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TIINA KORKEA-AHO

Epidermal Papillomatosis in Roach (*Rutilus rutilus*) as an Indicator of Environmental Stressors

Doctoral dissertation

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ABSTRACT

Epidermal papillomatosis is a disease often observed in feral and cultured fish species. Epidermal papilloma is a benign tumor which can be seen growing usually on the skin of fish. Several studies have noted that epidermal papillomatosis in fish is affected by contaminants in the aquatic habitat and it has been proposed as a bioindicator of environmental health in the aquatic habitats of some fish species. This thesis aims to evaluate the feasibility of using epidermal papillomatosis in roach as a bioindicator of environmental stressors. The thesis includes diagnostic studies which confirm histopathologically epidermal papillomatosis in roach. Field studies were conducted to determine the occurrence and prevalence of papillomatosis, as well as to reveal all confounding factors affecting these in feral roach populations, including environmental stressors. A methodology was developed to study epidermal papillomatosis in roach in experimental and field studies. Finally, the possible connection between environmental stressors and papillomatosis in roach was also investigated by experimental studies under laboratory conditions.

The histological results of this thesis show that epidermal papillomatosis in roach is a common epidermal hyperplasia and papilloma in fish, a benign neoplasm. Furthermore, field studies revealed that papillomatosis was highly aggregated in feral roach populations and with its occurrence following the theory of island biogeography in Finnish lakes. These results suggest that epidermal papillomatosis represent an infectious disease in roach. A field study that took in to account all confounding factors, including as spatial and temporal patterns of the disease, as well as fish length and sex, showed that epidermal papillomatosis in roach could provide a useful indicator of environmental stressors. The field study showed an average papillomatosis prevalence of 16.6 % in impact populations (mainly affected by industrial and sewage effluent) and 5.8 % in reference populations. Epidermal papillomatosis in roach was shown to be affected by abiotic stressors in an experimental study in which the intensity of papillomatosis was intensified by hypoxia and fluctuating water temperature. Furthermore, the intensity of epidermal papillomatosis was greater in male roach when exposed to effluents under laboratory conditions. These results confirm that the roach-papillomatosis system has potential for use as a bioindicator in the monitoring of environmental stressors.

Universal Decimal Classification: 591.2, 597.551.2, 639.2.09, 502.51, 504.5

CAB Thesaurus: fish diseases; skin; skin diseases; neoplasms; hyperplasia; papilloma; *Rutilus rutilus*; biological indicators; water pollution; effluents; pollutants; aquatic environment; stress; hypoxia; water temperature



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LIST OF ORIGINAL PUBLICATIONS

- I Korkea-aho, T.L., Vainikka, A., and Taskinen, J. 2006. Factors affecting the intensity of epidermal papillomatosis in populations of roach, *Rutilus rutilus* L., estimated as scale coverage. *J. Fish Dis.* **29**: 115-122.
- II Korkea-aho, T.L., Vainikka, A., Kortet, R., and Taskinen, J. 2007. Factors affecting between-lake variation in the occurrence of epidermal papillomatosis in roach (Cyprinidae). Submitted manuscript.
- III Korkea-aho, T.L., Partanen, J.M., Kiviniemi, V., Vainikka, A., and Taskinen, J. 2006. Association between environmental stress and epidermal papillomatosis of roach (*Rutilus rutilus* L.). *Dis. Aquat. Org.* **72**: 1-8.
- IV Korkea-aho, T.L., Partanen, J.M., Kukkonen J.V.K., and Taskinen J. 2007. Hypoxia increases intensity of epidermal papillomatosis in roach (*Rutilus rutilus*). *Dis. Aquat. Org.* In press.
- V Korkea-aho, T.L., Vehniäinen, E.-R., Kukkonen, J.V.K., and Taskinen J. 2007. The effects of treated effluents on the intensity of papillomatosis and HSP70 expression in roach. Submitted manuscript.



1	INTRODUCTION	10
2	LITERATURE REVIEW	12
2.1	Epidermal papillomatosis in fish.....	12
2.2	The etiology and epidemiology of epidermal papillomatosis in fish	14
2.2.1	Pathogens in fish papillomatosis	15
2.2.2	Papillomatosis and the host	15
2.2.3	Environment and interactions affecting papillomatosis in fish	16
2.3	Epidermal papillomatosis of fish as a bioindicator of environmental stressors.....	19
2.3.1	HSP70 as biomarker of environmental stress.....	21
3	OBJECTIVES	23
4	MATERIALS AND METHODS	25
4.1	Histology.....	25
4.2	Field studies	25
4.2.1	Papillomatosis occurrence and infection patterns	26
4.2.2	Prevalence and intensity of papillomatosis	28
4.3	Experimental studies.....	28
4.3.1	Oxygen deficiency and temperature increase as stressors related to papillomatosis	29
4.3.1	Exposure to pulp mill and municipal effluents.....	29
4.3.1	Statistical analyses in the experimental studies.....	31
5	RESULTS	32
5.1	Macroscopic pathology and histology	32
5.2	Field studies	34
5.2.1	Papillomatosis occurrence and infection patterns	34
5.2.2	Papillomatosis prevalence and intensity	34
5.3	Experimental studies.....	36
5.3.1	Oxygen deficiency and temperature increase as stressors related to papillomatosis	36
5.3.2	Exposure to pulp mill and municipal effluents.....	36
6	DISCUSSION	37
6.1	Epidermal papillomatosis in roach	37
6.2	Papillomatosis occurrence and infection patterns in feral roach populations	37
6.3	Prevalence and intensity of papillomatosis in roach	39
6.4	Hypoxia, temperature changes and papillomatosis in roach.....	41
6.5	Effluents and papillomatosis in roach	43
7	CONCLUSIONS	45
8	REFERENCES	47

APPENDIX: ORIGINAL PUBLICATIONS AND MANUSCRIPTS

1 INTRODUCTION

Epidermal papilloma is a neoplasm growing on the skin of fish. Epidermal papillomatosis occurs in many fish species, both in farmed and feral fish. The first epidermal papillomatosis in fish was reported as early as 1563, when farmed carp were afflicted by papillomatosis in Europe (Hofer 1906). The first known reports of papillomatosis in feral fish appeared much later in 1900. A chemical etiology for papillomatosis in feral fish was already suspected (Russell and Kotin 1957). Today epidermal papillomas are known to be one of the most frequently occurring benign skin tumours in fish (Harshbarger and Clark 1990; Harshbarger and Slatick 2001).

Epidermal papillomatosis is found throughout the world in freshwater and marine environments (Harshbarger and Clark 1990; Dethlefsen et al. 2000). Although epidermal papillomatosis is common in many fish species and the appearance of papillomas is similar across the species, there are many differences in the disease etiology across the species studied (Table 1). The cause of papillomatosis seems to be most likely viral in some species, though the viral agent varies between species. In some species, no viral etiology has been found, though papillomas seem in other cases to be more affected by chemical contaminants (Harshbarger and Clark 1990). Epidermal papillomatosis does not usually cause mortality in adult fish (Table 1). Moreover, epidermal papillomatosis is easy and cost-effective to study in most of the fish species. For these reasons, epidermal papillomatosis could serve as a bioindicator of environmental stressors for many fish species in which papillomatosis is known to be promoted by contaminants and other environmental stressors (Vethaak et al. 1992; Baumann et al. 1996). Typically, these earlier field studies have compared prevalence of papillomatosis (percentage of diseased fish in population, %) in populations sampled from contaminated and more pristine reference sites (e.g., Baumann et al. 1996).

One of the possible bioindicator species for environmental stressors is roach (*Rutilus rutilus*). Roach is widely distributed in lakes and brackish water areas in Europe and western Asia. Furthermore, roach tolerates poor water quality and even polluted aquatic habitats and, as it is shown in the present thesis, papillomatosis is also frequently found in roach populations. The present thesis focuses on epidermal papillomatosis in roach and its possible use as a bioindicator of environmental stressors in aquatic habitats. Epidermal papillomatosis is

described and confirmed morphologically and histologically in roach. Several field studies were conducted to reveal factors affecting the occurrence of papillomatosis in roach within 34 Finnish lakes. This thesis proposes sampling methods developed for the field studies which take all confounding factors affecting the roach-papillomatosis system into account. Laboratory experiments were also conducted to investigate possible connections between epidermal papillomatosis and environmental stressors.

2 LITERATURE REVIEW

2.1 Epidermal papillomatosis in fish

Epidermal papillomatosis is a benign skin tumour, often also referred to as epidermal hyperplasia and skin neoplasia. Neoplasia is a common term used for abnormal hyperplasia of cells and is currently used as a synonym for tumours. More precisely, epidermal papillomatosis seems to grow and regress, with fish being affected by different stages of papillomas, forming a continuum from epidermal hyperplasia to benign papillomatosis (Smith et al. 1989a; Bucke et al. 1996). Epidermal papillomatosis is easy to diagnose by macroscopic inspection and palpating by hand on the skin of the fish. The histopathology of papillomas is used for confirmatory diagnosis of the disease (Smith et al. 1989a; Vethaak et al. 1992; Premdas et al. 1995). Although viral antigens have been detected from the papillomas of fish, they may not always be evident (Table 1). Papillomatosis of the common carp (*Cyprinus carpio*) is known to be caused by the cyprini herpesvirus (CHV) and has been detected by *in situ* hybridization (Sano et al. 1992). Conversely, Poulet et al. (1993) found no viral sequences from brown bullhead (*Ameiurus (= Ictalurus) nebulosus*) papillomas with bovine papillomavirus, cottontail rabbit papillomavirus, or fish retrovirus probes. Due to limitations in finding and recognising viral particles, as well as the variety of viral particles detected in the papillomas of fish (Table 1), molecular-based techniques have only rarely been used for diagnostic purposes in fish papillomatosis (Sano et al. 1992; Poulet et al. 1993).

In gross morphological observation papillomas are growing on the skin and scales of fish as raised pale, usually from translucent to white, single or multiple proliferations of epidermis. In some areas of fish, and for certain species, depending on the presence of pigment cells in the affected area, papillomas can also be pigmented from pink to brownish. In the early stages, only a few, slightly raised lesions are seen, while numerous large papillomas spread over large areas of the skin of fish can be observed in the advanced stages together with petechial hemorrhages on the papilloma (Bucke et al. 1996). For some fish species, certain types of papilloma lesions are more common. For example, in the European eel (*Anguilla anguilla*), the lesions are "cauliflower" like papillomas, which occur in and around the mouth (Peters 1977).

Table 1. Papillomatosis among various fish species. L = Larval, J = juvenile, A = adult, Su = summer, F = fall, W = winter, Sp = spring, VLP = Virus-like particles. + = noticed, 0 = no effect, ? = not known / studied.

Fish species	Life stage	Season	Virus	Mortality	Chemical promoters	Source
<i>Ameiurus (= Ictalurus) nebulosus</i> and <i>A. melas</i>	A	?	?	?	+	Grizzle et al. 1984; Baumann et al. 1996
<i>Anguilla anguilla</i>	A	Sp, Su, F	Rhabdovirus Herpesvirus	0	+	Anders and Yoshimizu 1994; Getchell et al. 1998;
<i>Barbus fluviatilis</i>	?	Sp	?	?	?	Anders and Yoshimizu 1994
<i>Catostomus commersoni</i>	A	0	?	0	+	Getchell et al. 1998; Mikaelian et al. 2000 Sano et al. 1993a;
<i>Cyprinus carpio</i>	J, A	F,W,Sp	Herpesvirus	+ J	0	Anders and Yoshimizu 1994; Getchell et al. 1998
<i>Esox lucius</i> and <i>E. masquinongy</i>	A	F,W,Sp	Retro-VLP	?	0	Anders and Yoshimizu 1994; Getchell et al. 1998
<i>Gadus morhua</i>	?	?	Adeno-VLP	?	0	Anders and Yoshimizu 1994
<i>Genyonemus lineatus</i>	?	?	?	?	+	Harshbarger and Clark 1990
<i>Hippoglossus hippoglossus</i>	?	?	?	?	?	Ottesen et al. 2007
<i>Leuciscus idus</i>	?	Sp	Herpesvirus	?	0	Anders and Yoshimizu 1994
<i>Limanda limanda</i>	A	Sp	Adeno-VLP	?	+	Vethaak et al. 1992; Anders and Yoshimizu 1994
<i>Merlangius merlangus</i>	?	?	VLP	?	?	Anders and Yoshimizu 1994
<i>Oncorhynchus masou</i>	J, A	?	Herpesvirus, Rhabdovirus, Birnavirus	+	0	Kimura and Yoshimizu 1991; Anders and Yoshimizu 1994
<i>Osmerus eperlanus</i>	A	F, W, Sp	Herpesvirus, Retro-VLP, Picorna-VLP	0	0	Anders and Yoshimizu 1994; Getchell et al. 1998
<i>Paralichthys olivaceus</i>	L, J	?	Herpesvirus	+	0	Kimura and Yoshimizu 1991
<i>Perca flavescens</i>	A	?	0	?	?	Anders and Yoshimizu 1994; Getchell et al. 1998
<i>Pleuronectes vetulus</i>	J	Sp, Su, F	0	?	?	Getchell et al. 1998
<i>Rutilus rutilus</i>	A	W, Sp	?	0	+	Kortet et al. 2002, 2003b, I - V
<i>Salmo salar</i>	J	Su, F	Retro-VLP, Herpes-VLP	0	0	Bylund et al. 1980; Anders and Yoshimizu 1994
<i>Salvelinus namaycush</i>	J	?	Herpesvirus	+	0	Anders and Yoshimizu 1994
<i>Silurus glanis</i>	?	?	Herpesvirus	?	?	Anders and Yoshimizu 1994
<i>Sparus aurata</i>	?	?	VLP	?	?	Anders and Yoshimizu 1994
<i>Stizostedion vitreum</i>	A	W,Sp	Herpesvirus	?	0	Anders and Yoshimizu 1994; Getchell et al. 1998

In certain species, some areas of skin are more commonly affected than others, such as lip papilloma in white sucker (*Catostomus commersoni*) (Smith et al. 1989a; Premdas et al. 1995) and papillomas in the fins of smelt (*Osmerus eperlanus*) (Anders and Möller 1985; Lee and Whitfield 1992). Similarly, two types of papillomas have been recognised in white sucker. Although all these types of papillomas are histologically similar, the gross morphology of one type is more like the papilloma described above, while the other resembles more a mucoid plaque (Smith et al. 1989a; Premdas et al. 1995). The prevalence of mucoid plaques seems to correlate with papilloma prevalence, though they respond to antibiotic treatment, which is not the case with true neoplasms (Premdas et al. 1995).

Regardless of the species or anatomical location of the papillomatous growth, they all seem to be histologically distinct, showing a continuum from epidermal hyperplasia to benign papilloma (Smith et al. 1989a; Poulet et al. 1994; Premdas et al. 1995). In the case of epidermal hyperplasia, thickened epidermis and hyperplasia of Malpighian cells can be seen microscopically. Papilloma seems to develop from this stage as a continuum, characterized by an intensive hyperplasia of Malpighian cells, with few or no mucous and club cells, nor any other secondary cells. Additionally, the basement membrane (basal layer) of the papilloma thickens to form pegs, and the vasculature of the papilloma increases as the papilloma grows. Papillomas are not usually invasive, nor do they ever metastasize due to their benignity (Smith et al. 1989a; Poulet et al. 1994; Premdas et al. 1995).

2.2 The etiology and epidemiology of epidermal papillomatosis in fish

The etiology and epidemiology of epidermal papillomatosis seems to be complex and is thought to be multifactorial in several fish species (Harshbarger and Clark 1990; Anders and Yoshimizu 1994; Baumann et al. 1996; Getchell et al. 1998). Three major components are known to encompass and interact with the etiology and epidemiology of fish diseases: pathogen, host, and environment (Snieszko 1974). In epidermal papillomatosis, the pathogen is viral, and host is a fish whose gender, age / length, species, immunology and endocrinal factors seem to affect papillomatosis. Furthermore, both the pathogen and host are further affected by environmental such factors as season, physical stressors (temperature, oxygen) and contaminants (e.g., Getchell et al. 1998).

2.2.1 Pathogens in fish papillomatosis

A viral etiology of papillomatosis has been suspected for several fish species, with the herpesvirus being most commonly found in the papillomas of fish. However, rhabdovirus, adenovirus, birnavirus, retrovirus, picornavirus and unrecognised viral-like particles have also been reported from the papillomas of fish (Table 1) (for reviews see Anders and Yoshimizu 1994; Getchell et al. 1998). Nevertheless, only two herpesviruses have been recognized to be oncogenic, i.e. inducing neoplasia, for fish. These oncogenic viruses are herpesvirus cyprini (CHV) and oncorhynchus masou virus (OMV) (Kimura and Yoshimizu 1991; Sano et al. 1991; Anders and Yoshimizu 1994). Both of these viruses are lethal, CHV mainly for juveniles, while OMV also causes mortality in adult fish. Although successful experimental transmission of epidermal papillomatosis has been demonstrated for white sucker, the same study found no viral particles using electron microscopy (Premdas and Metcalfe 1996). In the case of CHV, the viral genome has been found from several organs of fish in a latent infection: however, at this stage, no virus could be isolated. Nevertheless, when papillomas appeared during acute infection, both the virus and viral genome were detected (Sano et al. 1993a). In most cases of fish papillomatosis the viral infection seems to be the promoting or causative agent of papillomatosis (Table 1).

2.2.2 Papillomatosis and the host

Although the etiology of papillomatosis may vary between fish species (Table 1), some general patterns can be found for most of fish species. Several field studies have indicated that epidermal papillomatosis is more commonly observed in larger fish (Smith et al. 1989a; Lee and Whitfield 1992; Poulet et al. 1994; Mikaelian et al. 2000; Kortet et al. 2002), as well as in older fish (Smith et al. 1989a; Mellergaard and Nielsen 1995, 1997). This could be partly due to the effect of sex hormones on papillomatosis (Premdas et al. 2001) and the rare occurrence of papillomatosis in immature fish (Table 1). Field studies have also revealed that gender may affect susceptibility to papillomatosis. The form of papillomatosis is more frequent and severe in roach male than in the female (Kortet et al. 2002). This is most likely due to the higher hormonal levels of cortisol in male roach than female roach and higher testosterone levels in diseased than undiseased male roach during the spawning time (Kortet et al. 2003a; Vainikka et al. 2004). Moreover, Dethlefsen et al. (2000) noted in their study that epidermal

Kuopio Univ. Publ. C. Nat. and Environ. Sci. 223:1-53 (2007)

papillomatosis was more frequently observed in male than in female dab (*Limanda limanda*). In contrast, Møllergaard and Nielsen (1995, 1997) found that female dab was more often affected by papillomatosis than was the male. This was assumed to be due to the bad condition of female dabs as the sampling was done in the post-spawning period. However, no statistical significance was found between the condition factor of fish and papillomatosis prevalence in these studies (Møllergaard and Nielsen 1995, 1997). In addition, the observation that female dab in general were larger in size than male dab could partially explain the higher prevalence of papillomatosis in females (Møllergaard and Nielsen 1997).

Fish condition, measured as a condition factor, seems to have no systematic effect on papillomatosis. In white sucker, a significant trend was noticed only in one out of the four locations studied (Mikaelian et al. 2000). Furthermore, epidermal papillomatosis rarely cause mortality in adult fish (Table 1). Population level factors, especially population density, are known to affect the transmission and persistence of wildlife diseases (Swinton et al. 2002). Thus, Premdas and Metcalfe (1994) noted that papillomas of white sucker kept in the laboratory increased under crowded conditions. However, no correlation was found between stock density and papillomatosis prevalence in dab neither in a 9-year epidemiological survey (Møllergaard and Nielsen 1995) nor in occasional disease surveys at the German Bight (Vethaak et al. 1992; Møllergaard and Nielsen 1997). It seems clear that fish endocrine factors, such as stress-related and reproductive hormones, can affect papillomatosis in fish. Because these endocrine factors interact with environmental factors, they are discussed further in the next section.

2.2.3 Environment and interactions affecting papillomatosis in fish

Seasonal variation in the occurrence and prevalence of papillomatosis is well documented among several fish species (Harsbarger and Clark 1990; Getchell et al. 1998). In this cyclical appearance, the prevalence of papillomatosis peaks at a certain time of the year and then regresses, often sloughing off without leaving a mark (Getchell et al. 1998; Kortet et al. 2002). In contrast, no clear seasonality has been noted in the white sucker, though it has long been suspected (Mikaelian et al. 2000). In addition, papillomas in the white sucker are known to regress and proliferate seemingly spontaneously when observed in the laboratory (Smith and Zajdlik 1987; Premdas and Metcalfe 1994). Seasonal variation can be affected by

fluctuations in water temperature. For example, Peters (1977) found that in the European eel, papillomas grew in size at 15 °C and 22 °C, but did not grow or even regressed at 8 °C under experimental conditions. Furthermore, the European eel in the wild had the highest incidence of papillomas in summer during the highest ambient water temperature (Peters 1977). Some studies have suggested that the seasonal cycle of endocrine activity may play a dominant role in inducing papillomatosis (Lee and Whitfield 1992; Anders and Yoshimizu 1994; Kortet et al. 2002). It is likely that both temperature and hormonal patterns could be causal factors affecting the occurrence of papillomatosis. Endocrine control of spawning is mediated by environmental cues, including changes in ambient water temperature (Jobling 1995). Temperature also affects both the immunity of fish as well as the pathogenicity of the virus. Sano et al. (1993b) noted that the *in vitro* optimal temperature for CHV replication was 15 - 20 °C, while the mortality of *in vivo* CHV-inoculated carp was high at 15 °C, though mortality decreased and papillomas regressed or disappeared at temperatures of 20 °C and above. This was most likely due to the fish immunological defence mechanisms, which become more effective at higher temperatures in the carp. Induction of papillomatosis, after experimental infection with CHV, was noted in fish kept at 15 °C, 20 °C and 25 °C, but not at 30 °C (Sano et al. 1993b). In contrast, Sano et al. (1993a) observed that carp papillomas reappeared in the fall, when water temperature declined (7.5 °C), and papillomas regressed at higher temperatures (20 - 25 °C), appearing as latent infection. These results might suggest that in the normal seasonal cycle temperature may not comprise a prominent factor inducing papillomatosis in carp. Often, the papillomas appear on fish at the time of spawning, sexual maturation or smoltification (Table 1) (Lee and Whitfield 1992; Anders and Yoshimizu 1994). These are all life stages of fish also involving endocrinal changes. Epidermal papillomatosis has been induced and increased experimentally by 17 β -oestradiol and testosterone injections in white sucker (Premdas et al. 2001). Similarly, Kortet et al. (2003a) found that papillomatosis was associated with high concentrations of testosterone in the feral male roach. Sex steroids are known to depress the immunologic defence mechanisms of fish (Pickering 1986; Watanuki et al. 2002), and spawning stress itself has further consequences for the condition and immunity of fish.

Stressors are intrinsic or extrinsic stimuli and stress is the organism's response when its homeostasis is disturbed or threatened by these stimulations (Wendelaar Bonga 1997). In the literature integrated stress responses are often divided as primary, secondary and tertiary stress responses. The primary stress response in fish is to release of catecholamines and

Kuopio Univ. Publ. C. Nat. and Environ. Sci. 223:1-53 (2007)

corticosteroids into the circulatory system, thus affecting a series of biochemical and physiological changes referred to as a secondary stress response. Although the secondary stress response mainly influences the metabolization of energy reserves and corticosteroids, especially cortisol, it also affects the immunity of fish during acute stress through lymphocytopenia (Anderson 1990; Espelid et al. 1996; Wendelaar Bonga 1997). A tertiary stress response occurs when the stress becomes chronic. This is followed by various organism-level responses, including an impairment of reproductive success, growth rate and disease resistance. Furthermore, distinction should be made between impairment and disability when using physiological changes of organisms as an indicator of environmental stressors (Depledge 1989). Impairment in homeostasis of organism is detected earlier than the greater disability and disease.

Environmental stressors seem to affect on papillomatosis in fish. Mellergaard and Nielsen (1995, 1997) identified a peak in papilloma prevalence in dab populations after oxygen deficiency in a sampling area in the North Sea. Similarly, higher papilloma prevalences have been noted in contaminated areas (e.g., Harshbarger and Clark 1990). It is likely that during stress, immunosuppression is one of the elements promoting an outbreak of papillomatosis in fish. Moreover, some environmental pollutants are potentially immunotoxic, thus suppressing fish immunity more directly (Wester et al. 1994). In aquatic environments, carcinogenic chemicals may also be present, thereby promoting the tumour development of epidermal papillomatosis in fish. Bauman (1998) listed several locations where papillomas and other neoplasms have been found in fish in North America. The sediment or water of these habitats was frequently contaminated with genotoxins in especially with polycyclic aromatic hydrocarbons (PAHs). Black (1983) induced papillomas experimentally in brown bullheads by exposure to an extract of sediment containing PAHs. Moreover, epidermal papilloma was experimentally induced in adult male zebrafish (*Danio rerio*) by ethylnitrosourea (ENU), an alkylating agent and well known cause of tumours in a variety of animals (Beckwith et al. 2000).

2.3 Epidermal papillomatosis of fish as a bioindicator of environmental stressors

Aquatic environments have been seriously altered due to anthropogenic wastes and contaminants. Bioindicators are needed to observe, monitor and predict the adverse effects of human impact on aquatic environments. Bioindicators are organisms or biological systems that reveal the toxic effects of and / or exposure to environmental contaminants (Au 2004; Hutchinson et al. 2006). In a review article, Au (2004) lists the advantages of epidermal hyperplasia as a bioindicator. It is cost-effective and has high sensitivity and notable dose-response relationship for certain contaminants. However, it has limitations, including lack of specificity, since it responds to a variety of stressors and technical difficulties, since all the confounding factors are not yet clearly understood. Monitoring epidermal papillomatosis is ecologically relevant because it gives information concerning realistic changes in natural fish populations, while posing no actual threat to most fish species (Au 2004). Furthermore, epidermal papillomatosis can especially act as an indicator of chronic exposure to environmentally relevant and fluctuating concentration of contaminants.

The International Council for the Exploration of the Sea (ICES) uses epidermal papillomatosis among other fish diseases, for monitoring the effects of environmental changes in dab in the North Atlantic (Bucke et al. 1996). Vethaak et al. (1992) noted in a survey at the German Bight that epidermal papillomatosis decreased with decreasing contaminant concentrations in water and in the liver of dab. Furthermore, the main effects and interactions of length, age and sex were included in interpreting the results of papillomatosis prevalence in the survey by using a linear logistic model (Vethaak et al. 1992). Similarly, Mellergaard and Nielsen (1995, 1997) found in a long-term disease survey that the papilloma prevalence of papillomatosis in dab increased after previous years of oxygen deficiency in the areas sampled. However, caution should be taken in interpreting these results, as dab are quite mobile, and conclusions are difficult due to the spatial and temporal changes in sampling sites (Vethaak et al. 1992).

In North America, especially in the Great Lakes area, epidermal papillomatosis in brown bullhead and white sucker have been proposed as bioindicators of environmental degradation (Smith et al. 1989a; Baumann et al. 1996). Higher papilloma prevalence has been observed in *Kuopio Univ. Publ. C. Nat. and Environ. Sci. 223:1-53 (2007)*

several studies on white sucker in industrialised and polluted areas of the Great Lakes when compared to more pristine reference sites. (Smith et al. 1989a; Hayes et al. 1990; Premdas et al. 1995; Baumann et al. 1996; Mikaelian et al. 2000). Furthermore, comparisons between the size and sex of fish and season of capture were also taken account, mainly in sampling procedures by comparing these factors in samples between polluted and reference sites.

Bauman et al. (1996) concluded that over 20 % prevalences in papillomatosis in white sucker were found only in lower (and more industrialized) areas of the Great Lakes. Moreover, papilloma prevalence was found to correlate with elevated contaminants in the muscle tissue of white sucker at a polluted site (Premdas et al. 1995). Smith et al. (1989a) found leukocytic inflammation in papillomatosis histopathology of white sucker. Similarly, Hayes et al. (1990) showed elevated glutathione peroxidase and glutathione reductase activities in papillomas, indicating tissue injury. It was concluded that the peroxidative mechanism in skin might be one of the promoting factors in papillomas of the white sucker. Crowded conditions in fish tanks have elevated papillomatosis in white sucker, while fish kept under the normal conditions showed no induction of papillomas, or regression of the papillomas (Premdas and Metcalfe 1994). However, injection of organic contaminants had no effect on induction or growth of papillomatosis when tested experimentally (Premdas and Metcalfe 1996). Interestingly, 17 β -oestradiol and testosterone injections induced papillomatosis and tamoxifen, which competes with oestradiol from its own receptor, mainly resulting in the regression of papillomas in white sucker (Premdas et al. 2001).

Similarly, several field studies have revealed elevated papilloma prevalences at contaminated sites in brown bullheads (Smith et al. 1989a; Baumann et al. 1996; Yang et al. 2003; Pinkney et al. 2004a, 2004b). Moreover, Yang et al. (2003) found a significant correlation between biliary PAH metabolites and the prevalence of barbel abnormalities and papillomas in brown bullhead. Pinkney et al. (2004a) noticed higher papilloma prevalence in contaminated sites, while at a reference site no papillomatosis were detected in brown bullhead. They also detected the highest ethoxyresorufin-*O*-deethylase (EROD; a widely used biomarker of environmental contaminant exposure) activity, muscle polychlorinated biphenyls (PCB) and chlordanes in fish tissue as well as the highest concentrations of PAHs and chlordanes in the sediment of the site where the highest papilloma prevalence (12 %) was observed, while the second highest mean benzo(a)pyrene-like bile metabolite concentrations were observed at the same site (Pinkney et al. 2004a). Pinkney et al. (2004a) argued that bile metabolites detected

in the fish might not always be comparable for tumor prevalence in fish because metabolite describes recent exposure occurring within days, whereas tumor initiation may take several months or even years. In contrast, biliary PAH-like metabolites and liver DNA adduct concentrations were elevated in bullheads at the sites exhibiting higher papilloma prevalence than at the reference site (Pinkney et al. 2004b), thus providing strong evidence for the tumour promoting effect of PAHs. Experimental exposures have further revealed that in brown bullheads papillomas were induced when the fish skin was painted with sediment extract containing PAHs (Black 1983). Moreover, black bullhead (*Ameiurus* (= *Ictalurus*) *melas*) papillomas were also induced when caged in a final oxidation pond of chlorinated wastewater effluent (Grizzle et al. 1984).

Experimental evidence also exists for a connection between environmental stressors and induction of papillomatosis in fish species that have not been used as bioindicators, or have not yet been studied in the field. Most recently, induction of papillomatosis has been described in zebra fish from exposure to ethylnitrosourea (ENU) (Beckwith et al. 2000). Furthermore, a fluctuation in water salinity and temperature has been noted to affect papillomatosis in European eel under experimental conditions (Peters and Peters 1979; Peters 1977). However, more experimental results are needed regarding the effect of environmental stressors on fish papillomatosis in controlled environments. The species studied should also be taken into account, since a successful environmental bioindicator fish species should be widely distributed and original to the area, rather sedentary throughout its life, its habitat characteristics and recruitments should be well known, and the species should be suitable both to field studies and laboratory experimentation (Munkittrick and Dixon 1989).

2.3.1 HSP70 as biomarker of environmental stress

For assessing suitable bioindicators, it is valuable to compare the proposed bioindicator to other possible environmental stress endpoints in fish. However, association between Heat shock protein 70 (HSP70) and the development of papillomatosis in fish has not been previously studied. HSP70 belongs to a highly conserved class of proteins called HSPs. These proteins can be classified into five major families according to their molecular mass, with HSP70 belonging to a protein family with a molecular mass ranging from 70 to 72 kDa (for reviews see Iwama et al. 1998; Basu et al. 2002). Expression of HSPs was first discovered in

cells following heat shock (Ritossa 1962) and has since been described as responding to a wide variety of abiotic and biotic stressors. HSPs are found constitutively in cells, though some families of HSPs are highly induced under stress and are involved in the adaptation of the cell to cope with stressors (Iwama et al. 1998). Particularly, HSP70 family expression is enhanced under stressful conditions and has therefore been used extensively as a biomarker of cellular-level stress. In studies of environmental stress in fish HSP70 has been shown to express when impacted with bleached kraft pulp mill effluents (BKME) (Janz et al. 1997; Vijayan et al. 1998), heavy metals (Williams et al. 1996), herbicides (Hassanein et al. 1999), sodium dodecylsulphate (SDS) (Vijayan et al. 1998), and general contamination in the field (Schröder et al. 2000). Furthermore, *in vitro* studies of fish cell lines have shown enhanced HSP70 expression when exposed to such contaminants as heavy metals and organochlorines (Deane and Woo 2006).

Although several studies have used HSP70 expression in fish as a biomarker of environmental stress and contamination, the results are sometimes inconsistent and caution should thus be exercised when interpreting HSP70 expression results (Iwama et al. 2004). Several explanations have been proposed for the inconsistency of these results. For example, differences between species may result in different responses to HSP70 expression (Häkkinen et al. 2004; Vehniäinen et al. 2003). Physiological factors of the fish as such seem to also affect HSP70 expression, as diseases and gender are known to affect HSP70 expression in fish (Ackerman and Iwama 2001; Afonso et al. 2003). Furthermore, the expression of HSP70 has been shown to sometimes decrease (Häkkinen et al. 2004) or end (Vehniäinen et al. 2003) following increased exposure. This suggests that HSP70 has a threshold for expression, rather than increasing in a dose response manner with exposure.

3 OBJECTIVES

The objective of this study is to investigate the possibility of applying the roach-papillomatosis system to indicate environmental stressors in aquatic habitats. Papillomatosis in roach might provide a suitable tool for the assessment and monitoring of freshwater ecosystem health. The study is also topical as new tools are required by the European Union's Water Framework Directive for determining and monitoring the ecological status of lakes. The objective will be approached by performing field studies and laboratory experiments to provide sufficient background to enable future use of the roach-papillomatosis system as a bioindicator.

The specific aims of the study were:

- 1) To confirm the diagnosis of epidermal hyperplasia / papillomatosis in roach by microscopic inspection of histological samples of papillomas and to report the histopathology of epidermal hyperplasia and papillomatosis for the first time in roach.
- 2) To investigate the occurrence of papillomatosis in roach populations in a number of Finnish lakes and further investigate possible infection patterns of epidermal papillomatosis in natural roach populations. (I, II)
- 3) To explore the possible connection between environmental stressors and papillomatosis in roach populations based on field studies as well as other factors affecting papillomatosis in natural roach populations beside the environmental stressors, such as, season of sampling, fish size and sex. (III)
- 4) To develop a methodology for the practical use of roach papillomatosis as a bioindicator. A scale coverage method was evolved to determine the intensity of the disease in roach papillomatosis, in addition to prevalence, for use in experimental and field studies. Furthermore, practical guidelines, such as sampling regimes and statistical analyses, suitable for field studies were further developed to take into account all the confounding factors in affecting roach papillomatosis. (I - III)

5) To study experimentally the effects of environmental stressors on the development of papillomatosis in roach. Roach were exposed to abiotic stressors by introducing fish to fluctuating temperature and oxygen deficiency. Furthermore, fish were exposed to different concentrations of treated municipal and pulp mill effluents in the laboratory to reveal their response on papillomatosis and dose-response relationship of municipal effluents on papillomatosis induction. In this experiment, the connection between papillomatosis induction and possible cellular level stress was further explored by analyzing HSP70 expression in exposed and control fish. (IV, V)

4 MATERIALS AND METHODS

4.1 Histology

The skin lesions of roach were histopathologically examined to confirm and describe the disease diagnosis. In May 2003 two diseased roach, male and female, were caught from Lake Jyväsjärvi. Only two fish were chosen for these confirming purposes, because papilloma diseases has been identified histologically from roach before in the Veterinary Research Institute of Finland (EELA, nowadays EVIRA) by Dr. Eija Rimaila-Pärnänen (Kortet et al. 2002). Both fish were sacrificed, and histopathological samples were taken of the diseased skin. The fish had several papilloma-like changes all over the body surface and several papilloma-like growths were studied. The female had a large papillomatous tumour on the skin of the head. This tumour had a large prominent vascular surface. All samples of skin lesions were fixed either in Bouin's fixative or in 10% formaldehyde. Fixed tissues were embedded in paraffin and sectioned at 5 µm with a microtome. After clearing and rehydration, the sections were stained with haematoxylin and eosin (H & E) according to standard methods (e.g., Bancroft and Stevens 1996). Photography was performed using an Olympus PX41 microscope with attached digital camera (Olympus CAMEDIA C-3040ZOOM).

4.2 Field studies

For field studies, population samples of roach were collected from southern Finland, mainly from the River Kymijoki and River Vuoksi waterways, both draining into the Baltic Sea (Table 2) (I - III). For study III roach were collected in spring between 2004 and 2005. Studies I and II also used fish collected in 2000 and 2003. Some of the samples from the year 2000 were previously used in a study by Kortet et al. (2002). Details of the samples are given in Table 2. Additionally, several diseased roach samples were collected in spring 2003 and 2004 to study the dependence of papillomatosis intensity on the size and sex of the fish, as well as the test repeatability of the scale coverage method (I). These fish samples were from Lake Kallavesi and from Lake Jyväsjärvi, with some of the samples being previously used in the study of Vainikka et al. (2004).

Fish were sampled mainly by ice fishing (Table 2), killed immediately after capture and transported to the laboratory. The length, sex and intensity (number and size of the tumors among the diseased fish) were recorded from each fish in the laboratory using the scale coverage method, and papillomatosis prevalence (percentage of diseased fish in population, %) was determined for every population sample (I - III). The prevalence and occurrence of papillomatosis in the population was compared to the characteristics of lake (II) and locations of environmental impact in lake (III). Information concerning point source locations and the morphological characteristics of the lakes were mainly obtained from the Environmental Information System - HERTTA (available from: <http://www.environment.fi/default.asp?node=14812&lan=en>) and literature from Ekholm (1993). All the statistical analyses in field studies were performed by SPSS for windows 13.0 (SPSS Inc.), if not otherwise mentioned.

4.2.1 Papillomatosis occurrence and infection patterns

Factors affecting the occurrence of papillomatosis in feral roach populations were investigated in study II (Table 2). Discriminant analysis was used to discriminate between the variables which representing morphological factors describing the lake, population factors concerning the fish and sampling procedure in order to study whether the occurrence of papillomatosis is consistent with the theory of island biogeography (MacArthur and Wilson 1967) in infection patterns (II).

Papillomatosis aggregation in study I was examined using five population samples (Table 2). Aggregation in the roach population was analysed using the frequency distribution of papillomatosis intensity in the population and comparing this to fit a Poisson distribution and negative binomial distribution. Furthermore, the aggregation parameters m (abundance of papillomatosis) and k (inverse measure of aggregation) were calculated for each population using the maximum likelihood method. Aggregation analyses were performed with the SAS system for Windows release 8.2 (SAS Institute Inc.) (I).

Table 2. Population samples collected and used in this thesis. Name of the lake. Site of the population sampling, if several populations from the same lake are sampled. Identification number for the population referred in the given article. The month, year and method of sampling. N = number of fish sampled, P % = prevalence of papillomatosis.

Lake	Site	Identification Number	Year	Method	N	P %
Jyväsjärvi		2 ^a , 2 ^b , 1A ^c	March 2004	Ice fishing	103	22
Iso Kuhajärvi		1 ^a , 8 ^b	April 2003	Ice fishing	100	3
Palvajärvi		3 ^a , 3 ^b	April 2004	Ice fishing	75	11
Nilakka		4 ^a , 15 ^b , 3B ^c	April 2004	Ice fishing	78	9
Iisvesi	Iisvesi	7 ^a , 14 ^b , 2A ^c	April 2004	Ice fishing	20	30
	Rasvanki	5 ^a , 14 ^b , 3A ^c	April 2004	Ice fishing	72	9
	Virmasvesi	6 ^a , 14 ^b , 2B ^c	April 2004	Ice fishing	81	5
Pihlajavesi		8 ^a , 29 ^b , 8A ^c	April 2004	Ice fishing	50	14
Unnukka		9 ^a , 26 ^b , 7B ^c	April 2004	Ice fishing	53	6
North Kallavesi	Autioranta	18 ^b , 4B ^c	April 2004	Ice fishing	51	3.9
	Neulaniemi	14 ^a , 18 ^b , 4A ^c	March 2004	Ice fishing	244	10
South Kallavesi	Siikalahti	13 ^a , 19 ^b , 5A ^c	March 2004	Ice fishing	301	11
	Haaslahti	12 ^a , 19 ^b , 6A ^c	April 2004	Ice fishing	39	21
	Litmalähti	11 ^a , 19 ^b , 6B ^c	April 2004	Ice fishing	93	10
Juurusvesi	Iso-Jälä	10 ^a , 20 ^b	April 2004	Ice fishing	99	5
	Melavesi	20 ^b , 5B ^c	April 2004	Ice fishing	102	2
Pyhäselkä	Napaluoto	15 ^a , 31 ^b , 9B ^c	May 2005	Fish trap	27	15
	Joensuu	16 ^a , 31 ^b , 9A ^c	May 2005	Fish trap	26	31
	Noljakansaaret	17 ^a , 31 ^b , 10A ^c	May 2005	Fish trap	144	12
	Perhesaaret	18 ^a , 31 ^b , 10B ^c	May 2005	Fish trap	32	9
	Liperi	19 ^a , 31 ^b	May 2005	Fish trap	106	22
Päijänne		1 ^b , 1B ^c	April 2004	Ice fishing	58	1.7
Haukivesi		27 ^b , 7A ^c	April 2004	Ice fishing	82	2.4
Joutenvesi		28 ^b , 8B ^c	April 2004	Ice fishing	58	4.8
Symäjärvi		30 ^b	April 2004	Ice fishing	45	0
Tallusjärvi		16 ^b	April 2004	Ice fishing	91	0
Onkivesi		17 ^b	April 2004	Ice fishing	98	1
Sulkavanjärvi		21 ^b	April 2004	Ice fishing	81	0
Ylä-Pieksä		22 ^b	April 2004	Ice fishing	97	0
Kumpunen		23 ^b	April 2004	Ice fishing	68	0
Pieni Vehkalahti		24 ^b	April 2004	Ice fishing	86	0
Kolmisoppi		25 ^b	April 2004	Ice fishing	66	0
Hietajärvi		32 ^b	March 2005	Ice fishing	132	0
Rahkeenvesi		33 ^b	March - June 2005	Netting	42	0
Pielinen		34 ^b	June 2005	Netting	10	10
Vuojärvi		9 ^b	April 2003	Ice fishing	15	0
Peurunka		11 ^b	June 2000	Netting	149	6
Koivujärvi		4 ^{b,d}	May 2000	Netting	63	6.3
Tuomiojärvi		5 ^{b,d}	May 2000	Netting	54	22.2
Lahnajärvi		6 ^{b,d}	May 2000	Netting	152	0
Vihtajärvi		7 ^{b,d}	May 2000	Netting	116	0.9
Saravesi		10 ^{b,d}	May 2000	Netting	108	5.6
Kuuhankavesi		12 ^{b,d}	May 2000	Netting	101	0
Konnevesi		13 ^{b,d}	May 2000	Netting	81	6.2

^a Study I, ^b Study II, ^c Study III, ^d Data from study Kortet et al. (2002)

4.2.2 Prevalence and intensity of papillomatosis

To investigate the affect of environmental stressors on papillomatosis prevalence in the roach sampled for study III, 20 population samples were collected (Table 2). These samples were taken as 10 matched pairs. Population pairs were sampled usually from the same lake so that the impact population sample was taken from a site which was influenced by industrial and / or sewage effluents, and the reference population sample was taken upstream from that site, a more pristine site. To avoid seasonal differences in disease status, population pairs were collected on the same day or on average 5 days later from reference site. For statistical tests, the Generalized Linear Mixed Model (GLMM) was used to analyse differences in papillomatosis prevalence between impact and reference populations, and the fish size and gender were used as covariates in the model (III). In addition to the environmental impact, effect of fish size and gender on papillomatosis prevalence was also assessed using the Linear Mixed Model (LMM) and GLMM, respectively. LMM and GLMM analyses were performed using the SAS system for Windows release 8.2 (SAS Institute Inc.) (III).

The intensity of papillomatosis in the roach was also studied (I, III). A scale coverage method for estimating of disease intensity was developed and the reliability of the method was tested between persons and single-person repeats using the intraclass correlation coefficient (ICC) (I). The sex and size dependence of the intensity was tested with diseased roach samples from Lake Kallavesi and Lake Jyväsjärvi by analysis of covariance (ANCOVA) (I) and the effect of environmental stressors on papillomatosis intensity in 5 population pairs was tested by LMM (III). Correlation of the prevalence and intensity of papillomatosis in 19 roach populations was tested using Pearson correlation analysis (I).

4.3 Experimental studies

To investigate the possible connection between environmental stressors and epidermal papillomatosis, three experiments were conducted under laboratory conditions. In the first experiment, fish were exposed to abiotic stressors caused by temperature changes and oxygen deficiency in spring 2004 in study IV. In spring 2005, fish were exposed experimentally to treated municipal for 38 days and pulp mill effluents for 19 days, and in spring 2006 to different concentrations of treated municipal effluents for 22 days in study V. The fish used in these experiments were caught each spring mainly using fish traps from Lake Kallavesi (IV-

V). Only diseased and mature fish were selected for experiments. At the beginning of each experiment, fish were anaesthetised (MS-222, Sigma), individually marked by fin cutting, measured for length and / or weighed, papillomatosis intensity was recorded, and the fish were transferred to 70-l plastic tanks, which received a constant water flow (IV) or effluent diluted standing water (V) from Lake Kallavesi. Every tank contained 15 fish, and every exposure group had at least one replicate tank (Table 3). During the experiments, temperature and oxygen content was monitored (OxyGuard[®] oxygen electrode) from each tank separately, and the fish were fed to excess with commercial pellets. In the effluent exposure experiment, the pH of the water in the tanks was also measured (Consort P901 pH meter). At the end of each experiment, the fish were killed with a blow on the head, weighed, length measured, the intensity of papillomatosis was recorded, and the sex determined from the gonads.

4.3.1 Oxygen deficiency and temperature increase as stressors related to papillomatosis

The exposure for abiotic stressors in study IV was done near the roach spawning season at the end of March. The experiment lasted for 17 days with two exposure periods during days 1-8. During the exposure periods fish were exposed to temperature fluctuations and periodic oxygen deficiency (OT group). In another group, the fish were exposed only to temperature fluctuations (T group), and a control group was kept under conditions of constant temperature and oxygen content (Table 3). At the end of experiment, three physiological indices were recorded from the fish: (1) gonado-somatic index (GSI), (2) condition factor (K) and (3) haematocrit by a haematocrit centrifuge (Compur M 1100).

4.3.1 Exposure to pulp mill and municipal effluents

In the effluent exposure experiments (V), effluents were diluted with Lake Kallavesi water, and the control tanks contained standing Lake Kallavesi water. An internal biological filter (Magic Jet 380, Resun[®]) was used to provide aeration, and mechanical and biological filtering of the water was provided in all the tanks. Pulp mill effluent was provided by Powerflute Oy Savon Sellu and municipal effluent by the wastewater treatment plant of city of Kuopio (V). Both plants treat the effluents mainly mechanically by clarification and biologically by activated sludge-treatment prior to discharge into recipient water.

Table 3. Results of experimental exposures of roach to abiotic stressors and effluents. Number of fish in the beginning of experiment (N). Exposure regime or concentration. Duration of exposure (Time). Mortality during the experiment. Change in the intensity of papillomatosis during the experiment, measured as scale coverage of papillomas (male / female). Gonadosomatic index (GSI), Haematocrit (Hkt). Condition factor (K). Relative amount of heat shock protein 70 (HSP).

	N	Sex	Exposure	Time	Mortality	Papillo	GSI	Hkt	K	HSP
<u>Exposure to abiotic stressors (IV)</u>										
Hypoxia and temperature	29	m	13.5-0.3 mg O ₂ l ⁻¹ 10.1-0.9 °C	6 days	28 %	29.3	7.0	36.4	0.9	-
Temperature	30	m	9.6-0.9 °C	6 days	0 %	19.5	7.2	38.1	1.0	-
Control	30	m	-	0 days	3 %	2.1	8.0	35.5	1.0	-
<u>Exposure to effluents (V)</u>										
Pulp mill	30	m/f	10 %	19 days	0.03%	9.6 / 6.1	-	-	-	-
Control	30	m/f	0 %	19 days	0.03%	9.9 / 10.0	-	-	-	-
Municipal year 2005	30	m/f	10 %	38 day	0.03 %	5.0 / 5.4	-	-	-	-
Control	30	m/f	0 %	38 day	0.03 %	-0.6 / 7.1	-	-	-	-
Municipal year 2006	45	m/f	15 %	22 days	0 %	11.6 / 8.9	-	-	-	139
Municipal year 2006	45	m/f	1.5 %	22 days	0 %	7.7 / 9.6	-	-	-	161
Control	30	m/f	0 %	22 days	0 %	8.2 / 9.1	-	-	-	118

Results are presented as mean values per group, for detailed results with standard deviations and exact numbers of fish used in analysis see corresponding articles. m = male, f = female, - = not measured

For Experiment 1, in spring 2005, roach were exposed to 10 % treated pulp mill effluent for 19 days and 10 % treated municipal effluent for 38 days. For Experiment 2, in spring 2006, roach were exposed to 1.5 % and 15 % of treated municipal effluents (Table 3) (V).

HSP70 western immunoblot analysis was performed for the roach exposed to treated municipal effluents in spring 2006 (Experiment 2), using methods described by Vehniäinen et al. (2003). The expression of HSP 70 was measured from roach gills, which were homogenized and the protein was then separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli 1970) and transferred to a nitrocellulose membrane with a Mini-Protean II apparatus (Bio-Rad). HSP70 was detected by HSP70 monoclonal antibody (MA3-006, Affinity BioReagents Inc.) and goat anti-mouse total immunoglobulin G (IgG) peroxidase conjugate (DC08L, Calbiochem). HSP70 was visualized by chemifluorescence of blot with Typhoon 9400 Imager (Amersham Biosciences) and semi-quantified by image analysis software ImageQuant TL v2005 (Amersham Biosciences).

4.3.1 Statistical analyses in the experimental studies

Differences in mortality between exposure groups were tested by χ^2 -test. All other statistical analyses for the experimental studies were performed separately for males and females (V) or solely for males (IV). Differences between treatments were revealed by ANCOVA, in which fish length and papillomatosis intensity at the beginning of the experiment were used as covariates. In addition, differences in physiological incidences in exposure to temperature and oxygen stress as well as heat shock protein 70 (HSP70) expressions between 15 %, 1.5 % and 0 % municipal effluent exposure were determined by analysis of variances (ANOVA), except for the comparison of GSI, which was performed using ANCOVA. The Bonferroni post hoc test was further used if ANOVA or ANCOVA revealed significant differences between treatments. Statistical analyses were performed by SPSS for windows 13.0 (SPSS Inc.) (IV, V).

5 RESULTS

5.1 Macroscopic pathology and histology

Papillomas of roach are visible and palpable. The colour of the tumours ranges from brownish and yellowish to white. Some of the tumours are translucent nodules on the scales of the roach skin. The consistency is smooth with the papillomas appearing as multiple or single discrete focuses on the scales of the roach (I). When the fish are heavily affected, tumours seem to proliferate and spread all over the roach skin, head and fins. Occasionally, in large papillomas, haemorrhages were already macroscopically observed. In pure epidermal hyperplasia, the epidermis is thickened in general or by forming plaques.

The normal skin of roach is thin and composed of two layers, the outer epidermis and the inner dermis or chorium. These together contain four to twenty layers of cells. The top layer contains stratified squamous epithelial cells, with underlying layers usually consisting of cupoidal Malpighian, mucous and club cells (Fig. 1A). Immediately below the dermis is the basal layer and scales. Pigment cells are situated in dermis of the non-scaled area of the fish.

Histopathological sections of papilloma reveal extensive hyperplasia of the Malpighian cells, with few or no secondary cells being present (Fig. 1B). These hyperplastic Malpighian cells are usually smaller in size, tightly packed, and the nucleus seems to be more prominent in H&E staining than in normal Malpighian cells. As epidermal hyperplasia increases, the basal layer seems to form pegs and is slightly scalloping (Fig. 1B).

Sagittal sections of the large papilloma show proliferation of the stroma and angiogenesis beside the prominent hyperplasia of the Malpighian cells (Fig. 1C). Mucous cells seem to displace themselves as far as possible, away from the proliferating stroma, and are packed in central areas of the tumour (Fig. 1C). None of the roach papillomas studied were either invasive or metastatic.

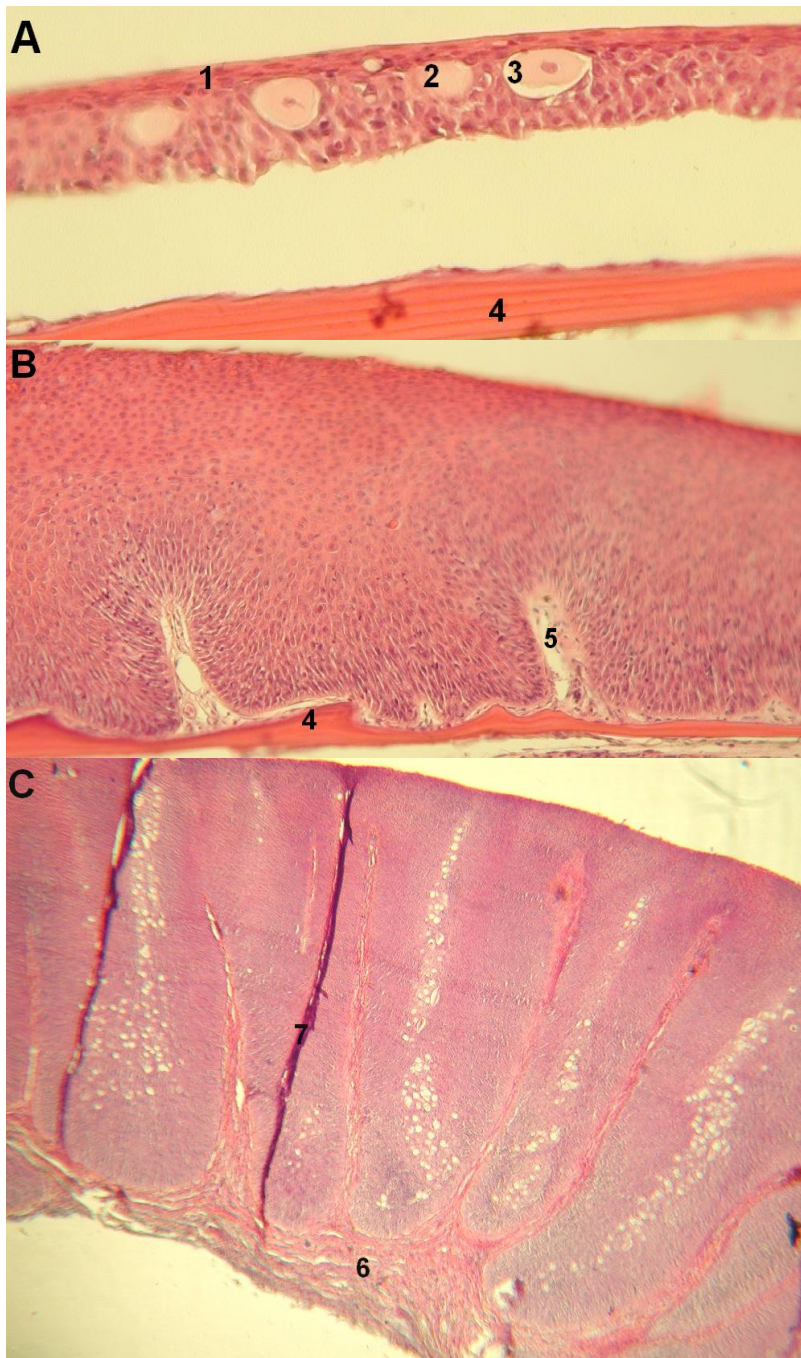


Figure 1. Histological sections from the skin of the roach. (A) Normal epidermis of roach, composed of (1) squamous epithelial cells, (2) mucous cells, (3) club cells and predominantly cupoidal Malpighian cells. (4) Scale detached from skin at this section (H&E, x 400). (B) Epidermal hyperplasia, composed from extensive hyperplasia of Malpighian cells, absence of secondary cells and (5) peg formation of basal layer (H&E, x 200). (C) Benign papillomatosis with (6) stroma and (7) vasculature (H&E, x 40).

5.2 Field studies

5.2.1 Papillomatosis occurrence and infection patterns

Epidermal papillomatosis in roach was found from 65 % of the 34 lakes examined in study II (Table 2). Discriminant analysis revealed that the strongest discriminating variables for papillomatosis occurrence were the characteristics of the lake, especially the maximum depth of the lake, the percentage of lakes in the vicinity of the lake, and the altitude of the lake. Fish-related variables, such as the mean length of the fish in the sample, proportion of males in the sample or the date of fish collection (date count), were not statistically significant factors discriminating between diseased and undiseased lakes. The classification function further determined that probability of the disease occurring in the lake increased with increasing lake depth and percentage of lakes in the vicinity of the lake, and decreased with increasing altitude. Furthermore, lake area correlated with the discriminating variables, indicating that a larger lake area increases the probability of the disease occurring in that lake.

Papillomatosis aggregation was analysed for five feral roach populations (I). Aggregated distribution of the intensity of papillomatosis, measured as scale coverage, was observed in all five populations, as they fitted better to the negative binomial distribution than to the Poisson distribution.

5.2.2 Papillomatosis prevalence and intensity

Results of GLMM indicated that the risk of being diseased with epidermal papillomatosis was 2.7 times higher in the impact populations than in the reference populations. The mean prevalence was 16.6 % for the impact and 5.8 % for the reference populations (III). The prevalence of papillomatosis was higher in the impact populations in all population pairs studied, except for population pair number 7 (Fig. 2). Furthermore, the risk of papillomatosis was 7.5 times higher in males than females and increased with fish length (III). The influence of fish length and gender was eliminated from the population pair comparison by statistical methods.

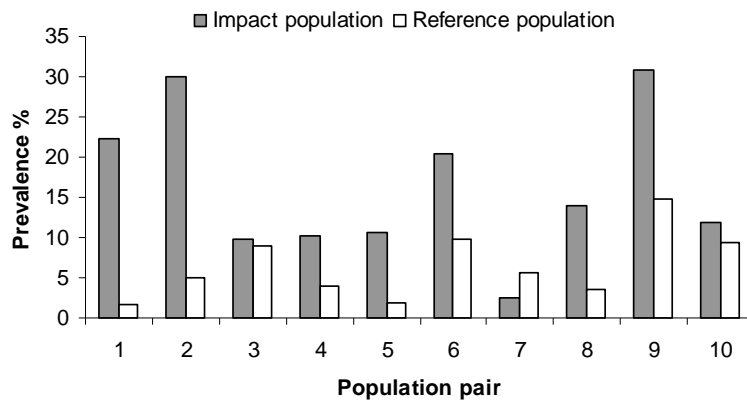


Figure 2. Prevalence of papillomatosis (%) in 10 matched pairs of roach population samples. Sources of contamination in the site of impact population as follows: 1 = pulp and paper industry, PCB, sewage effluents, 2 = saw mill and peat industry, sewage effluents, 3 = fish farming, sewage effluents, 4 = rubbish dumping site on the land, sewage effluents, 5 = pulp and paper industry, oil storage, snow dumping site on the land, 6 = mining industry, sewage effluents, 7 = pulp and paper industry, thermal effluents, 8 = pulp and paper industry, sewage effluents, 9 = saw mill and pulp and paper industry, sewage effluents, 10 = pulp and paper industry, sewage effluents.

In population pair 7, the impact site was found to be affected by thermal effluents which had consequences for the development of papillomatosis (see Discussion). Therefore, if we exclude the data from population pair 7, the variation in the prevalence values for impact sites was between 9.7 – 30.8 %, and the variation in the prevalence values among reference sites was 1.7 – 14.8 %, respectively (Fig. 2) (III). In eight of the nine impact sites, the prevalence of papillomatosis was above 10 %. In addition, in eight of the nine reference sites the prevalence of the disease was below 10 %.

Epidermal papillomas on roach are easy to observe visually and by palpating with the hand. We developed a methodology for estimating the intensity (severity) of papillomatosis by counting the scales covered by papilloma tumors on the roach (I). The reliability of the method was high both between two investigators as well as with single investigator repeats. The intensity of papillomatosis was affected by the gender and size of the fish, with the intensity being higher in males and larger fish (I). However, papillomatosis intensity seemed not to be affected by environmental stressors (III), neither did it correlate with papillomatosis prevalence in the 19 populations compared (I).

5.3 Experimental studies

5.3.1 Oxygen deficiency and temperature increase as stressors related to papillomatosis

The highest increase in the intensity of papillomatosis was in male roach exposed to periodical oxygen deficiency and temperature fluctuations (OT group), the second highest intensity change was in the group exposed only to temperature fluctuations (T group) and the lowest intensity change was found in the control group, with the changes being statistically significant between groups (Table 3) (IV). Mortality was highest and the condition factor the lowest in the OT group (Table 3). Fish in the OT group also showed behavioural signs of stress (Baroudy and Elliott 1994), which were not observed among T and control-group fish. Other physiological measurements did not differ statistically significantly between exposure groups.

5.3.2 Exposure to pulp mill and municipal effluents

In roach exposed to 10 % and 0 % treated pulp mill and municipal effluents in spring 2005 (Experiment 1), no significant differences in papillomatosis intensity change was observed (Table 3). In roach exposed to 0 %, 1.5 % and 15 % treated municipal effluent (Experiment 2), the highest papillomatosis intensity change was observed in males exposed to 15 % concentrations of the effluent (V). The difference in papillomatosis intensity changes was statistically different between the 15 % and 1.5 % exposure groups in males, though neither of these were statistically significantly different from the control group. Interestingly, no changes were noticed in the intensity of papillomatosis in female roach exposed to municipal effluent neither in 2005 or 2006. Moreover, in all the effluent experiments average papillomatosis intensity change among female roach was consistently higher in the control group than among groups exposed to effluents, though this was not a statistically significant difference. There were no statistical differences observed in relative amount of HSP70 between control and exposure groups.

6 DISCUSSION

6.1 Epidermal papillomatosis in roach

Histological observations confirmed that raised lesions observed mainly during the spring time in roach skin are epidermal hyperplasias and papillomas. Roach papillomatosis resembled histologically the papillomas observed in brown bullhead and white sucker, featuring a continuum ranging from epidermal hyperplasia to papillomatosis (Smith et al. 1989a; Poulet et al. 1994; Premdas et al. 1995). Although the papillomas in the present study represented different stages of development, none of the papillomas of two roach studied were invasive or metastatic. Thus, this indicates that papillomas are benign tumors. This is a common observation in the histology of fish papillomatosis (Bylund et al. 1980; Premdas et al. 1995; Beckwith et al. 2000). Only Smith et al. (1989a) has reported invasive papillomas in brown bullhead and white sucker. In addition, inflammatory infiltrates have been observed in large papillomas from white sucker histopathology (Smith et al. 1989b; Premdas et al. 1995), and the inflammation itself in papilloma could be a promoting factor for growth of the papilloma. However, no leukocytes were observed in any of the histological sections studied from roach in this study.

Epidermal papillomatosis in roach is occasionally observed all over the body, head and fin epidermis of fish. Furthermore, papillomas in roach are easy to diagnose even by gross observation and palpating by hand (I). However, several other skin tumours could be similar with papillomas macroscopically, such as basalioma or fibromas, and caution should be taken when diagnosing roach papillomas. Additional investigation of the other organs for the signs of metastasis should be performed with the histology in the future studies of roach papillomatosis. This would provide valuable extra information of roach tumors in question.

6.2 Papillomatosis occurrence and infection patterns in feral roach populations

The highest probability of epidermal papillomatosis occurring was found in deep, large lakes, of close vicinity to other lakes at low altitude. These results are in accordance with island

biogeography theory, which predicts that the probability of a species to occupying an island increases with island size and decreases with distance to the mainland, i.e. dynamic equilibrium between extinction and colonization rates determines the number of species on the island (MacArthur and Wilson 1967). In disease epidemiology, one important factor is also population density, as denser populations seem to promote transmission and persistence of the disease within a population (Swinton et al. 2002). However, Vethaak et al. (1992) or Møllergaard and Nielsen (1995, 1997) found no correlation between stock density and papillomatosis prevalence in their field studies with dab. We did not have information on the density of the roach populations in the study lakes. Thus, more research is needed to reveal the influence of population size on the transmission of roach papillomatosis. However, the population structure variables male percentage and mean length of fish in population sample were used as explaining variables for discriminant analysis, since papillomatosis in roach was known to be affected by fish sex and size (Kortet et al. 2002). Furthermore, date count was included in the discriminant model, as papillomatosis is known to change seasonally (Kortet et al. 2002). These variables related to population structure and season did not discriminate in papillomatosis occurring between the diseased and undiseased lakes.

No geographical gradient was observed in the occurrence of epidermal papillomatosis in roach along the River Kymijoki or River Vuoksi waterways studied. It seems that epidermal papillomatosis in feral roach populations occurs mainly in large inter-connected lakes in Finland, where it is possible for the disease to persist through the high colonization rate and low extinction rate of both the roach and the disease. This information is important for the use of the roach-papillomatosis system as a possible bioindicator in these lakes. Moreover, these results confirm that lake morphology is an important factor to take into account when using papillomatosis as an indicator of environmental stressors, as the absence of the disease does not necessarily mean that the lake is pristine.

High aggregation was noted in the disease intensity within the feral roach populations studied, i.e. most of the individuals in the population were undiseased or had few papillomas, while a small number of fish in the population have a high intensity of papillomatosis (I). Aggregation is commonly observed in parasite studies of wild host populations, and it has been proposed the pattern may occur due to variation in individual susceptibility and exposure to parasite infection (Wilson et al. 2002).

Seasonal occurrence and viral etiology is common for epidermal papillomatosis in fish (for a review see Getchell et al. 1998). Clear seasonality has also been observed in the papillomatosis of roach, with the prevalence and intensity of papillomas peaking during the spawning season in spring (Kortet et al. 2002). In addition, the present results suggest that papillomatosis occurrence follows the island biogeography theory (II) and is highly aggregated in wild roach populations (I), which is characteristic of infectious diseases (Hess et al. 2002). Thus, the results suggest that a virus could be possible cofactor for the papillomatosis of roach, although viral particles have not been discerned from roach papillomas.

6.3 Prevalence and intensity of papillomatosis in roach

The association between environmental stressors and epidermal papillomatosis was studied by comparing 10 matched roach population pairs in which impacted population were collected from a site influenced mainly by industrial and / or sewage effluents and compared to a reference population from a more pristine site (III). The use of field studies in which fish parasites and diseases are indicators of environmental stressors needs to be well designed to overcome confounding spatial and temporal variation in the environment (Lafferty 1997). Therefore, the sampling and statistical methods were designed to take into consideration the confounding factors which may affect papillomatosis in roach: (1) population samples were taken only from lakes where the papillomas were known to occur and a possible infectious agent was present, (2) matching population pairs (impact population and reference population) were sampled to ensure that the reference population was chosen upstream of the impact population, usually from the same lake, thus eliminating any possible between-lake variation, (3) the sampling was usually made within the same day or slightly later for the reference population in order to avoid confounding temporal patterns in papillomatosis prevalence between the compared population pair, (4) fish size and sex ratios were used as covariates in statistical analyses, if they differentiated between impact and reference population, as roach papillomatosis is known to be more common in male and large fish (Kortet et al. 2002).

In the study III, the prevalence of epidermal papillomatosis was higher in the impact roach populations than in the reference populations. This result is in concordance with several other

field studies which propose an association between environmental stressors and elevated fish papillomatosis prevalence (Smith et al. 1989a; Hayes et al. 1990; Vethaak et al. 1992; Premdas et al. 1995; Baumann et al. 1996; Mikaelian et al. 2000; Kortet et al. 2002; Pinkney et al. 2004a). The present results also confirm that the prevalence of papillomatosis in roach populations could be used as an indicator of environmental stressors. Exceptionally, population pair no. 7 showed a lower papillomatosis prevalence at the impact site than in the reference population. This was most likely due to the thermal effluents present in the impact site, which had an influence on the spawning of roach and confused the seasonal cycle of fish and papillomatosis. This result underlines that the nature of impact also needs to be taken into account when using the roach-papillomatosis system as an environmental indicator.

If population pair 7 is excluded due to the thermal effluents which had advanced the reproductive cycle of the roach and the seasonal cycle for papillomatosis, all prevalence values at the reference sites were below 15 %, and in eight of the nine impact sites, the prevalence of papillomatosis was above 10 %. Baumann et al. (1996) noted that papillomatosis prevalence was always of over 20 % in brown bullhead populations at contaminated sites in the Great Lakes, but the papillomatosis prevalence was never greater than 20 % in more pristine reference sites. In light of the present results, prevalence values above 15 % in roach populations most probably indicate that the fish are subject to environmental stressors and it should be investigated further.

Contrary to papillomatosis prevalence, papillomatosis intensity has only rarely been used for field or experimental studies of papillomatosis in fish, although it might provide additional information on the impact. Moreover, the results of papillomatosis intensity and environmental stressors are inconsistent (Premdas, et al 1995; Mikaelian, et al 2000;), which could also be due to the variation in the methodology used to measure the intensity of papillomatosis in fish (Premdas et al. 1995; Mikaelian et al. 2000; Kortet et al. 2002; Vainikka et al. 2004). We further developed a methodology to measure the intensity of papillomatosis (I), as described in Vainikka et al. (2004), to count the scales covered by the papilloma tumors. This method proved to be reliable and repeatable in fish with distinct scales, such as roach. Furthermore, the fish scales increase in size as the fish grows, giving a relative size-related measure of papillomatosis intensity. The methodology used to count the scales covered by papillomas is also fairly easy and quick, making it convenient to use in experimental and field conditions. Male and larger fish had higher papillomatosis intensity

than females or smaller fish. This was in concordance with other studies on the prevalence of papillomatosis (III, Kortet et al. 2002). Papillomatosis intensity did not correlate with prevalence in the 19 roach populations compared in our study (I), neither was papillomatosis intensity affected by environmental stressors (III). This infers that the dynamics of papillomatosis intensity and prevalence differ in natural populations. Furthermore, in white sucker, Mikaelian et al (2000) found no differences in papillomatosis intensity between contaminated and reference sites: nevertheless, Premdas et al. (1995) found that multiple papillomas were more common at contaminated sites.

In our experimental studies (IV-V), the intensity of papillomatosis regressed and proliferated even in the control fish. Moreover, the intensity of papillomatosis increased more in the fish which already had higher intensity at the beginning of the experiment. This was taken into account in experimental studies by using papilloma intensity from the beginning as a covariate in statistical analyses. Seemingly spontaneously regressing and proliferating tumors have also been observed in other fish species (Smith and Zajdlik 1987; Premdas and Metcalfe 1994), and it seems that multiple foci of tumors, i.e. high disease intensity, mean more tumors to proliferate or regress. Thus, papillomatosis prevalence has been shown to increase in tandem with papillomatosis intensity within the same experimental studies (Premdas and Metcalfe 1994; Premdas et al. 2001). Furthermore, we noted that papillomatosis intensity responded to different environmental stressors in laboratory experiments (IV, V), but the undefined development of papillomas needs to be taken in consideration in experimental studies of epidermal papillomatosis in fish.

6.4 Hypoxia, temperature changes and papillomatosis in roach

Hypoxia and periodic temperature changes increased the papillomatosis intensity in roach (IV). Oxygen deficiency poses severe stress for feral fish, especially due to increasing anthropogenic nutrient inputs and eutrophication (Wu 2002). Furthermore, in shallow eutrophicated Finnish lakes, periodic hypoxia is a relatively common phenomena (Lehtonen and Rask 2004). Oxygen deficiency is known to cause embryonic malformation, impaired reproduction, enhanced susceptibility to diseases and mortality for fish (Wu 2002; Wu et al. 2003; Shang and Wu 2004). The present results suggest that hypoxia affects also papilloma disease in roach. This is consistent with the field studies of Møllergaard and Nielsen (1995, 1997), who reported a peak in papillomatosis prevalence in dab after oxygen depletion in

areas along the coast of Denmark. Hypoxia has also been shown to increase bacterial concentrations in the organs and tissues of fish as well as to increase the mortality of diseased fish (e.g., Mqolomba and Plumb 1992; Fukuda et al. 1997).

Papillomatosis disease has not been reported to affect the survival of adult roach (Kortet et al. 2003b). Thus, the highest mortality in the OT group observed in study IV was probably caused by the oxygen deficiency. Moreover, fish in the OT group showed behavioural signs of stress, such as loss of equilibrium (Baroudy and Elliott 1994). During severe stress, fish try to maintain their homeostasis by undergoing physiological changes, further reducing its overall fitness (Wendelaar Bonga 1997). The condition factor at the end of the experiment was lowest in the OT group in the experiment study IV. The condition factor has rarely been found to correlate with papillomatosis prevalence in field studies (Møllergaard and Nielsen 1997; Mikaelian et al. 2000). Stress, as such, can also affect the immunity of fish, thus making them susceptible to diseases (Anderson 1990). During acute stress, the most commonly observed effect is the release of cortisol hormone in fish, resulting in the further depletion of circulating lymphocytes (Espelid et al. 1996). Although immunosuppression due to the stress caused by oxygen deficiency has been observed, other controversial results exist concerning the relationship between low oxygen concentrations and disease outbreaks in fish (Fevolden et al. 1993; Mesa et al. 2000). In our experiment (IV), the stress caused by hypoxia was most likely the main cause of the higher disease intensity, either due to the direct consequences of stress on the physiology of the fish or through immunosuppression of the fish resulting from stress.

Rising temperatures are also known to cause stress and increase susceptibility to pathogens in fish (e.g., Engelsma et al. 2003). Moreover, the ambient water temperature of ectothermic fish modulates the immune system and the virulence of pathogens as such (Le Morvan et al. 1998; Slater and Schreck 1998). This was also noted for the papilloma inducing virus of carp, herpesvirus cyprini, the optimal incubation temperature for which was 15 - 20 °C *in vitro*, but papillomas of carp regressed in 20 °C water *in vivo* (Sano et al. 1993b). This was most likely due to the more efficient immunity of carp in higher water temperatures. The results of increasing papillomatosis change in fish exposed to fluctuating temperature indicate that temperature is one of the factors affecting papillomatosis in fish. Peters (1977) earlier noted that papillomatosis increased in European eels (*Anguilla anguilla*) kept at high temperatures (15 - 22 °C), while at low temperatures (5 - 16 °C) papilloma growth was inhibited. In our

experiment (IV), the fish exposed to elevated temperatures (T group) had higher disease intensity than did those in control group.

6.5 Effluents and papillomatosis in roach

The results of field study III show that effluents could be one of the major factors contributing to the development of papillomatosis in roach. In field studies, fish could be exposed to several natural or chemical stressors simultaneously. Thus, we wanted to further explore the possible link between effluents and roach papillomatosis under laboratory conditions in study V. The experimental results suggest that treated municipal effluent can be one of the multifactorial elements contributing to roach papillomatosis. Several field studies have suggested that environmental contaminants, including industrial and municipal effluents are connected to elevated papilloma prevalence in fish populations (Smith et al. 1989a; Hayes et al. 1990; Vethaak et al. 1992; Premdas et al. 1995; Baumann et al. 1996; Mikaelian et al. 2000; Kortet et al. 2002), but only Grizzle et al. (1984) have experimentally studied the effect of effluents on fish. They noticed that the prevalence of papillomatosis increased in black bullheads caged in a wastewater pond with increasing chlorination of domestic wastewater (Grizzle et al. 1984). In the effluents used in our studies (V), chlorination was not used in the treatment of effluents but we can not rule out the possibility that our effluents contained immunotoxic or tumor promoting genotoxic substances, which might have a further effect on susceptibility to papillomatosis in fish (Price et al. 1997; Baumann 1998; White and Rasmussen 1998).

Some studies have reported immunosuppression in fish after exposure to effluents (Aaltonen et al. 2000; Fatima et al. 2001; Hoeger et al. 2005). Studies by Aaltonen et al. (2000) and Hoeger et al. (2005) suggest that immunosuppression could be due to the endocrine disrupting substances present in effluents, as males and females were affected differently. In our experiments (V), the difference in papillomatosis intensity change between the sexes was consistent in all the experiments, with treated municipal effluents found to have a significant effect only on male roach exposed to the municipal effluent in Experiment 2. Endocrine disrupting substances have frequently been discovered in different industrial and municipal effluents and are known to act as androgenic and estrogenic agonists and antagonists (Jobling et al. 1998; Tyler et al. 1998; Parks et al. 2001). Moreover, epidermal papillomatosis has been

induced experimentally in white sucker by testosterone and 17 β -oestradiol injections (Premdas et al. 2001), and higher circulating testosterone concentrations have been found in papillomatosis-infected male roach than in undiseased male roach (Kortet et al 2003a). Unfortunately, no potential endocrine disrupting substances were analysed from the effluent in our studies, and we can not rule out the possibility that roach males are simply more susceptible to the disease.

Effluents present in water can cause stress and render fish susceptible to immunosuppression and diseases (for review see Anderson 1990). In our effluent experiments, no clear indications of fish stress were observed, as the survival of the fish was high, and no significant differences were found in HSP70 expression levels between exposure groups (V). HSP70 is known to express cellular-level stress and has been induced by environmental contaminants in fish (Basu et al. 2002). However, the suitability of using HSP70 as a stress indicator is not always so straightforward (Iwama et al. 2004). For example, in longear sunfish (*Lepomis megalotis*) exposed to municipal effluents, physiological alterations caused by exposure have been documented, though HSP70 expression was not affected (Porter and Janz 2003).

7 CONCLUSIONS

Diagnosis of epidermal papillomatosis in roach was confirmed by histopathological observations of tumors. Furthermore, the occurrence of papillomatosis in Finnish lakes was consistent with the theory of island biogeography, with the papillomatosis intensity pattern being found to be highly aggregated in diseased roach populations. These observations suggest that epidermal papillomatosis of roach is likely an infectious disease and a viral particle could be cofactor in the development of papillomatosis in roach.

The matched pair study of 20 roach population indicated that papillomatosis prevalence is higher in roach populations from those sites impacted by contaminants than in the reference populations from more pristine sites. The methodology of the field study was designed to ensure that all confounding factors, such as spatial and temporal patterns of disease as well as the length and sex of the fish, were taken account in the sampling methods and statistical analysis. The study confirms that the prevalence of epidermal papillomatosis in feral roach populations residing in large lakes, where the disease is known to occur, could be used as a bioindicator or for the monitoring of environmental stressors. Furthermore, our field study suggest that a 15 % papillomatosis prevalence and over in roach populations is indicative of environmental stressors, when mature specimens are sampled in large lakes during the spring.

Roach exposed to hypoxia and fluctuating temperature demonstrated severe stress as well as the highest increase in papillomatosis intensity during the experiment. Furthermore, roach male exposed to a concentration of 15 % treated municipal effluent showed the highest increase in epidermal papillomatosis intensity. No obvious signs of stress, measured in terms of mortality and HSP70 expression, were observed in fish exposed to treated effluents.

The roach is a potential bioindicator species. It is a common, widespread native cyprinid in brackish and freshwaters in Europe and Western Asia, it occupies even poor quality aquatic habitats, and it is rather sedentary throughout its life. Furthermore, roach are also suitable for both field and laboratory experimentation. The present thesis suggests that the roach-papillomatosis system would offer an ecologically relevant bioindicator, as it expresses the natural changes resulting from exposure to environmental stressors in fish populations. Moreover, the roach-papillomatosis system is a cost-effective and easy bioindicator to use. In

the future, more research is needed of the pathogen evolved with roach papillomatosis, the distribution of papillomatosis in roach populations in different lakes and the dose-response relationships of roach papillomatosis and contaminants. Especially possible connection between endocrine disrupting agents and papillomatosis development in roach needs to be further investigated.

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