, phosphatases) are probably released onto the epithelium where they contribute to further injury or sensitization of the cell layers. A corresponding release of unidentified substances from *G.salaris* parasitizing the Atlantic salmon was noted by Mo (1994). According to Ozerov *et al.,* (2010) *G. salaris* has never been reported in Estonian rivers or in fish farms.

The large variations in mean epidermal thickness, mucus production, and mucous and club cell density between groups of fish suggest that the epidermis is very plastic in its response to environmental insults. To more fully understand epidermal cells functions and functional interactions, studies should conduct more extensive sampling from all parts of the morphology and mucosal immunology of fish. Therefore, an in-depth study of the integument, which forms the outer barrier against aquatic environment, could increase our knowledge and ability to manage and reduce the risk of diseases in aquaculture (Ottesen *et al.,* 2010) and in the wild. Sublethal infection pathogenesis, combined with clinical signs, histological features of epidermal tissue and remarks in the literature, refer to possible activation triggers for mucous and club cells in the damaged epidermis of fish.

3. AIMS OF THE STUDY

This study focuses on the alterations of epidermal club cell activity and on the integration with mucous cells in common carp epidermis by ichthyophthiriosis and elucidates processes involved in epidermal papillomatosis regeneration of koi carp also mucous cells activity in triploid Atlantic salmon by gyrodactylosis.

The specific aims of the thesis were:
1) examine preventing relationships between epidermal regeneration and changes in the cellular structure in the final phase of hyperplasia healing in koi carp (*Cyprinus carpio* L) in warm water (**I**);

2) describe changes in club and mucous cell activity due to *Ichthyophthirius multifiliis* F. infection for test the club cells anti-parasite hypothesis (Smith 1992, Magurran *et al.,* 1996, Chivers *et al.,* 2007, Halbgewachs *et al.* 2009). If club cells have anti-parasite attributes it can be 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 (**I, II**);

3) demonstrate that triploid Atlantic salmon (*Salmo salar* L) mucous cell activity is not as effective at preventing *Gyrodactylus salaris* M. infection as their diploid counterparts (**III**).

4. MATERIALS AND METHODS

Study I

On September 2007, when the water temperature decreased to 9 °C, specific pox symptoms were detected on fins and body tissue of koi carp bred in cages in a pond in South Estonia. The stocking density of fish in this period was ca1 kg/m³. Large waxy coatings covered the body surface and both sides of the caudal fin (Figure 3). A total of 14 affected (seven fish) and unaffected (seven control fish) immature male koi carp (sex ratio was determined after euthanasia by autopsy) with body weights of 130–450 g where put into a 700 litre plastic tank filled with tap water. During 13 days of acclimatization in laboratory conditions the water temperature was raised up to 18.5 ± 1.3 °C. During the study the fish where fed *ad libitum* with a commercial koi feed (Danafeed DAN-EX 0333). The scarcity of experimental material ensured that the study was designed in such a way that allowed observation of the final phase of regression. To study the cellular structure of caudal fin skin comparatively in unaffected and affected (U & A) fish, samples for observation by light microscopy were taken at days 1 (2U+2A fish) (Figure 4), 62 (3U+3A fish) (Figure 5, 6, 7, 8) and 78 (2U+2A fish) (Figure 8,9,10). For sampling, fish were netted from the tank into 60 L of aquarium water containing 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 six samples per fish were taken (three from damaged areas and three from undamaged areas of affected fish and six from unaffected fish in longitudinal sections). For light microscopy, samples were fixed in 10% neutral buffered formalin, dehydrated in ethanol and embedded carefully in paraffin. Sections of 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 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.

Study II

On September 2009, two hundred four-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 water at a temperature of 17–22 ºC. Fish were acclimatized for one month in plastic tanks filled with aerated and well-filtered tap water at 20 ºC under natural photoperiod conditions.

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 (2,000–2,500 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 (2,000–2,500 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 NaHCO₃. After three minutes, all anaesthetized fish were euthanized by cervical dislocation.

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 within a 500 μm perimeter (Figure 11, 12, 13, 14).

The results are presented as means ± standard deviation. To compare the club cell densities, mucous cell densities, and epidermis thicknesses on different days and between groups, the Wilcoxon test was performed using the SAS system. The correlation of cell densities between club cells and mucous cells was analysed using Pearson correlation analyses (Figure 15, 16).

Study III

On 21 February 2008, a small number of *Gyrodactylus sp.* parasites were found during a routine parasitological inspection in a freshwater fish hatchery in northern Estonia. On 27–28 February 2008, a total of 586 hatchery-reared juvenile salmon (1+ yr old), comprising 49 full-sib and half-sib families, were sampled from the aforementioned 2 m diameter fish tank. This tank consisted of Atlantic salmon of the smallest size, as these fish had been size-selected following routine hatchery practice (mean length: 24.12 ± 9.94 mm; mean weight: 12.95 ± 1.94 g). The fish were killed using an MS-222 overdose, and the right pectoral fin of every individual was stored separately in 96% ethanol. The total numbers of parasites present on both sides of the alcohol-preserved pectoral fin were counted under a dissection microscope (10× magnification). In addition, a small number of pectoral and pelvic fins (n = 32) were also examined from the fresh tissue using light microscopy (Leica CME) from 400× to 1000× magnification.

5. RESULTS AND DISCUSSION

Previous results have suggested that epidermal club cells have a generic role in response to injury, and that they display intense phagocytic activity, having an anti-parasitic function in the host.

5.1. Hyperplasia healing

In study (**I**) mature club cell 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) (Figure 6). Loss of epithelial integrity and evacuation of decomposed club cells from the top layer and sloughing off from the surface was observed in the distal areas of hyperplasias. Desquamation of hyperplastic areas was observed in line at a distance of 105 (± 5) µm from the basal layer. Mature mucous cells were present only inside lesions at a distance of 32 (± 4) to 110 (± 13) µm from the basal layer (32 µm in the middle and 110 µm in the distal areas of lesioned tissue) (Figure 6). In epidermis areas, after desquamation of the surface, a large number of mucous cells in the surface region and high secretory activity of superficial layer epidermal cells was observed (Figure 8). Extensive new mucous cell differentiation was observed beneath the club cells proliferation layer to the basement membrane. A mean of 118 (± 8) club cells and 63 (± 5) mucous cells were counted per 1 mm of length and total thickness of sectioned epidermis. In the epidermal tissue, from earlier peeled areas to the apex of the fins (Figure 9, 10), a normal density of epithelial cells was detected (mucous 21 (± 6) and club cells 9 (± 3) per 1 mm of length and total thickness) Table 1, (**I**).

5.2. Club cell anti-parasite hypothesis

In contrary to a previous study (James *et al.,* 2009) study **II** has shown that invasion of *Ichthyophthirius multifiliis* parasites activated epidermal club cells (Figure 11). It certainly needs clarification whether this 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 parasitic damage

of host epidermis (Smith, 1992; Chivers *et al.,* 2007; Halbgewachs *et al.,* 2009).

A local reduction of mucous cells occurred after theront invasion-induced proliferation, and increased club cell density around the ciliate *I. multifiliis* during the growth of trophonts (Figure 11). After parasites left the skin due to salt-water treatment, a decrease in the number of club cells was detected. During reinvasion no decrease in parasite activity in areas of club cell proliferation was observed (Figure 14). It was found that giant mature club cells were opened to the surface (**II**), (Figure 12, 13).

It was 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, has often been seen as a response to injury (Ottesen *et al.,* 2010, Ozerov *et al.,* 2010 (**III**)). 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 (Figure 11, 12, 13, 14, 15) in common carp.

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 fresh water study (**II**). 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 (Figure 14). Nevertheless the possibly beneficial mechanical pressure of these cells at the beginning of the free-living stage is not excluded (Figure 12). As pointed out by Selosse and Rowland (1990), the salt (in study **II)**, 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 the epidermis was diminished in carp, maybe by salt water together with a high proliferation of club cells, and not because of the activity of club cells alone (**II**).

Table 1. Microscope data of cellular types of epidermis (epid-s) (per 1 mm of length and total thickness) and epidermal thickness in normal, affected and healed koi carp caudal fins.

Number of cells	Normal epid-s (control group)	Hyperplastic epid-s (day 1)	Regenerating epid-s (*) (day 62)	Regenerating epid-s (**) (day 62)	Regenerating epid-s (***) (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

(*) – From hyperplastic areas (Figure 6)
(**) – From post-hyperplastic areas after desquamation of surface (Figure 7, 8)
(***) – From regenerating areas.

However, differentiation and activation of club cells associated with mechanical and proteolytic damage by ciliates are like replacements of damaged epithelial cells (**II**). At the same time, it is not 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 (epithelial) cells are capable of engulfing foreign material, and thus may function as scavenger cells of Atlantic salmon, *Salmo salar.*

5.3. Mucous cells activity

Mucus secretion has been shown to increase under stressful living conditions in carp (Iger and Abraham, 1997), during parasitic infection in Atlantic salmon (Ross *et al.,* 2000) and during incorrect medical treatment (Buchmann, 2004) in rainbow trout. Gyrodactylosis is a parasitic disease of salmonid fishes, caused by the viviparous ectoparasite monogenean flatworm *Gyrodactylus salaris. G. salaris* damages fish not only by consuming mucus and epithelial cells of the host, but also by piercing the fish epidermis with its hooklets, compromising the osmoregulatory function of the epidermis, and leaving it vulnerable to fungal and bacterial infection. In studies **I** and **II**, severe mucous cell hyperplastic area, as described by Ottesen *et al.,* (2010) in Atlantic halibut (*Hippoglossus hippoglossus*) and mucous production, as described in triploid Atlantic salmon *Salmo salar* (**III**, see video of live *G. salaris* in Supplement 1, available at **www.int-res.com/articles/suppl/d091p129_suppl/**) was not observed in koi carp, a but high cell turnover and increased mucous cell secretory activity were identified after desquamation in epidermis (**I**), and at two days after *I. multifiliis* invasion (**II**) (Figure 8, 11). Mucous cell exhaustion with reduced numbers of active cells is often seen as a response to injuries (Buchmann *et al.,* 2004). Nevertheless, high numbers of active mucous cells after desquamation in our samples may indicate active protection against undesirable environmental factors by the healed epithelium (Figure 8, **I**).

This work identified triploid Atlantic salmon of Baltic origin as more susceptible to *G. salaris* infection than their diploid counterparts, possibly due to compromised mucosal immunity pathways in triploid salmon, because the high mucus (mucous compounds) production did not diminish parasite activity (**III**).

5.4. Discussion

Club cell proliferation under the mucous cells line indicates clearly that the high density of club cells compensates of an overall low density of mucous cells inside the epidermis as an adaptation for effective healing and/or protective and/or compensation and mechanisms (**I, II**) (Figure 8, 11, 15).

Earlier works have 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) or communicate only when the epidermis has been ruptured by predation (Smith, 1992). In studies **I** and **II**, club cells on the surface of the epidermis released a viscous „secretion" from the fourth to the eleventh experimental days **(II),** and day 62 **(I)** (Figure 6, 12, 13). There are no data to date in the literature about the chemical content of club cells (Zaccone *et al.,* 1990; 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 by Rombout *et al.,* the epithelium of carp skin and mature club cells is indicated by intensive staining and expression of polymeric immunoglobulin receptor (pIgR) around the nuclei (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.
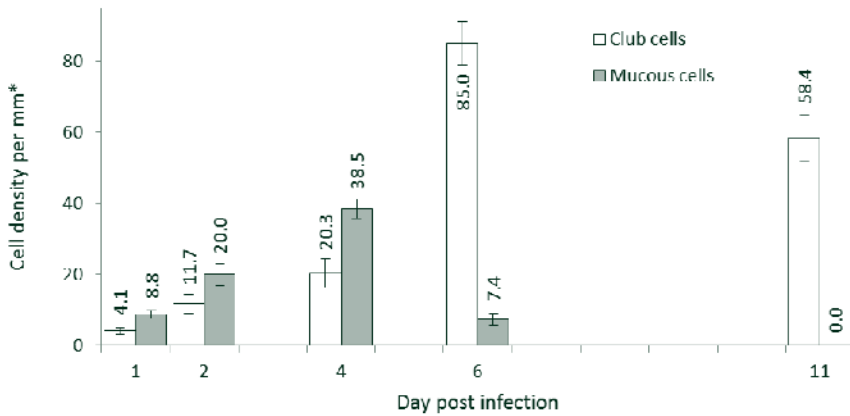
Figure 15. Densities of club- and mucous cells (mean ± standard deviation) in the epidermis of *Cyprinus carpio* in salt water days 1–11 post infection with *Ichthyophthirius multifiliis*; *determined on a 1-mm long transect covering the entire thickness of the epidermis.

The genesis of club cells seems to be an evolutionary adaptation to living in muddy/standing waters which contain more potentially harmful substances compared to clean waters. Parasite-host interactions, together with environmental 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 the healing and protection mechanisms of skin epidermis during post-predation and post-infection recovery. But club cells are not unique among the *Ostariophysi*. Fishes in the Percidae (*Acanthopterygii*) also possess club cells with similar histological properties to the club cells in the Ostariophysi (see Smith, 1992; Wisenden *et al.,* 2004; Chivers *et al.,* 2007). Nevertheless, the activation of club cells in carp is induced by skin damage caused by skin parasites. Finally, this study, which was the first of its kind performed in Estonia, demonstrated that there are several areas where further studies are required. The results demonstrate the potential role of club cells in the healing process during and after damage in carp. For better understanding of club and mucous cell integrated functions, further studies should include more extensive sampling from all parts of the skin biology of freshwater fish.

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

6. CONCLUSIONS

1. Club- and mucous cells are integrated into epidermal cell line physical protection mechanisms in the epidermis of common carp and koi *Cyprinus carpio* **(I, II)**. The activation of club cells in common carp is induced by skin damage caused by parasites after increased proliferation of mucous cells **(II)**. The high density of club cells in the epidermis compensates for the absence of mucous cells in common and koi carp **(I, II)**.

2. Erosion and desquamation, beginning from the line of new epidermis (the newly differentiated mucous cell line), was observed in the final phase of regression of epidermal hyperplasia (carp pox). Investigations showed a clear relationship between epidermal regeneration and the number of club cells. Results of investigations demonstrate the potential role of club cells in the healing process during and after hyperplasia in koi carp *C. carpio* **(I)**. Normal epidermis regenerates under the hyperplastic lesions.

3. After proliferation some giant mature club cells were opened to the surface of the epidermis and released a viscous „secretion". It can be hardly concluded that the function of club cells is the phagocytic removal of cell debris **(I, II)**.

4. The reinvading parasites *I. multifiliis* did not diminish the activity of club cells, and previously activated club cells did not diminish the activity of newly invading theronts. The growth of trophonts of *I. multifiliis* in the epidermis of the common carp *C. carpio* was diminished by a combination of salt water treatment together with a high proliferation of club cells, not because of the activity of club cells alone **(II)**. The proliferation of club cells indicates that club cells do not provide primary protection against *I. multifiliis* in the common carp *C. carpio*, nor do they inhibit the growth of the parasite; club cells do not have an anti-parasite function *per se* **(II)**.

5. The high production of mucus induced by skin damage caused by *G. salaris* in triploid Atlantic salmon *Salmo salar* and *Ichthyophthirius multifiliis* in naïve common carp *C. carpio* **(II, III)** is not an effective defence.

6. Triploid Atlantic salmon *Salmo salar* were more susceptible to *Gyrodactylus salaris* infection than their diploid counterparts, probably due to compromised mucosal immunity pathways **(III).**

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SUMMARY IN ESTONIAN

NAKKUSTE POOLT PÕHJUSTATUD EPIDERMISE RAKKUDE TALITLUSLIKU AKTIIVSUSE MUUTUSED KALADEL

Ohtlike kalahaiguste, nagu gürodaktüloos, ihtüoftirioos, vähemal määral papillomatoos, poolt tekitatud majanduslik kahju on suur. Ühelt poolt on teave kalade naha kaitsemehhanismide toimimisest tähtis kalade nakkushaiguste ärahoidmiseks ja profülaktikameetodite täiustamiseks, teisalt pakub saadav informatsioon huvi dermatoloogidele ja kalabioloogidele.

Tänapäeva selgroogsete naha fenotüübiline rikkus on evolutsiooniliselt pärit veekeskkonnast. Ühest küljest on vesi hea lahusti, aga samas ka hea elukeskkond erinevatele selgrootutele, kellest paljud võivad teatud oludes koos lahustunud sooladega mõjutada kalade kehapinda, seega tervist ja heaolu laiemalt. Kujundlikult võib öelda, et kalad ujuvad patogeenide ookeanis. Seega on otsene kontakt oma ümbritseva agressiivse keskkonnaga lubanud kaladel välja arendada elupaigaspetsiifilised tunnused ja efektiivsed naha kaitsemehhanismid.

Mitmekihilisel lame-epiteelil (nahal nimetatakse seda epidermiseks e marraskiks) on peamiselt katte- ja kaitseülesanne, teistel pinnaepiteeli liikidel lisanduvad sellele veel mõned kõrvalfunktsioonid. Võrreldes maismaaselgroogsetega koosneb kalade epidermis elusatest rakkudest. Elusa koena täidab kalade nahk keemilise ja füüsikalise kaitse funktsioone ning on tähtis patogeenidevastase spetsiifilise ja mittespetsiifilise immuunsuse tagaja. Just see bioloogiliselt ühine aspekt imetajatega võimaldab kalu kasutada mudelorganismina inimeste nahahaiguste kliinilistes ja rakenduslikes uuringutes. Kuigi kalade ja imetajate viimane ühine kopskalast eellane võis elada maal 350 miljonit aastat ja rohkemgi (nt vöötdaanio 420 milj.) tagasi, siis tänapäeval nende naha arhitektuuris põhimõttelisi erinevusi ei ole. Nahk koosneb kolmest kihist, välisest marraskist (*epidermis*), sisemisest pärisnahast (*dermis*) ja alusnahast (*hypodermis*). Kalade kogu kehapind ja uimed on kaetud avaskulaarse epidermisega. Selles limarakkude poolt toodetud lima on tähtis esmase kaitse osa.

Kalade epidermis, nagu maismaaloomadelgi, on arenenud mitmekihiliseks, mida pärisnahast eraldab basaalmembraan. Tõsi, kalade nahk on keratiniseerumata limaskesta süsteem (võiks kasutada nimetust limask), mis ei ole kaetud surnud rakkudega ega ole kuiv nagu hiirel või inimesel. Vigastamata pindmised soomusjad epiteelirakud tagavad epidermise rakulise struktuuri stabiilsuse. Koos teiste rakkudega moodustavad nad ühe kolmest epidermise kihist välimise (*stratum superficiale*), milles avanevad eelmainitud (PAS-positiivsed) limarakud. Lisaks sellele on epidermise struktuuris keskmine diferentseerunud rakkude kiht e ogakiht (*stratum spinosum*) ja basaalmembraaniga külgnev basaalkiht (*stratum basale*), mis on epidermise kasvukihiks. Diferentseerumata epidermaalsed tüvirakud asuvad basaalmembraanil, prolifereeruvad ja diferentseeruvad ogakihis ning moodustavad seejärel välimise epiteelkihi. Kasvukihi uuenemise arvel toimub epidermise füsioloogiline regeneratsioon. Seega on epidermis modelleeritud hoidma täpset tasakaalu rakkude proliferatsiooni ja diferentseerumise vahel. Sõltuvalt kalaliigist koosneb epidermis pindmistest soomusjatest (skvamoosetest), süvamistest kuboidaalsetest epiteelirakkudest, mida varem nimetati filamendi e Malpighi rakkudeks, limarakkudest, nuirakkudest, infiltreerunud leukotsüütidest ja lümfotsüütidest. Epidermise paksus (ca 100 μm) ja rakukihtide arv (5-20) erineb kalaliigiti ja kehapiirkonniti ning võib teatud määral sõltuda aastaajast, kalade soost ja elukeskkonna seisundist.

Paljudel kalaliikidel paiknevad epidermise ogakihis suured, ühe või kahetuumalised eosinofiilsed nuirakud, millede mõõtmed ulatuvad 40 μm. Ajalooliselt on neid rakke kutsutud „*Schreckstoffzellen*" e alarmrakkudeks. Uuringute põhjal on kirjeldatud, et need rakud aktiveeruvad pärast epidermise kahjustusi, on fagotsütoosivõimelised kuid ei oma kontakti väliskeskkonnaga nagu see on limarakkudel. Arusaamine nende rakkude funktsioonist on aja jooksul muutunud, mis algas ca 70 aastat tagasi alarm-, lõppedes täna immuunfunktsiooniga. Kuna nuirakkude funktsioone pole tänaseni täpselt kirjeldatud, kasutatakse nende iseloomustamiseks väljendit multifunktsionaalne rakk. Paradoksaalselt pole tänapäeval laialt tsiteeritud nuirakkude omadused olnud mitte algallikate uurimistulemuseks vaid diskussiooni osaks. Viimased uuringud näitavad, et nuirakkudel võib olla teatud roll epidermise struktuuri ennistumisel ja tervenemisel pärast vigastusi ning nad omavad teatud mõju sellesse tunginud välisparasiitidesse.

Eesti jaoks uudse kalade patomorfoloogiat puudutava töö eesmärgiks oli, kasutades histoloogia ja histokeemia meetodeid, selgitada haigustekitajate

poolt põhjustatud rakulise struktuuri ja aktiivsuse muutusi kalade epidermises. Käesolev väitekiri on ülevaateartikkel, mis tugineb koos kaasautoritega avaldatud kolmele teadusartiklile (**I, II, III**).

Valkjasroosasid sügisesi kasveid karpkalade kehapinnal ja uimedel on kirjeldatud alates 16. saj. kui karpkalade rõugeid „*Karpfenpocke*" (ik *carp pox, candle wax disease*), hiljem naha papillomatoosina (ik *epithelioma papillosum, epizootic cutaneous papillomatosis*). See ajalooliselt kõige esimesena kirjeldatud kalade haigus (*Historiae Animalium Liber* IIII, Zurich: Tiguri. 1558) on tänapäeval probleemiks peamiselt dekoratiivkalakasvatuses, sporaadiliselt ka karpkalakasvatuses, sest patoloogia sümptomid vähendavad oluliselt just kalade kaubanduslikku välimust. Haiguse tekitajaks peetakse CyHV-1 (*Cyprinide herpesvirus* 1). Põhjapiirkondades ilmuvad haigusnähud 2- 3 mm diameetriga nähtavate ja palpeeritavate kõrgenditena sügisel kui veetemperatuur langeb alla 14 °C, mis ekstreemsetel juhtudel võivad laatuda ja katta kogu kehapinna. Kevadel kui veetemperatuur tõuseb, kasved kaovad ja epidermis arvatavasti terveneb. Käesoleva töö eesmärk oli uurida rakulise struktuuri muutusi kasvaja taandumisel soojas vees, selgitamaks varem mittekirjeldatud epidermise funktsionaalse struktuuri ennistumise mehhanisme. Karpkalade rõugeid pole enne käesolevat uuringut Eestis kasvatavatel karpkaladel diagnoositud (**I**).

Ihtüoftirioos on epidermise kahjustustega kulgev mageveekalade invasioonihaigus, mille tekitajaks on ümmargune kuni 1 mm läbimõõduga infusoori *Ichthyophthirius multifiliis* trofont. Haigust diagnoositakse kliiniliste tunnuste (valkjad täpid kehapinnal, limaeritus) ja keha pinnalt võetud kaabete mikroskoopilise uuringu leiu alusel. Käesolevaks ajaks haiguse efektiivsed tõrjevahendid puuduvad. See seletub trofondi parasiteerimisega teda „kaitsva" epidermise sees. Käesolevas töös jälgiti tabandunud epidermise rakulise struktuuri muutusi parasiidi ümber nii magedas kui ka soolases vees (**II**), selgitamaks parasiidi mõju nui- ja limarakkude funktsionaalsele struktuurile ning teisalt nende rakkude mõju parasiitse trofondi aktiivsusele.

Gürodaktüloos on kalade kehapinna, uimede ning lõpuste kahjustusega kulgev invasioonihaigus, mille tekitajaiks on ainupõlvsed imiussid perekonnast *Gyrodactylus*. Gürodaktülused toituvad epiteelkihis, tekitades sellele vigastusi, mis raske tabandumise korral ja sekundaarse mikrofloora toel süvenevad nahanekroosiks. Haiguspuhangud esinevad

augustis ja märtsis-aprillis. Haigust on seniajani diagnoositud kliiniliste tunnuste ja haigustekitajate leiu alusel. Ohtliku parasiidi *Gyrodactylus salaris* poolt põhjustatud haigust pole Eesti kalafarmides ja looduslikes kalapopulatsioonides enne käesolevat uuringut diagnoositud (**III**).

Katsed sisebasseinides viidi läbi aastatel 2007–2009 EMÜ, Veterinaarmeditsiini ja loomakasvatuse instituudi, kalakasvatuse ja morfoloogia osakondades ning 2008 Põlula Kalakasvatuskeskuses ja Turu Ülikoolis. Tulemused on avaldatud aastatel 2010 ja 2011 kolmes rahvusvahelise levikuga teadusajakirjas.

Valkajsroosad palpeeritavad kõrgendid ja laatunud vohandid tiigis peetud koi karpkalade kehal ning sabauimedel ilmnesid septembris, kui veetemperatuur pärast väga kuuma augustit järsult langes (**I**). Veetempetaruuril 10 ºC võetud koetükid näitasid epidermaalset hüperplaasiat (papillomatoos, karpkalade rõuged) (Joonis 3, 4). Kuna selle haiguse taandumise käigus toimuvaid rakulise struktuuri muutusi soojas vees pole varem kirjeldatud, püüti haigustunnustega kalad kinni ja paigutati pärast aklimatiseerimist sisebasseinidesse. Kogu katse ajal vett filtreeriti, aereeriti ja kalu söödeti karpkalade kommertssöödaga *ad libitum*. Pärast veetemperatuuri tõstmist võetud proovides nähti 62. päeval ulatuslikku nuirakkude proliferatsiooni ja 78. päeval pärast kasve pindmise kihi irdumist ennistunud (funktsioneerivat) epidermist. Katse tulemus näitab, et nuirakud täidavad olulist rolli epidermise tervenemisel ja seda, et nuirakud asendavad ogakihis välispinnal avanenud limarakke. Selgelt on nähtav ka nuirakkude, varem mittekirjeldatud avanemine keha välispinnale (Joonis 6, 7, 8, 9, 10). Viimased andmed (Chivers *et al.,* 2007, Halbgewachs *et al.,* 2009) viitavad nuirakkude võimalikule rollile kalade naha immuunsüsteemis. Kui nuirakud omavad teatud mõju epidermisesse tunginud parasiitidesse, siis peaks see olema nähatav ka ohtliku parasiidi *I. multifiliis* invasiooni korral.

Katsesse (**II**) võeti 200 mitteimmuunset samasuvist karpkala kaaluga 65 ± 5 g. Pärast aklimatiseerimist jagati kalad viide gruppi. Epidermise seisundi hindamiseks surmati 40 kala MS 222 üledoosiga seda koeproovide võtmiseks. Esimese ja teise katsegrupi kalad nakatati *I. multifiliis* trofontidega. Esimese grupi kalad, vältimaks reinvasiooni ja sekundaarse mikrofloora võimalikku häirivat mõju katsetulemustele, paigutati seejärel soolasesse vette (5g/l), teise grupi kalad magedasse vette. Kolmanda grupi

kalad paigutati magedasse vette ja neljanda grupi kalad soolaveelisse (5g/l) basseini. Katse tehti temperatuuril 20 ºC. Kõigist gruppidest võeti 10 kala ja surmati 2., 4., 6. ja 11. päeval. Võetud histoloogiline materjal fikseeriti puhverdatud formaliinis ja värvustati histoloogiliste ja histokeemiliste H&E ja PAS meetoditega. Nui- ja limarakkude arv loendati 500 μm raadiuses parasiitide ümber. Fotode tegemisel kasutati digikaamerat AxioCam HRc (Saksamaa). Statistilises andmetöötluses kasutati Wilcoxon testi SAS süsteemi ja Pearsoni korrelatsioonianalüüsi. Esimese grupi kalade (soolane vesi) preparaatides oli näha parasiitide kasv koos samaaegse nuirakkude arvu suurenemise ja limarakkude arvu drastilise vähenemisega (Joonis 11). Samal ajal võis täheldada parasiitide kasvus teatud mahajäämust võrreldes parasiitidega mageda vee katsekaladel.

Teise grupi kalade epidermises täheldati esialgset nuirakkude arvu suurenemist ja limarakkude arvu vähenemist, mis sarnanes olukorraga soolases vees tehtud katsega. Selgesti on näha reinvasiooni poolt põhjustatud kahjustused, kuid mingit muutust parasiitide aktiivsuses, vaatamata varasemale nuirakkude proliferatsioonile, ei täheldatud (Joonis14). Nuirakud avanevad mõlema katsegrupi kaladel keha pinnale (Joonis 12, 13). Kolmanda ja neljanda grupi kalade epidermise rakuline struktuur ja paksus, võrreldes katsete algusega, oluliselt ei muutunud. Selle katse tulemused näitavad, et nuirakud aktiveeruvad parasiitide ründe tulemusena ja täidavad olulist rolli epidermise tervenemisel ning seda, et nuirakud asendavad ogakihis proliferatsiooni lõpetanud ja välispinnale avanenud ning seejärel surnud limarakke. Kasvetest tervenemise faasis (uuring **I**) on samuti selgelt nähtav nuirakkude avanemine keha välispinnale. Vaatamata parasiitide mõjule nuirakkude proliferatsioonis, ei anna see alust arvata, et nuirakkudel on võime tõkestada infusoori noorvormide (hulkurrakk e theront) invasiooni või takistada parasiitse trofondi kasvu pärast invadeerumist. Parasiitide kasvu aeglustumine soolases vees ei ole tingitud otseselt nuirakkude mõjust vaid soola teatud antiparasiitsest toimest isegi epidermise sees. Selle katse tulemuste põhjal võib öelda, et nuirakud siiski ei oma antiparasiitset efekti *per se*, kuigi ulatusliku proliferatsiooni survestav mõju parasiitide epidermisest „väljalükkamisele" on võimalik (Joonis 12). Nuirakkude proliferatsiooni ala on võib-olla veespetsiifiline mäda analoog.

Aktiveerunud ja välispinnale avanenud suurtest nuirakkudest võib keskkonda sattuda nende rakkude poolt toodetud immunoglobuliini

retseptor R (ik *polymeric immunoglobulin receptor* pIgR), mis põhjalike alarmreaktsiooni uuringute taustal, viitab paradoksaalselt asjaolule, et olfaktoorsüsteemi (laiemalt meeleorganite ja aju ning edasi käitumise) areng võib olla seotud immuunsüsteemi evolutsiooniga ja mõjutatud selle poolt.

Tugev *G. salaris* tabandumine avastati rutiinse ihtüopatoloogilise uuringu käigus Balti merest pärit triploidsetel atlandi lõhedel. Kuigi triploidseid kalu on kasvatatud üle kolmekümne aasta, võis tabandumise intensiivsus viidata sellele, et triploidsed lõhed on võrreldes diploidsete liigikaaslastega sellele parasiidile vastuvõtlikumad. Tabandumise intensiivsust rinnauimel vaata **www.int-res.com/articles/suppl/d091p129_suppl/**. Tabandunud kalad surmati MS-222 üledoosiga. Surnud kaladelt lõigati ära üks rinnauim, mis fikseeriti alkoholis järgnevateks geneetilisteks uuringuteks, teiselt äralõigatud rinnauimelt loeti parasiitide koguarv stereomikroskoobi all ja määrati morfoloogiliste tunnuste abil parasiidi liik. Järgnenud uuringud näitasid, et triploidsed lõhed on parasiidi *G. salaris* suhtes vastuvõtlikumad. Põhjus võib olla selles, et vaatamata tugevale limaeritusele ei ole selle komponendid efektiivsed parasiitide ründe takistajad või nad puuduvad. Vastupidi – suur lima hulk võib olla parasiitide paljunemist soodustavaks või toetavaks faktoriks.

Järeldused

1. Uuringu tulemus näitab, et nuirakud täidavad olulist rolli epidermise tervenemisel ja seda, et nuirakud asendavad ogakihis prolifereerumise lõpetanud ja välispinnale avanenud limarakke. Selgelt on nähtav nuirakkude varem mittekirjeldatud avanemine keha välispinnale (Joonis 6, 7, 8, 9, 10).

2. Uuringu tulemuseks võib lugeda ka seda, et katsete käigus ei täheldatud prolifereerunud nuirakkude varemkirjeldatud fagotsüteerivaid omadusi.

3. Reinvadeerunud parasiitidele (theront) ei avalda esimese ründe tulemusena varem prolifereerunud nuirakud mingit tuntavat mõju, seega võib arvata, et need ei oma antiparasiitseid omadusi *per se*.

4. Epidermaalse hüperplaasia tervenemine soojas vees toimub kasve pindmise osa irdumisega (erosioon, koordumine) ennistunud struktuuriga epidermiselt ja kasvete all uuesti diferentseerunud limarakkude kihilt, mis viib pärast nuirakkude uut proliferatsiooni epidermise funktsiooni ja loomuliku regeneratsiooni taastumiseni ning kalade tervenemiseni kasvajast.

5. Triploidsete lõhede lima võib olla soodsaks keskkonnaks *G. salaris* massilisele paljunemisele. Samuti ei ole efektiivne mitteimmuunsete karpkalade ohtralt toodetud lima, takistamaks infusoori *I. multifiliis* hulkurrakkude (theront) rünnet.

6. Triploidsed atlandi lõhed on vastuvõtlikumad parasiidi *G. salaris* ründele võrreldes normaalsete diploidsete liigikaaslastega. Põhjuseks võib olla triploidsete kalade limas ja epidermises puuduvad või vähese aktiivsusega mittespetsiifilised komponendid (IL-1).

Tuginedes tehtud uuringutele võib kokkuvõtvalt öelda, et epidermise rakuline struktuur ja rakkude funktsionaalne aktiivsus on väga muutlikud ja otseses seoses haigustekitajate ja keskkonna mõjuga. See ei tähenda üheselt siiski seda, et epidermises toimuvate regenereerumisprotsesside käigus oleks alati tagatud kaitse parasiitide reinvasioonile. Tulemused ühelt poolt kinnitavad aga teiselt poolt lükkavad ümber rea varemavaldatud seisukohti ja hüpoteese nuirakkude funktsioonist. Käesolev töö on osa laiemast kalade naha kaitsemehhanismide ja bioloogia uurimisest tulevikus.

ACKNOWLEDGEMENTS

I

LIST OF PUBLICATIONS AND
ORIGINAL FIGURES

Päkk P., Hussar P., Järveots T., Paaver T. (2011). Club cells active role in epidermal regeneration after skin hyperplasia of koi carp *Cyprinus carpio. AACL Bioflux,* **4** (4), 455–462.

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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 14ºC, 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).
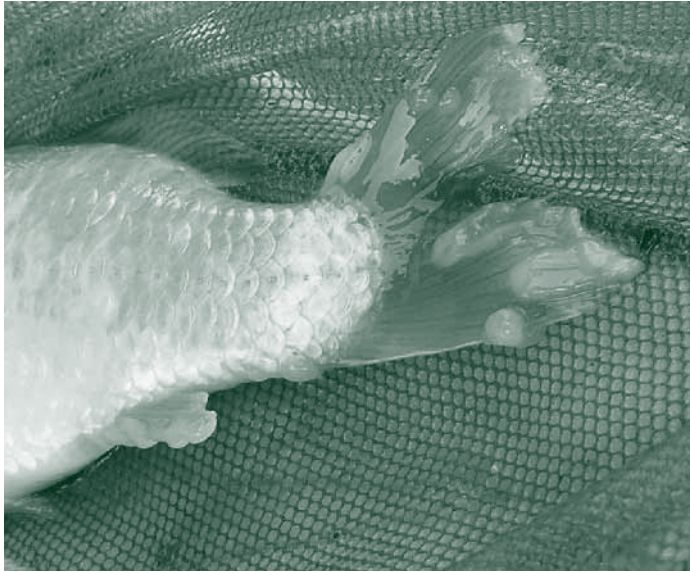
456

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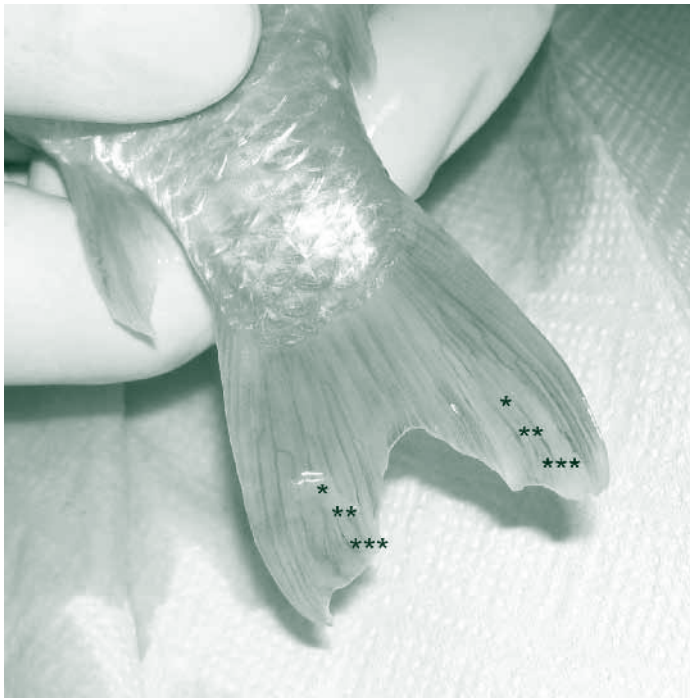*Cyprinus carpio* (first day of study).

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).

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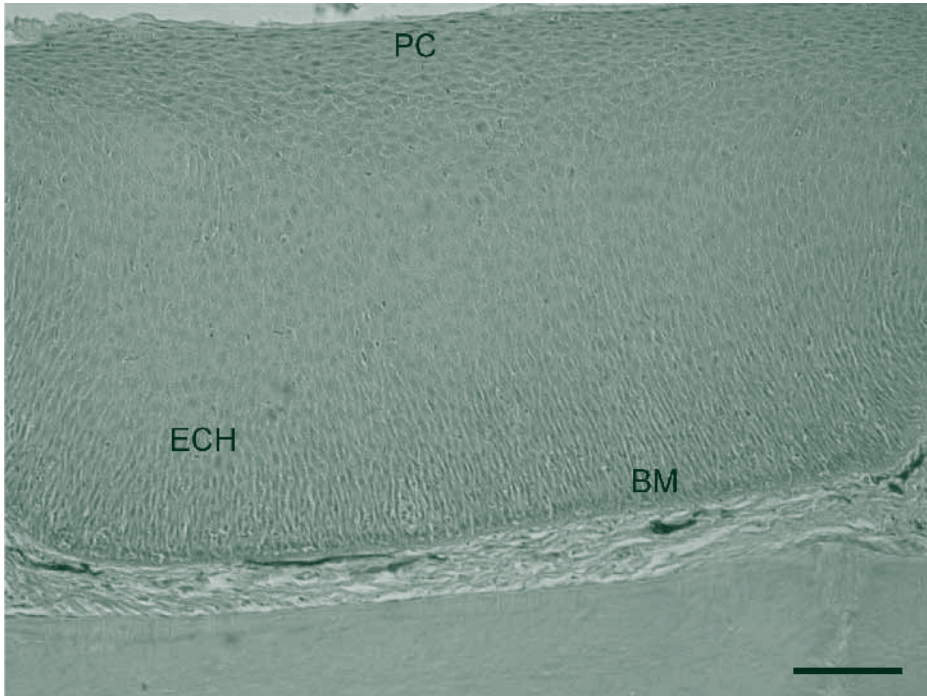
Figure 3. Extensive hyperplasia of epithelial cells (EC) in caudal fin of koi carp *Cyprinus carpio*. Note the absence of club cells and mucous cells. Epithelial cells are smaller in size and tightly packed (PC). The basal layer (BM) forms pegs and is slightly scalloping. PC- epithelial cells. First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

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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).
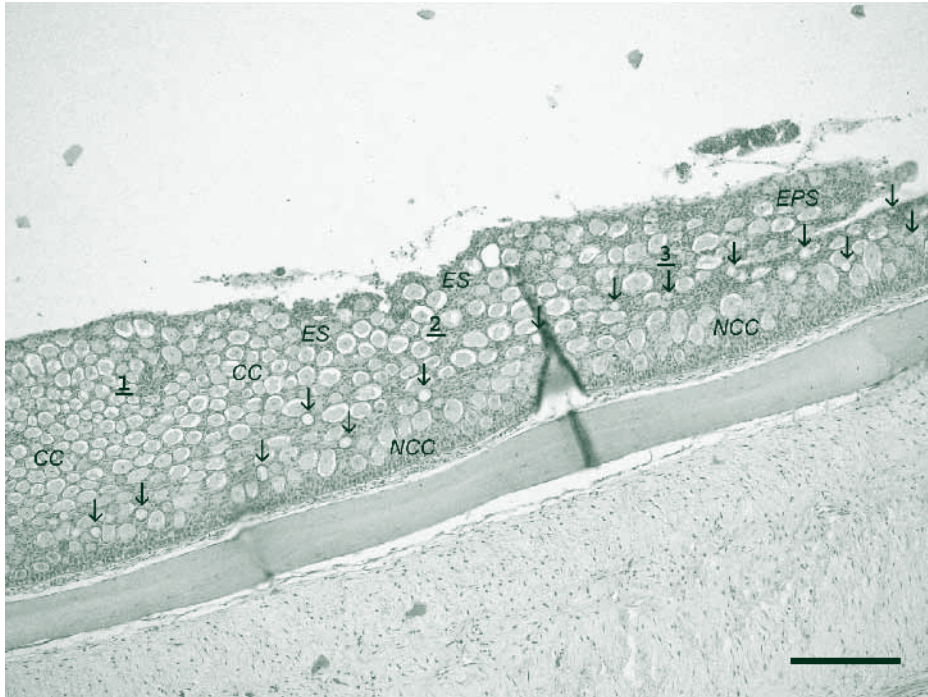
Figure 4. Epidermal hyperplasia medial area of caudal fin of koi carp *Cyprinus carpio* 62 days after rising water temperature. Loss of epithelial integrity (ES) and eventual desquamation (EPS): 1. - section of hyperplasia with high proliferation of eosinophilic enlarged club cells (CC) and undamaged 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.
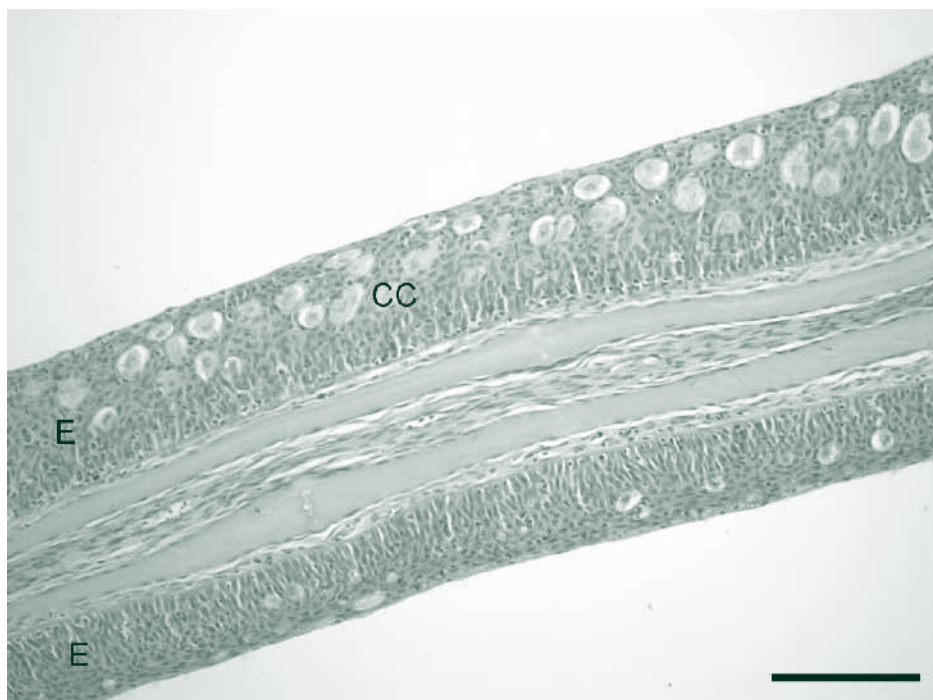
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Figure 5. Epidermal hyperplasia area after desquamation of surface of caudal fin of koi carp *Cyprinus carpio* 62 days after rising water temperature. E- Epidermis. CC- Club cells. Bar = 100µm (H&E).

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

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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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II

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, Smith 1992, 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-

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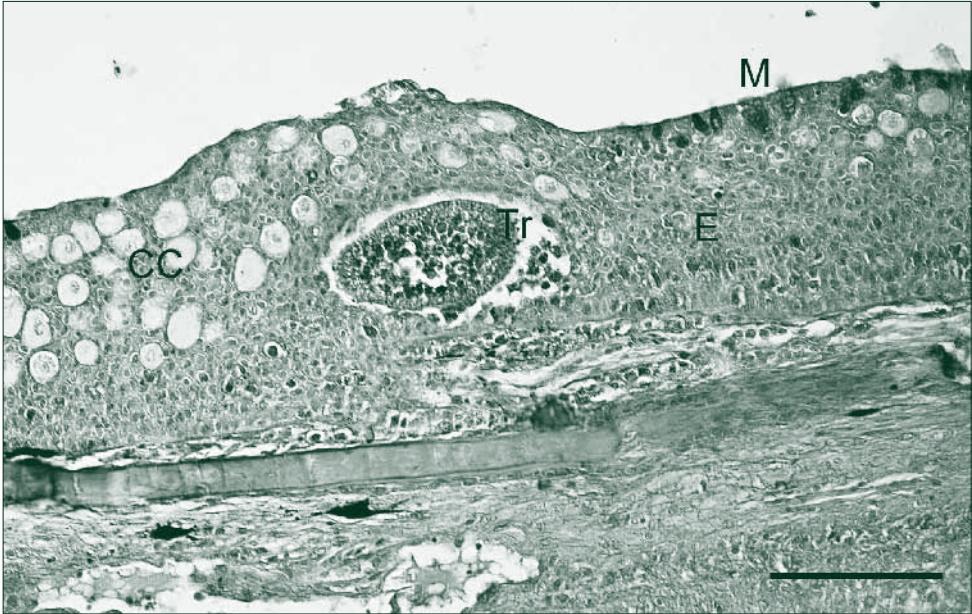
Fig. 1. Skin of common carp, *Cyprinus carpio*, four days after infection; E = epidermis, Tr = trophont of *Ichthyophthirius multifiliis*, CC = club cells, M = mucous cells; PAS staining, scale bar = 150 μm
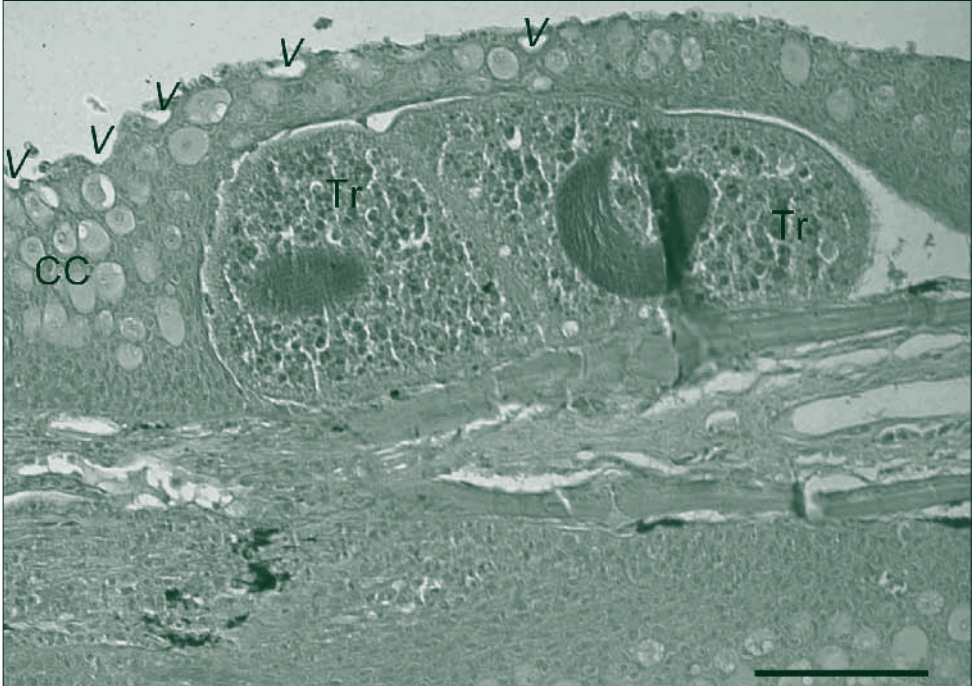

Fig. 2. Skin of *Cyprinus carpio* six days after infection in salt water; E = epidermis, CC = club cells, Tr = trophonts of *Ichthyophthirius multifiliis*, V = opening of club cell; H&E staining; scale bar = 150 μm

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
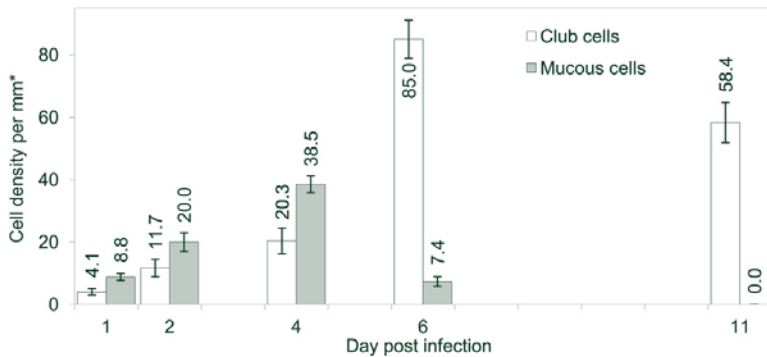

Fig. 3. Densities of club- and mucous cells (mean ± standard deviation) in the epidermis of *Cyprinus carpio* in salt water days 1–11 post infection with *Ichthyophthirius multifiliis*; *determined on 1-mm long transect covering the entire thickness of the epidermis
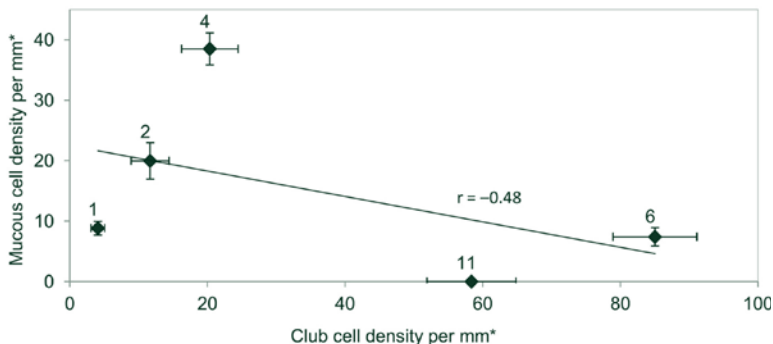

Fig. 4. Correlation on cell densities between club- and mucous cells of *Cyprinus carpio* (mean ± standard deviation) in sequential days post infection with *Ichthyophthirius multifiliis* (corresponding day shown above the marker); *determined on 1-mm long transect covering the entire thickness of the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

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ABSTRACT: We describe an unusually high infection rate of *Gyrodactylus salaris* Malmberg in juvenile Atlantic salmon *Salmo salar* L. of Baltic Sea origin, which are generally believed to be more resistant to *G. salaris* than East Atlantic salmon populations. Based on analyses of mitochondrial (complete cytochrome oxidase 1 [CO1] gene, 1548 bp) and nuclear (ADNAM1, 435 bp; internal transcribed spacer [ITS] rDNA region, 1232 bp) DNA fragments, the closest relatives of the characterized Estonian *G. salaris* strain were parasites found off the Swedish west coast and in Raasakka hatchery, Iijoki (Baltic Sea, Finland). Analyses of 14 microsatellite loci of the host *S. salar* revealed that approximately 40% of studied fish were triploids. We subsequently identified triploid Atlantic salmon of Baltic origin as more susceptible to *G. salaris* infection than their diploid counterparts, possibly due to compromised complement-dependent immune pathways in triploid salmon. This is in accordance with earlier studies that have shown elevated susceptibility of triploids to various viral or bacterial pathogens, and represents one of the first reports of increased susceptibility of triploid salmonid fish to an ectoparasite. However, further experimental work is needed to determine whether triploid Atlantic salmon is generally more susceptible to *G. salaris* compared to their diploid counterparts, irrespective of the particular triploidization method and population of origin.

KEY WORDS: Atlantic salmon · *Gyrodactylus salaris* · Pathogen susceptibility · Triploid · Microsatellites · Baltic Sea

INTRODUCTION

Culture of triploid fish (i.e. individuals carrying 3 sets of chromosomes) has been used over the last 30 yr as effective way of controlling sexual development in the fish farming industry (Dunham 2004). In salmonid aquaculture, triploidy has 2 main benefits: (1) it increases growth, carcass yield, and flesh quality by inhibiting gonadal development (Chourrout 1986, Dunham 1996, Gregory 2007); (2) in combination with other genetic manipulations it can prevent crossing of native wild fish with genetically modified or non-native stock (Dunham 2004, Gregory 2007). However, triploid salmon often exhibit higher mortality rates and higher incidence of deformities (e.g. shortened opercula, jaw abnormalities), reduced gill surface area, and impaired oxygen carrying capacity due to the altered size and shape of the red blood cells (O'Flynn et al. 1997, Sadler et al. 2001). Triploidy can also affect the pathogen susceptibility/resistance, and, in general, triploid salmonids appear to be less resistant to various pathogens than diploid fish (Dunham 2004). For example, triploid rainbow trout *Oncorhynchus mykiss* Walbaum are more susceptible to bacterial gill disease compared to diploid trout (Yamamoto & Iida 1994). Similarly, Jhingan et al. (2003) found that triploid coho salmon *O. kisutch* Walbaum were more susceptible to vibriosis than their diploid counterpart. However, several studies have failed to de-

tect significant differences in susceptibility between diploid and triploid fish. For example, both diploid and triploid rainbow trout exposed to *Vibrio ordalii*, *Aeromonas salmonicida*, or infectious hematopoietic necrosis virus demonstrated similar mortality rates (Yamamoto & Iida 1995). Similar mortality rates between diploid and triploid Atlantic salmon were also observed after experimental infection with *Renibacterium salmoninarum* (Bruno & Johnstone 1990).

Gyrodactylosis is a parasitic disease of salmonid fishes caused by the viviparous ectoparasite monogenean flatworm *Gyrodactylus salaris*, which belongs to the *G. wageneri* species-group, of the subgenus *Limnonephrotus* (family Gyrodactylidae, Malmberg, 1957). Due to 'hyperviviparity' (also known as a 'Russian doll' style of reproduction), combined with a rapid generation time, it reproduces fast, and in a matter of weeks a single worm can produce thousands of progeny (Bakke et al. 2007, Buchmann 2008). *G. salaris* damages fish not only by consuming mucus and epithelial cells of the host but also by piercing the fish epithelium with its hooklets, compromising its osmoregulatory function, and leaving it vulnerable to fungal and bacterial infections. Importantly, Atlantic salmon populations exhibit marked differences in susceptibility to *G. salaris* infection, with populations from the Atlantic and the White Sea coasts exhibiting higher infection rates and increased mortality, while salmon populations from the Baltic basin are more resistant to the parasite (Bakke et al. 1992, 2002, Rintamäki-Kinnunen & Valtonen 1996, Dalgaard et al. 2003, Kuusela et al. 2009). The most severe *G. salaris* outbreaks have been observed in Norway, where epidemics of this parasite have devastated salmon stocks >46 rivers over the last 25 yr (Johnsen & Jensen 1991, Johnsen et al. 1999, Bakke et al. 2007, Kuusela et al. 2007, 2009, Buchmann 2008). Despite the fact that Baltic salmon are generally believed to be more resistant to *G. salaris*, the susceptibility level varies among populations. For example, salmon populations from the Luleälven and Indalsälven rivers in Sweden (Bakke et al. 2002, 2004, Dalgaard et al. 2003) have been shown to be more susceptible to *G. salaris* infection than salmon populations from the Neva river in Russia and Tornio/Torne river along the border of Finland and Sweden (Bakke et al. 1990, 1992, Anttila et al. 2008). Moreover, the severity of infection also depends on the particular parasite strain (Lindenstrøm et al. 2003, Jørgensen et al. 2007, Kuusela et al. 2007, Ziętara et al. 2010). For example, outbreaks associated with rare clones of *G. salaris* have been recorded on Baltic salmon in fish farms (e.g. Rintamäki-Kinnunen & Valtonen 1996, Kuusela et al. 2007). In a few cases it has been demonstrated that the farm parasites were species hybrids, such as *G. pomeraniae* × *G. lavareti* on

rainbow trout (Kuusela et al. 2008), or unusual backcross combinations of *G. salaris* in Denmark (Lindenstrøm et al. 2003), Poland, and Macedonia (Ziętara et al. 2010).

On 21 February 2008, a small number of *Gyrodactylus* sp. parasites were found during a routine parasitological inspection in a freshwater fish hatchery in northern Estonia among juvenile Atlantic salmon *Salmo salar* L. of 1+ yr old, originating from the Kunda river (Gulf of Finland, Baltic Sea). After 1 wk, the parasite prevalence reached 100%, and the intensity of infection was much higher (from 10s to several 100s of parasites per individual fin) than commonly observed in Baltic salmon (Rintamäki-Kinnunen & Valtonen 1996). This unusual level of *G. salaris* infection was alarming because of the possibility of a new, aggressively pathogenic parasite strain (Ziętara & Lumme 2002, Kuusela et al. 2007). We subsequently genetically characterized both the host and the parasite in an attempt to gain more detailed insights into this unusually high *G. salaris* infection in supposedly resistant Baltic salmon. Based on analyses of mitochondrial cytochrome oxidase 1 (CO1) fragments and the variable nuclear marker ADNAM1, the parasite was genetically closely related to the *G. salaris* strains from the Gulf of Bothnia (Baltic Sea), and Swedish west coast (North Sea). Analyses of 14 microsatellite markers of the host confirmed its Baltic origin; however, it also revealed that a large proportion (ca. 40%) of juvenile salmon carried 3 sets of chromosomes (i.e. were triploid) and the number of *G. salaris* found on triploid individuals was significantly higher than on their diploid counterparts.

MATERIALS AND METHODS

Sample collection and examination. On 21 February 2008, a small number of *Gyrodactylus* sp. parasites were found during a routine parasitological inspection in a freshwater fish hatchery in northern Estonia (n = 4). In one particular tank 10 parasites per pectoral fin were observed in 2 examined juvenile Atlantic salmon of 1+ yr old (Kunda river origin). On 27–28 February 2008, a total of 586 hatchery-reared juvenile salmon (1+ yr old) comprising 49 full-sib and half-sib families were sampled from the abovementioned 2 m diameter fish tank. This tank consisted of 1+ yr old Atlantic salmon of the smallest size, as these fish had been size-selected during routine hatchery practice (mean length: 24.12 ± 9.94 mm; mean weight: 12.95 ± 1.94 g). These fish were produced from the captive broodstock of Kunda river origin in autumn 2005 using a standard 'dry' artificial fertilization method. The fish were killed using MS-222 over-

dose, and the right pectoral fin of every individual was stored separately in 96 % ethanol, while the left pectoral fin was preserved in RNAlater® (Ambion) for future gene expression analysis. Pectoral fins were chosen for subsequent analysis because *G. salaris* is most frequently (80 %) found on pectoral fins in Baltic salmon (Rintamäki-Kinnunen & Valtonen 1996). The total number of parasites present on both sides of the alcohol-preserved pectoral fin was counted under a dissection microscope (10× magnification). In addition, a small number of pectoral and pelvic fins (n = 32) were also examined from the fresh tissue using light microscopy (Leica CME) at 400× to 1000× magnification (see video of live *G. salaris* in Supplement 1, available at www.int-res.com/articles/suppl/d091p129_supp1/).

DNA sequence analysis of parasite. DNA was isolated from single parasite specimens collected from individual hosts according to Ziętara et al. (2000). For molecular identification of the species and for phylogenetic characterization of the parasite, we sequenced the ITS rDNA with ITS1F and ITS2R primers (1232 bp), a 1623 bp long fragment encompassing complete mitochondrial cytochrome CO1 with Trp1F and Thr1R primers, and a 435 bp long variable nuclear marker ADNAM1 with InsF and InsR primers (Ziętara et al. 2006, Kuusela et al. 2007, 2009). These 3 loci have been successfully used to characterize both the inter- and intra-specific relationships within the genus *Gyrodactylus* (Ziętara & Lumme 2002, Kuusela et al. 2007). Altogether, 2 ind. parasites collected from 2 different hosts were sequenced for the ITS; 3 parasites collected from 3 different hosts were sequenced for mtDNA CO1; and 9 parasites collected from 5 hosts were sequenced for nuclear anonymous DNA marker (ADNAM1). In addition, 32 parasites collected from 8 salmon were screened for ADNAM1 using agarose gel electrophoresis (Ziętara et al. 2006).

The mitochondrial and variable nuclear marker sequences were compared with earlier published *Gyrodactylus salaris* CO1 and ADNAM1 sequences (Kuusela et al. 2007, 2009). For clarification of isolated parasite strain genetic relationships with other strains, a Neighbor-joining tree (NJ tree) based on mtDNA CO1 sequence using maximum composite likelihood distance was constructed, with 1000 bootstrap replicates. The calculations were performed using the MEGA 4 program package (Tamura et al. 2007). The tree includes all well-characterized strains (complete CO1) of *G. salaris* found on *Salmo salar*, and the most close relatives on rainbow trout *Oncorhynchus mykiss* and Ohrid trout *S. letnica* Karaman (Kuusela et al. 2007). The tree is rooted with a sequence from a parasite on European grayling *Thymallus thymallus* L. from Hnilec river, Slovakia (Plaisance et al. 2007). The se-

quences of *G. salaris* were deposited in GenBank under accession numbers GU187353 (ITS rDNA), GU187354 (mtDNA CO1), and GU187355 (ADNAM1).

Microsatellite DNA analysis of host. After the examination of the parasite, host DNA was extracted from 111 Atlantic salmon specimens using the right pectoral fin clips according to Elphinstone et al. (2003), with slight modifications as described in Tonteri et al. (2009). Three separate multiplex polymerase chain reactions (PCR) were used to amplify 14 polymorphic Atlantic salmon microsatellites as described in Tonteri et al. (2009). PCR products of each of the 3 multiplexes were pooled together and genotyped using an ABI Prism 3130xl Genetic Analyzer (Applied Biosystems). The internal size standard GS600LIZ (Applied Biosystems) was used to define microsatellite allele sizes. DNA fragments were analyzed and genotypes were scored with GENEMAPPER 4.1 software (Applied Biosystems). Based on the multi-locus genotype profiles of microsatellite loci, it was possible to efficiently separate diploid and triploid individuals, as diploid specimens possessed either 1 or 2 alleles per locus, while triploid fish exhibited 3 alleles in at least 1 locus out of 14 (Supplement 2, available at www.int-res.com/articles/suppl/d091p129_supp2.pdf). Importantly, we never observed more than 3 alleles per locus, which might indicate contamination of the samples (i.e. mixture of 2 or more diploid individuals). In addition, we repeated DNA isolation according to Laird et al. (1991) using the left pectoral fin and re-genotyped ca. 10 % of triploid individuals to evaluate the repeatability of multi-locus genotype profiles.

Statistical analyses. The difference in number of parasites on the upper and lower sides of the pectoral fin (n = 586), as well as on the pectoral and pelvic fin for a subset of salmon individuals (n = 32), was tested using a non-parametric Wilcoxon signed-rank test. The dependence of the intensity of infection (number of *Gyrodactylus salaris* on the right pelvic fin of the host) on the host ploidy level was tested using a non-parametric Mann-Whitney test. As the growth rates of diploid and triploid fish are rarely similar (Dunham 2004), we tested whether the size of diploid and triploid 1+ yr old salmon juveniles differ from each other using a non-parametric Mann-Whitney test. All tests were carried out using SPSS for Windows, version 11.

RESULTS

Parasite abundance

The estimated prevalence of infection was 100 %, and mean intensity of *Gyrodactylus salaris* was 85

parasites per pectoral fin. The total number of *G. salaris* counted on a single pectoral fin of the host varied from 1 to 855 across 586 ind., and 147 juvenile salmon (25%) had >100 parasites per fin (Fig. 1). The number of parasites was higher on the upper side of the pectoral fin compared to the lower side (median: 31 and 22, respectively; Wilcoxon signed-rank test, $p < 0.001$). There was also significant correlations between the number of parasites found on the upper and lower side of the pectoral fin (n = 586, Spearman's rank correlation coefficient $r_S = 0.402$, $p < 0.01$) and between the number of parasites on pelvic and pectoral fins (n = 32, Spearman's rank correlation coefficient $r_S = 0.430$, $p < 0.05$). More parasites were observed on pectoral fins compared to pelvic fins (n = 32) (median: 55 and 31, respectively; Wilcoxon signed-rank test, $p < 0.01$).
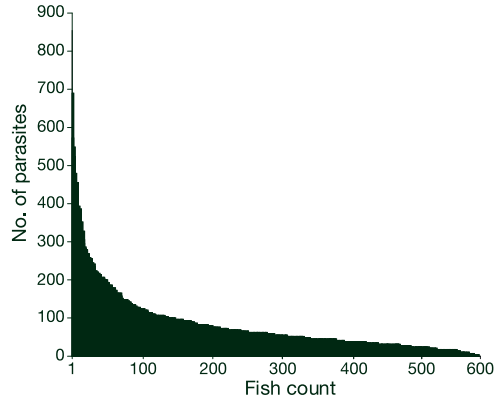
Fig. 1. Distribution of parasites in 586 studied Atlantic salmon (number of *Gyrodactylus salaris* on the right pelvic fin of the host)

Molecular identification and phylogenetic characterization of the parasite

Based on analyses of ITS rDNA sequence, the parasite was identified unambiguously as *Gyrodactylus salaris* Malmberg. When mitochondrial CO1 sequences were compared with earlier *G. salaris* sequences (Kuusela et al. 2007, 2009), the mtDNA CO1 haplotypes genetically closest to the Estonian *G. salaris* were parasite strains from Raasakka hatchery, Iijoki, Finland, and from the Genevadsån river, Swedish west coast (Fig. 2). The CO1 sequence of the Estonian *G. salaris* differed from these strains by only a single nucleotide. The nuclear ADNAM1 genotype of Estonian strain was S4 (TMRTYRTAT, consisting of alleles TCATTGTAT [BS5] and TAGTCATAT [WS3]). This ADNAM1 combination and the allele BS5 have been found before in parasites on the Swedish west coast (Fig. 2). *G. salaris* strains found in landlocked salmon populations from Lakes Ladoga and Onega, on the other hand, were genetically more distant from the Estonian strain (Kuusela et al. 2007, 2009). More detailed information about the relationships between different ADNAM1 alleles can be found in Kuusela et al. (2007).
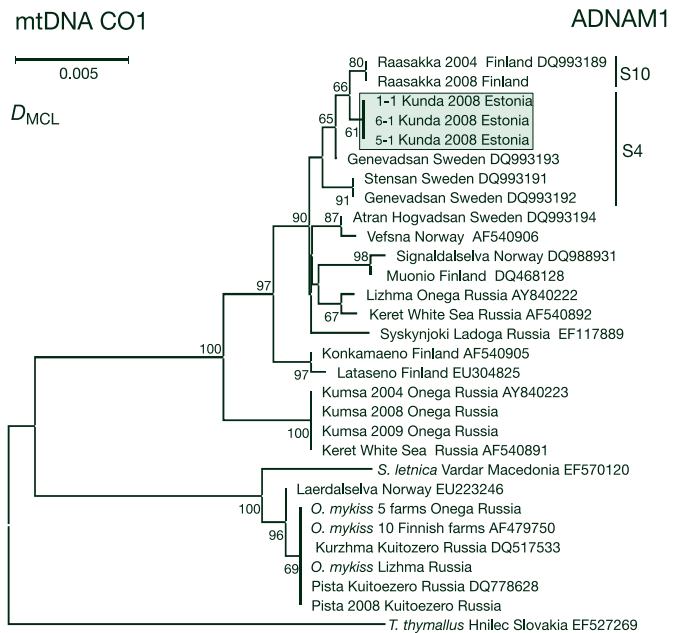

Fig. 2. Neighbor-joining tree based on maximum composite likelihood distance (D_{MCL}) of the mtDNA CO1 gene (1548 bp). Haplotypes of Estonian *Gyrodactylus salaris* strain are marked in grey. The tree includes all well-characterized strains of *G. salaris* found on *Salmo salar*, and the closest relatives on rainbow trout *Oncorhynchus mykiss* and Ohrid trout *S. letnica* (Kuusela et al. 2007). The tree is rooted with a sequence from a parasite on European grayling *Thymallus thymallus* from the Hnilec river, Slovakia (Plaisance et al. 2007). Two nuclear genotypes of ADNAM1 marker (S4 and S10) are shown in the upper right corner

Ploidy of host and elevated susceptibility of triploid Atlantic salmon

Microsatellite analyses of Atlantic salmon confirmed its Baltic origin (data not shown), but also revealed that about 40% of analyzed individuals possessed 3 alleles at least in 1 locus out of 14 (Supplement 2). Repeated DNA extraction and re-genotyping of about 10% of specimens indicated that these multi-locus genotype profiles were highly repeatable and never possessed >3 locus alleles per marker. As a result, we inferred that juvenile salmon exhibiting 3 alleles per locus most likely carry 3 sets of chromosomes (i.e. were triploid). Subsequent evaluation of multi-locus genotypes revealed that triploid individuals most likely originated from several families as they possessed a large number of different allele combinations (Supplement 2). For example, triploid individuals carried altogether 10 alleles at the most variable microsatellite locus SSOSL311, which is consistent with multiple triploid family scenario. The parasite load was significantly higher in triploid salmon than in diploid fish (Fig. 3; median: 65 and 46, respectively; Mann-Whitney test, p = 0.003). The length and weight, on the other hand, did not differ between triploid and diploid fish (Mann-Whitney test, p values of both tests > 0.16).

DISCUSSION

In the present study we genetically characterized both the host and parasite to further understand the unusually high level of *Gyrodactylus salaris* infection on Baltic salmon in an Estonian fish hatchery. Such elevated levels of parasite abundance were rather unexpected as Baltic salmon is generally believed to be re-

sistant to *G. salaris* (Bakke et al. 1992, 2002, Rintamäki-Kinnunen & Valtonen 1996, Anttila et al. 2008). For example, the number of parasites observed on Baltic salmon in Finnish hatcheries has been typically very low (1 to 5 per fish in most cases; Rintamäki-Kinnunen & Valtonen 1996). However, mean intensity of *G. salaris* observed in an Estonian fish hatchery was as high as 85 parasites per pectoral fin. The closest relatives of the Estonian *G. salaris* strain were parasites found in Genevadsån, Swedish west coast (North Sea), and in Raasakka hatchery, Iijoki (Gulf of Bothnia, Finland), showing one nucleotide difference in the CO1 region of mtDNA. Moreover, the combination of alleles at the nuclear ADNAM1 marker was identical to that found in parasites on the Swedish west coast. Therefore, it is likely that the described parasite strain is native to the Baltic Sea and exists in the wild salmon populations in Estonia, as the Atlantic west coast parasites found in Sweden are believed to originate from the Baltic (e.g. Hansen et al. 2003). Consequently, analyses of *G. salaris* collected in Estonian rivers could provide further information about distribution and prevalence of this strain. To our knowledge, *G. salaris* has never been reported in Estonian rivers, suggesting that it is generally harmless in the wild (Kuusela et al. 2009).

Subsequent analyses of microsatellite loci of the host *Salmo salar* revealed that about 40% of the analyzed individuals were triploids, and we observed highly significant differences in parasite load between triploid and diploid fish suggesting that high *Gyrodactylus salaris* infection rate in this particular case was most likely triggered by the triploidy of the host. Hence, it is possible that increased *G. salaris* susceptibility of triploid Atlantic salmon is related to compromised complement-dependent immune pathways in triploid fish, as several earlier studies have demonstrated the important role of complement (C3) in protection against *G. salaris* in salmonid fishes (Moore et al. 1994, Buchmann 1998, Harris et al. 1998, Bakke et al. 2002). As a result, it may take a longer time to recover complement activity for triploid salmon (Langston et al. 2001). This is also in accordance with earlier studies that have demonstrated elevated susceptibility of triploid salmonids to various viral or bacterial pathogens (Yamamoto & Iida 1994, 1995, Jhingan et al. 2003, Dunham 2004). However, triploidy of the host does not always result in increased parasite abundance, as similar susceptibility to *Gyrodactylus* sp. has been observed in diploid and triploid tench *Tinca tinca* L. (Piačková & Flajšhans 2006).

How can the occurrence of triploid fish in this hatchery be explained? Triploidy in fishes is commonly induced artificially by forcing retention of the second polar body after normal fertilization that yields fusion of nuclei from egg, sperm, and second polar body
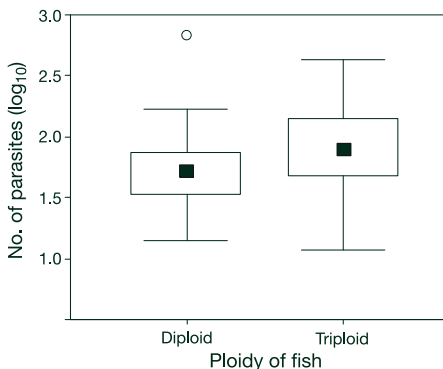

Fig. 3. Number of *Gyrodactylus salaris* parasites ($\log_{10}$ scale) in diploid and triploid host *Salmo salar* (Mann-Whitney test, p = 0.003). ■: median; ○: outlier. Open rectangle and whiskers 25th and 75th quartiles and non-outlier range, respectively

(Chourrout 1980, 1984, Lou & Purdom 1984). The number of different alleles at a specific locus in triploids depends on the genotype of both parents and the crossing-over frequency during the first meiotic division of primary oocytes. Thus, the triploids can possess either 1, 2, or even 3 alleles at a specific locus. Retention of the second polar body can be achieved by applying hydrostatic pressure, anesthetics, temperature, pH, or chemical shocks shortly after fertilization (Thorgaard et al. 1981, Chourrout & Itskovich 1983, Benfey & Sutterlin 1984, Cassani & Caton 1986, Curtis et al. 1987, Ueda et al. 1988, Johnstone et al. 1989). Alternatively, triploids can be obtained by fertilization of diploid eggs of tetraploid fish by haploid sperm of diploid fish (Chourrout et al. 1986). However, neither of these techniques has ever been used in the Estonian fish hatchery in question. Triploids can also be produced when hybridization and backcrossing occurs between Atlantic salmon and brown trout *Salmo trutta* L. (e.g. Johnson & Wright, 1986). This can be excluded, however, as we did not observe any trout-specific microsatellite alleles within the 1+ yr old fish, and additional genetic analyses of the parents (47 females and 33 males) did not reveal any alleles diagnostic for trout (data not shown). In addition, microsatellite analyses of the subsequent hatchery cohort (n = 722) created using the same parental fish (eggs fertilized in 2007) did not reveal any triploid specimens (data not shown). As a result, we were not able to identify the exact mechanism of 'spontaneous' production of triploid Atlantic salmon from the diploid salmon broodstock. However, a low frequency of 'spontaneous' occurrence of triploid individuals in fish has been previously reported (Chourrout 1980). As to the question of why the infection level was also elevated in diploid juveniles, we suggest that by continuous exchange of the worms, the more resistant diploid fish in the same tank were probably loaded by the surplus of parasites, which explains the high overall intensity of infection.

In the present study we used highly variable microsatellite markers to determine the ploidy of juvenile salmon, but there exist a large number of alternative methods that have been used previously for detection of triploid individuals. Traditionally, triploid fish have been identified using karyotyping, cell-size measurement with a Coulter Counter Channelyzer, or by using blood smears, silver staining of nucleoar organizing regions (NORs), or flow cytometry (Dunham 2004). Earlier works also demonstrated the utility of dimeric isozymes for determination of triploid individuals (Sugama et al. 1988, Crozier & Moffet 1990). The use of highly polymorphic microsatellite markers for detection of triploid individuals, however, has a number of advantages compared to less variable isozymes and other traditional methods, such as the ability to use fixed or old samples of any tissue for DNA isolation, the possibility for non-invasive sampling, and the availability of a large number of polymorphic markers in many species. On the other hand, the limitations of the use of microsatellite markers for triploid detection include potential misclassification of triploid fish as diploids (type II error) due to low variability or when a relatively low number of markers is used. However, in the present study we used 14 highly polymorphic microsatellite markers consisting in total of 100 alleles, and, therefore, it is unlikely that we have misclassified triploid fish as diploids. Furthermore, misclassification would make our results even more conservative, as this would reduce the difference in parasite abundance between triploid and diploid fish.

In conclusion, we described an unusual *Gyrodactylus salaris* infection in a hatchery population of Baltic salmon, phylogenetically characterized the *G. salaris* strain showing that it was genetically closest to other strains of Baltic origin, and demonstrated that triploid Atlantic salmon were more susceptible to *G. salaris* infection than their diploid counterparts, probably due to compromised complement-dependent immune pathway. This is in accordance with earlier studies that have shown elevated susceptibility of triploids to various viral or bacterial pathogens and represents one of the first reports of increased susceptibility of triploid salmonid host to an ectoparasite. However, future work is needed to determine the generality of our finding, e.g. whether triploid Atlanic salmon is generally more susceptible to *G. salaris* compared to their diploid counterparts, irrespective of the particular triploidization method (e.g. andro- or gynogenesis induced by physical or chemical factors) and population of origin.

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Figure 3. Epidermal hyperplasia in caudal fin of koi carp, *Cyprinus carpio*. First day of study.
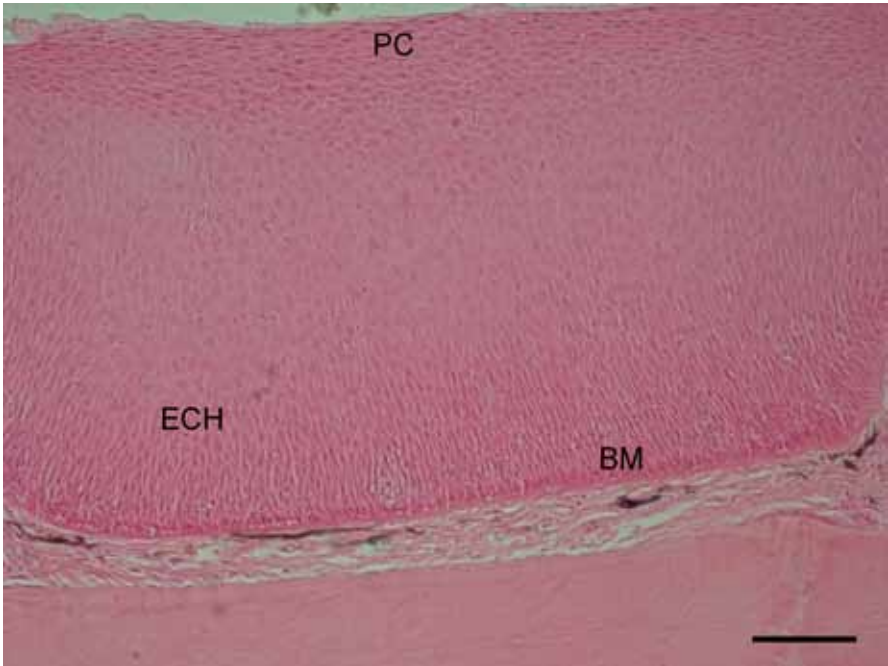
Figure 4. Extensive hyperplasia of epithelial cells in caudal fin of koi carp, *Cyprinus carpio*. Note the absence of club cells and mucous cells. Epithelial cells are smaller in size and tightly packed (ECH). The basal layer (BM) forms pegs and is slightly scalloping. PC– epithelial cells. First day of study. (H&E, bar = 50 μm).
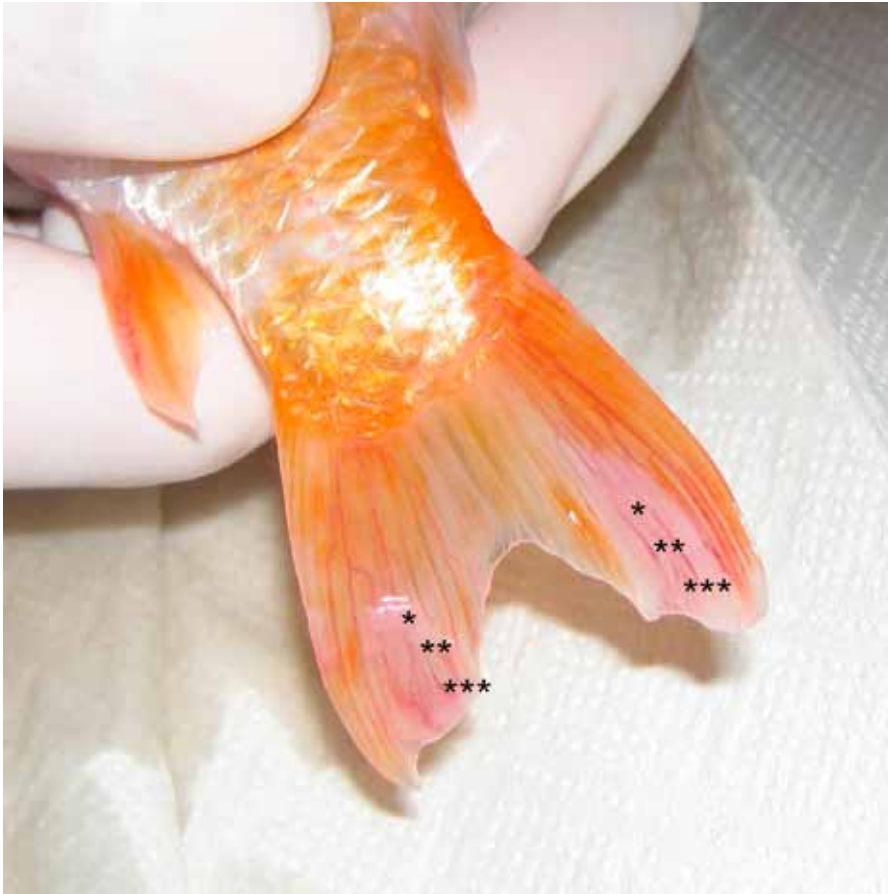
Figure 5. Regressed epidermal hyperplasia in warm water in caudal fin of koi carp, *Cyprinus carpio*, 62 days after rising water temperature (* Part of hyperplastic areas; ** Part of areas after desquamation of surface; *** Part of regenerating areas).
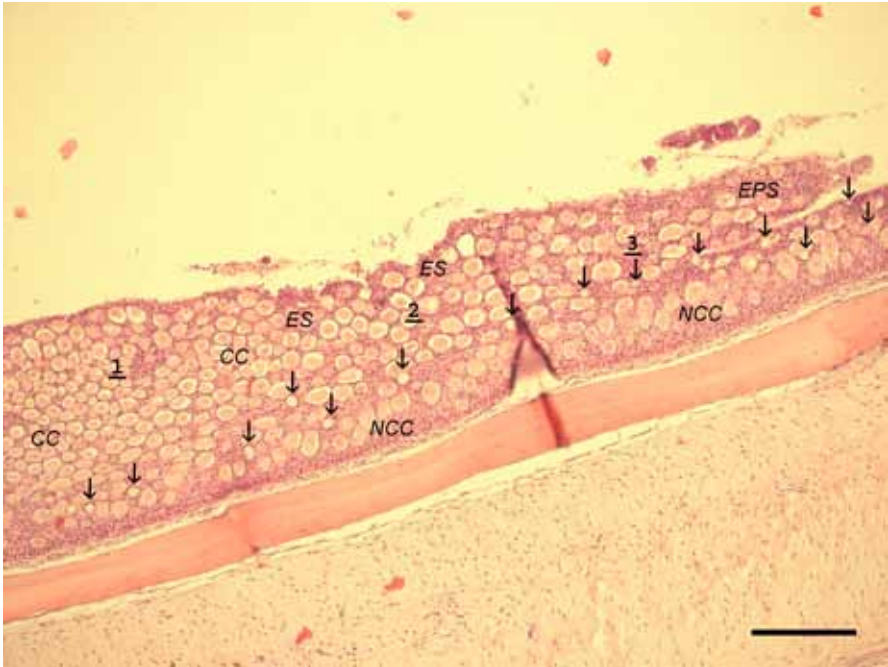
Figure 6. Epidermal hyperplasia medial area of caudal fin of koi carp, *Cyprinus carpio*, 62 days after rising water temperature. Loss of epithelial integrity (ES) and eventual desquamation (EPS): 1. – section of hyperplasia with high proliferation of eosinophilic enlarged club cells (CC) and undamaged 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). (H&E, bar = 100 μm).
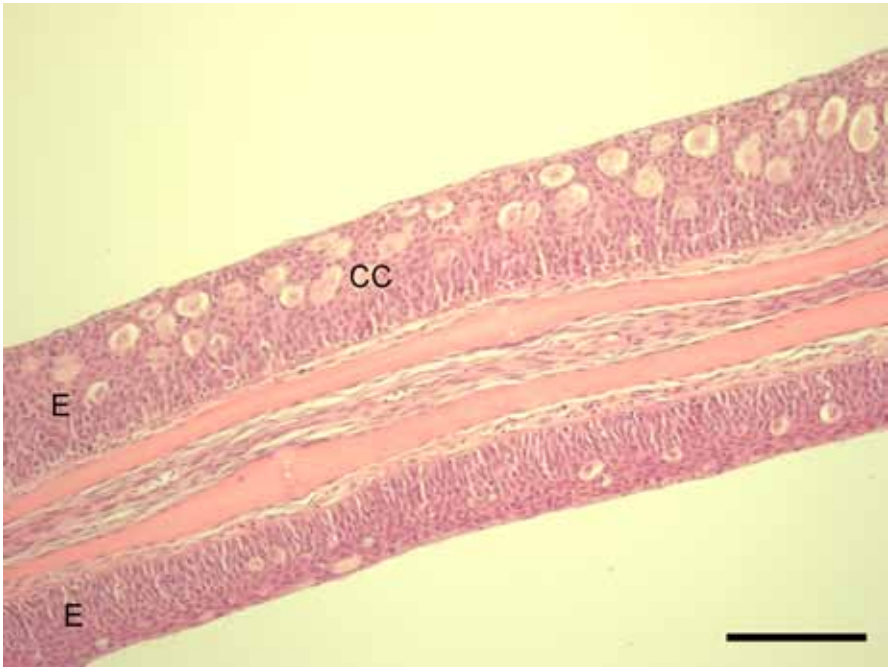
Figure 7. Epidermal hyperplasia area after desquamation of surface of caudal fin of koi carp, *Cyprinus carpio*, 62 days after rising water temperature. E- Epidermis. CC - Club cells. (H&E, bar= 100 μm).
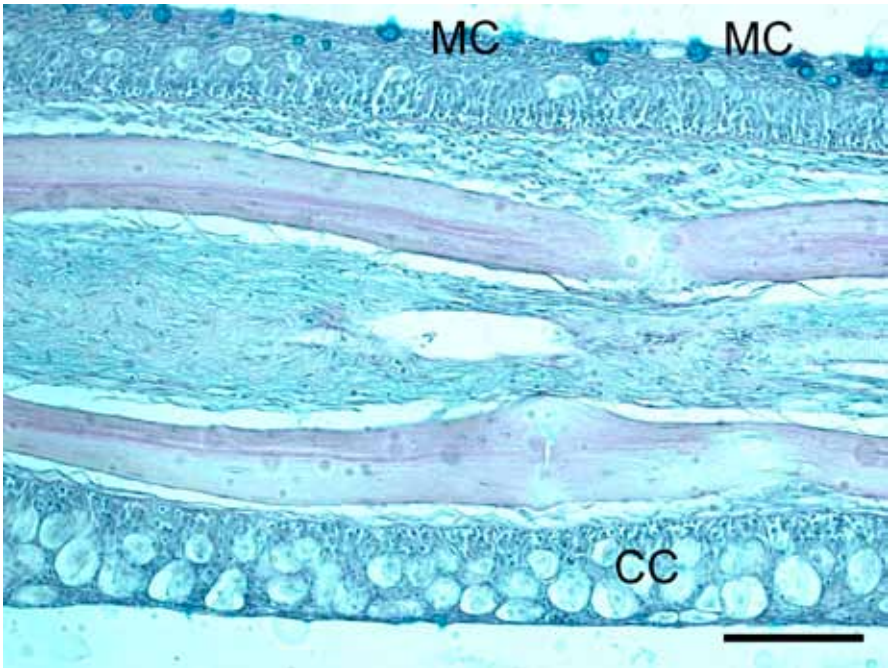
Figure 8. Epidermal hyperplasia area after desquamation of surface of caudal fin of koi carp, *Cyprinus carpio*, 62 days after rising water temperature. MC - PAS positive mucous cells, CC - Club cells. (PAS, bar = 100μm).

Figure 9. Epidermal hyperplasia area after desquamation, distal section of caudal fin of koi carp, *Cyprinus carpio*, 78 days after rising water temperature. (H&E, bar = 150 μm).

Figure 10. Epidermal tissue in apical section of caudal fin of koi carp, *Cyprinus carpio*, 78 days after rising water temperature. Epidermis with few mucous and club cells (H&E, bar = 150 µm).
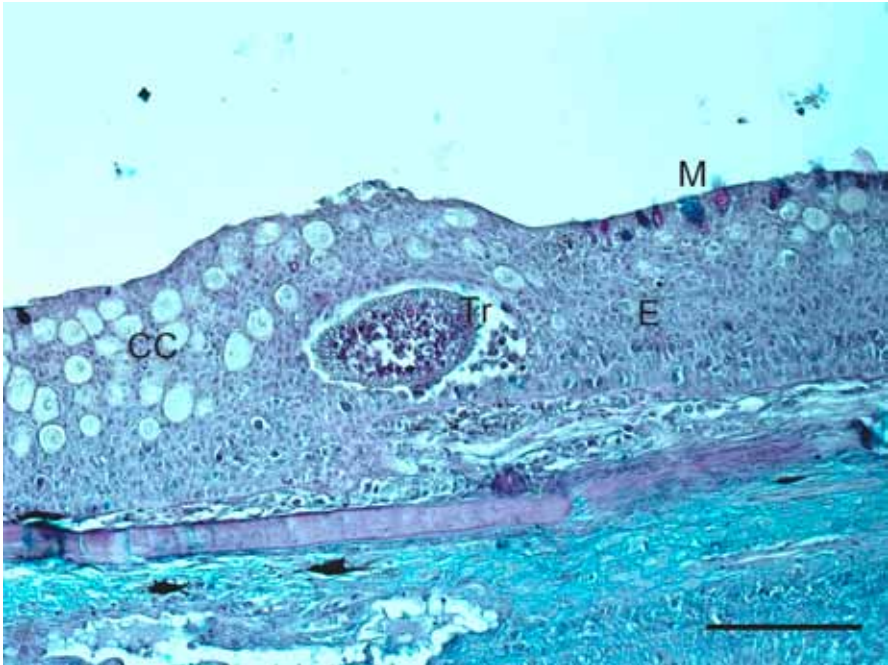
Figure 11. Skin of common carp, *Cyprinus carpio*, four days after infection; E = epidermis, Tr = trophont of Ichthyophthirius multifiliis, CC = club cells, M = mucous cells (PAS, bar = 150 µm).
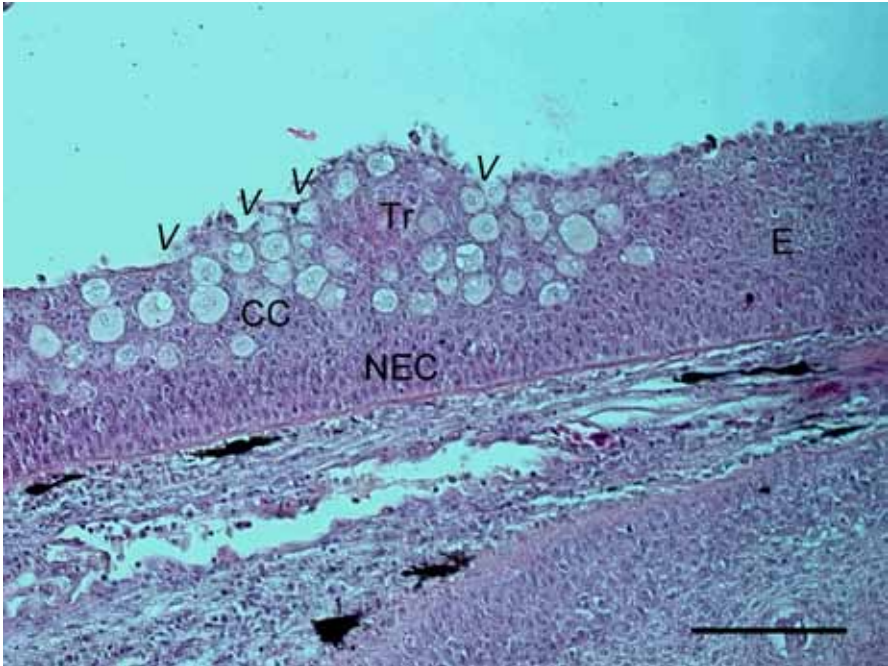
Figure 12. Skin of common carp, *Cyprinus carpio*, six days after infection in salt water; E = epidermis, CC = club cells, Tr = trophonts of *Ichthyophthirius multifiliis*, *V* = opening of club cell; Absence of mucous cells were seen. (PAS, bar = 150 µm).
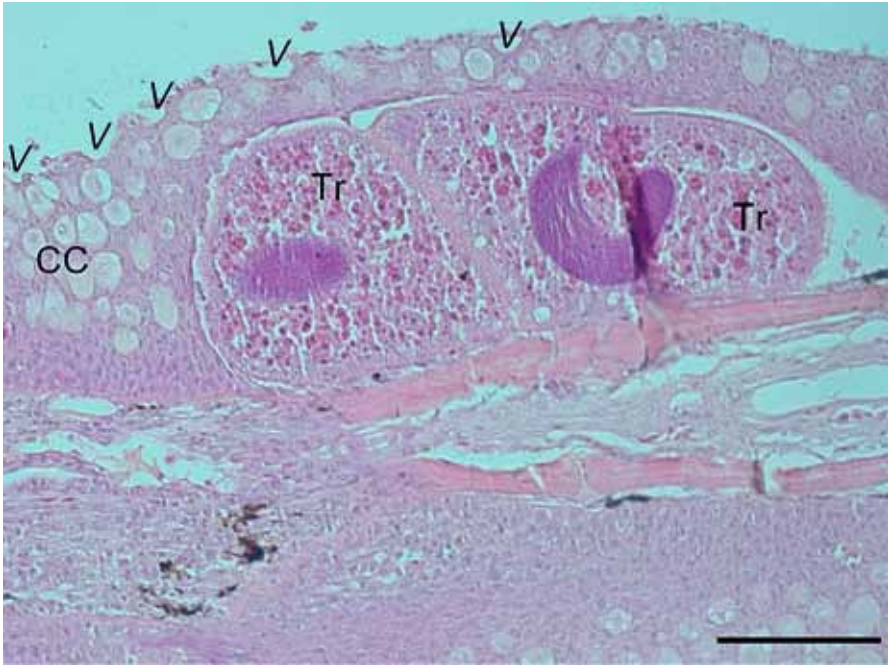
Figure 13. Skin of common carp, *Cyprinus carpio*, six days after infection in salt water; E = epidermis, CC = club cells, Tr = trophonts of *Ichthyophthirius multifiliis*, *V* = opening of club cell. (H&E, bar = 150 μm).
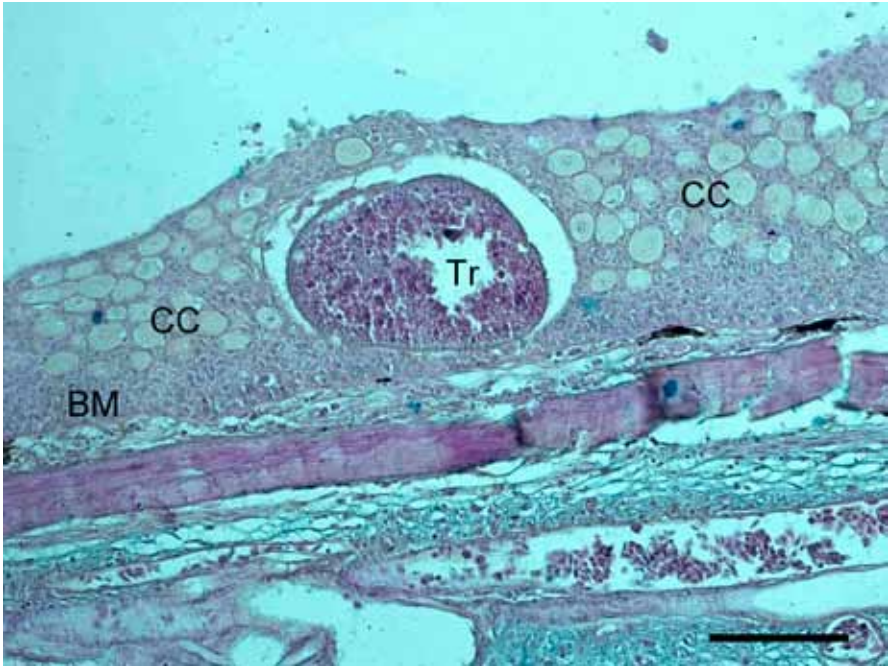
Figure 14. Skin of common carp, *Cyprinus carpio*, 11 days after first infection in fresh water; CC = previously activated club cells, Tr = newly reinvading trophont of *Ichthyophthirius multifiliis*; BM= basement membrane. (PAS, bar = 120 μm).

CURRICULUM VITAE

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Veterinary medicine Studies

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Russian: spoken (good), written (good)

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Supervised Masters theses:
Härmo Hiiemäe. 2010. Supervisors: Prof. Tiit Paaver, DVM Priit
Päkk. The effectivness of anesthetics to European eel (*Anguilla
anguilla*) tranquilization in aquaculture trials". Estonian University of
Life Sciences, Institute of Veterinary Medicine and Animale Sciences.

Supervised Diploma theses:
Triin Engmann.2011. Supervisor: DVM Priit Päkk „Cultivation of tropical
ornamental fish in Estonia". Estonian Maritime Academy; Marianne
Kiholane. 2011. Supervisor: DVM Priit Päkk. „First Experiences of
Holding the Doctor Fish (Garra rufa) in Estonia". Estonian Maritime
Academy.

Training activities:
VL.0144 Pet fish in aquarium;
VL.0180 Medicine of exotic animals;
VL.0827 Aquacultivation technologies, fish and crayfish diseases and hygiene;
VL.0351 Fish farming;
VL.0166 Ichtyopathology and Aquatic toxicology;
VL.0474 Fish farming;
Laboratory works (2005–2011).

Special courses:
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9–11 May.
Workshop "Common carp as a model organism for biological studies: propagation, husbandry and health control". Institute of Ichthyobiology and Aquaculture in Golusz, Poland.

2008
10–11 January.
The course in "Laboratory Animal Science, special focus on Fish". Bergen, Norway.

2007
22 January–8 February.
Laboratory animal Science: C-category competence course, University of Tartu.

2006
16–18 August.
Summer School "Teaching in University", Arossa villa, Võrumaa, Eesti.

List of pulications:

1.1. Articles indexed by Thomson Reuters Web of Science

Ozerov M.Y., Lumme J., **Päkk P.**, Ristamäki P., Zietara M.S., Barskaya Y., Lebedeva D., Saadre E., Gross R., Primmer C.R., Vasemägi A. (2010). High *Gyrodactylus salaris* infection rate in triploid Atlantic salmon *Salmo salar*. *Diseases of Aquatic Organisms*, 91(2), 129–136. DOI: 10.3354/dao02242

Päkk, P., Hussar, P., Paaver, T. (2011). Alterations of club cells activity in epidermi of common carp, *Cyprinus carpio*, due to infection by *Ichthyophthirius multifiliis. Acta Ichthyologica et Piscatoria*, 41 (3), 185–192. DOI: 10.3750/AIP2011.41.3.06

1.2. Articles published in other peer-reviewed international journals with a registred code:

Päkk P., Hussar P., Järveots T., Paaver T. (2011). Club cells active role in epidermal regeneration after skin hyperplasia of koi carp *Cyprinus carpio*. *AACL Bioflux* 4 (4), 455–462.

5.2. Published meeting abstracts, not indexed by Thomson Reuters Web of Science

Priit Päkk, Mariann Nõlvak, Tiit Paaver. (2009). Epidermal club cells: part of the innate immune system in common carp? In: Book of abstract: *Two day workshop on the ontogeny of fish immune system,* Copenhagen, Denmark, 4–5 November 2009, 19.

Päkk P., Paaver T., Järveots T. (2009). Cellular structure of epidermal hyperplasia (fish pox) in koi carp (*Cyprinus carpio* L) in final stage of long time regression in warm water. In: Abstract book: *13th European congress of ichthyology*, Klaipeda, Lithuania, 6–12 September 2009, 73.

Nõlvak M., **Päkk P.**, Paaver T. (2009). Variation in proliferation of the epidermal club cells in the epidermis of mirror carp *Cyprinus carpio* (L) by ichthyophthiriosis – part of innate defence or innate immunity system? In: Abstract book: *13th European congress of ichthyology*, Klaipeda, Lithuania, 6–12 September 2009, 86.

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Haridustee:

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1984–1990	Eesti Põllumajanduse Akadeemia, Veterinaaria teaduskond, veterinaaria
1980	Lähte Keskkool

Kraad: DVM

Teadustöö biosüstemaatika põhisuunad:
Bio- ja keskkonnateadused, ökoloogia ja füsioloogia
(Kalade füsioloogia ja tervishoid)

Juhendamisel kaitstud Magistritöö:
Härmo Hiiemäe. 2010.
Juhendajad: Prof. Tiit Paaver ja DVM Priit Päkk.
Anesteetikumide mõju Euroopa angerja (*Anguilla anguilla*) uinumisele kalakasvatuse katsetes. EMÜ, Veterinaarmeditsiini ja loomakasvatuse instituut

Juhendamisel kaitstud Diplomitööd:
Triin Engmann. 2011. Juhendaja: DVM Priit Päkk.
Dekoratiivkalade kasvatamine Eesti tingimustes. Eesti Mereakadeemia

Marianne Kiholane. 2011. Juhendaja: DVM Priit Päkk.
Esimestest kogemustest doktorkala (*Garra rufa*) kasvatamisest Eestis.
Eesti Mereakadeemia

Õppetöö:
VL.0144 Akvaariumiteadus;
VL.0180 Eksootiliste loomade meditsiin;
VL.0827 Vesiviljelustehnoloogiad, kalade ja vähkide haigused ning tervishoid;
VL.0351 Kalakasvatus;
VL.0166 Ihtüopatoloogia ja veeorgansmide toksikiloogia;
VL.0474 Kalakasvatus ja -varude rikastamine.
Laboritöö 2005–2011

Erialane enesetäiendus:
2011
9 –11.05   Tööseminar ”Karpkala kui mudelorganism
                          bioloogilistes uuringutes”. Golusz, Poola.

2008
10 –11.01   Katseloomateaduse kursus (katseloomad kalad).
                          Bergen, Norra.

2007
22.01 – 8.02   Katseloomateaduse kursus (C kompetents).
                          Tartu Ülikool, Tartu.

2006
16 –18.08   Suvekool „Õpetamine kõrgkoolis“.
                          Arossa villa, Võrumaa, Eesti.

VIIS VIIMAST KAITSMIST

MARIO ALBERTO LUNA DEL RISCO

BIOCHEMICAL METHANE POTENTIAL OF ESTONIAN SUBSTRATES AND
EVALUATION OF SOME INHIBITORS OF ANAEROBIC DIGESTION.
EESTI SUBSTRAATIDE BIOKEEMILISE METAANITOOTLIKKUSE POTENTSIAALI
MÄÄRAMINE JA ANAEROOBSE KÄÄRITAMISE MÕNEDE INHIBIITORITE UURIMINE.
Dr. **Kaja Orupõld**, prof. **Henri-Charles Dubouguier**, Dr. **Marta Carballa Arcos**
16. detsember 2011

VILLEM ARUOJA

ALGAE PSEUDOKIRCHERIELLA SUBCAPITATA IN ENVIRONMENTAL HAZARD
EVALUATION OF CHEMICALS AND SYNTHETIC NANOPARTICLES.
VETIKAD PSEUDOKIRCHERIELLA SUBCAPITATA KEMIKAALIDE JA SÜNTEETILISTE
NANOOSAKESTE KESKKONNAOHTLIKKUSE HINDAMISEL.
Dr. **Anne Kahru**, prof. **Henri-Charles Dubourguier**, prof. **Kalev Sepp**
19. detsember 2011

MARJE SÄREKANNO

ANALYSIS OF PRODUCTIVITY INDICATORS OF FIELD GROWN POTATO MERISTEM
PLANTS MULTIPLIED BY DIFFERENT METHODS.
ERI MEETODIL PALJUNDATUD JA AVAMAAL KASVATATUD KARTULI
MERISTEEMTAIMEDE PRODUKTIIVSUSE NÄITAJATE ANALÜÜS.
Dr. **Katrin Kotkas**, Dr. **Evelin Loit**
19. detsember 2011

VIVIKA MELTSOV

INFERRING FLORISTIC DIVERSITY FROM MODERN SEDIMENTARY POLLEN RECORDS.
TAIMESTIKU MITMEKESISUSE PEEGELDUMINE KAASAEGSETE
JÄRVESETETE ÕIETOLMUS.
Dr. **Anneli Poska**, Prof. **Tiiu Kull**
21. detsember 2011

PILLE SOOVÄLI

INTEGRATED PLANT DISEASE MANAGEMENT
IN SPRING BARLEY AND OAT PRODUCTION.
INTEGREERITUD TAIMEKAITSE KASUTAMINE ODRAL JA KAERAL.
Dr. **Mati Koppel**, Prof. **Marika Mänd**, Dr. **Ülle Lauk**
21. detsember 2011