Vol. 53: 257–262, 2003 | DISEASES OF AQUATIC ORGANISMS Dis Aquat Org | Published February 27

Immunohistochemistry, ultrastructure and pathology of gills of *Abramis brama* from Lake Mondsee, Austria, infected with *Ergasilus sieboldi* (Copepoda)

Bahram Sayyaf Dezfuli[1,*], Luisa Giari[1], Robert Konecny[2], Paul Jaeger[3], Maurizio Manera[4]

[1]Department of Biology, University of Ferrara, Via Borsari 46, 44100 Ferrara, Italy
[2]Department of Limnology, Institute of Ecology and Conservation Biology, University of Wien, Althanstraße 14, 1090 Wien, Austria
[3]Land Salzburg, Referat 13/04 — Gewässerschutz Postfach 527, 5010 Salzburg, Austria
[4]Department of Structure, Functions, Animal Pathology and Biotechnology, University of Teramo, Pizza Aldo Moro 45, 64100 Teramo, Italy

ABSTRACT: Immunohistochemical, ultrastructural and pathological studies were carried out on the gills of bream *Abramis brama* (L.) from Lake Mondsee, Austria, that were naturally infected with *Ergasilus sieboldi* Nordmann, 1832. Of a total of 14 specimens of bream examined, the gills of 7 (50%) were parasitized with this copepod and the intensity of infection ranged from 1 to 23 crustaceans per host. Histopathological investigations of infected gill showed extensive tissue damage due to attachment and feeding of the crustacean. Parasites attached close to the base of filaments near the gill arch. Pressure exerted by the ectoparasite attached to the lateral margin of the gill filaments induced atrophy of the secondary lamellae. Tissue reactions included hyperplasia and mucous cell proliferation of the respiratory epithelium. Mucous cells displayed an intense immunohistochemical reactivity with the anti-nitric oxide synthase antibody. In parasitized primary and secondary lamellae, a high number of eosinophilic granular cells and rodlet cells were noticed. Rodlet cells represent an inflammatory cell type closely linked to other piscine inflammatory cells. Presence of a high number of inflammatory cells at the site of *E. sieboldi* attachment is related to intense host cellular reaction.

KEY WORDS: *Ergasilus sieboldi* infection · Immunohistochemistry · Ultrastructure · Cellular responses · Gill · *Abramis brama*

INTRODUCTION

Crustacean ectoparasites of fishes show an impressive diversity of structural and functional adaptations, and belong to several diverse orders, including Copepoda, Isopoda, Amphipoda and Ostracoda; they can be extremely harmful to their host (Kabata 1984).

Species of *Ergasilus* are ubiquitous; 21 species have been described from different hosts and locations in Brazil (Motta Amado & Rocha 1995), while in Europe *E. sieboldi* has been recorded in 14 species of British freshwater fishes (Alston & Lewis 1994). In gills of *Tinca tinca* infected by *E. sieboldi*, haemorrhages and inflammation were accompanied by reactive granulocytosis connected with intensified hemopoiesis and also an accelerated process of destruction of these cells in the peripheral blood (Einszporn-Orecka 1973). Moreover, extensive tissue damage resulting from the feeding and attachment of this parasite was reported in several species of fishes (Abdelhalim 1990). In addition, it was suggested that lesions due to attachment and feeding of ergasilids may be secondarily infected with fungi, bacteria and viruses (Nigrelli 1950, Dogiel et al. 1958). In the present study, lamellar fusion due to the hyperplasia of the interlamellar epithelium, massive mucous cell proliferation as well as the occurrence of a high number of eosinophilic granular cells (EGCs) and rodlet cells (RCs) in gills of bream infected with *E.*

*Email: dzb@dns.unife.it

sieboldi were recorded. In other fish species, it has been suggested that RCs represent an inflammatory cell type closely linked to other piscine inflammatory cells (Dezfuli et al. 2000, Manera et al. 2001).

Increase or infiltration of different types of cells (e.g. granular cells, lymphocytes, mucous cells) at the copepod attachment site on gills of fishes was reported by Bennet & Bennet (1994, 2001), Leino (1996) and Roubal (1989, 1999). With regard to mucous cells, nitric oxide synthase (NOS) activity has been previously reported in mucous and epithelial cells in mammals and has been related to epithelial cell integrity or secretion (Brown et al. 1992, 1993, Kwon et al. 2001). NOS derived from inducible NOS is closely related to host defence mechanisms and is important as a toxic defence molecule against infectious organisms; furthermore, leukocyte-derived cytokines are known to stimulate its production at the site of inflammation (Coleman 2001, Kwon et al. 2001).

This investigation comprises immunohistochemical methods and observations carried out by light and transmission electron microscopy and is the first report of the occurrence and reaction of RCs in gills of a fish infected with an ectoparasite.

MATERIALS AND METHODS

In August 2001, 14 specimens of bream *Abramis brama*, ranging from 32 to 46 cm (38.23 ± 4.11, mean ± SE) in total length and from 345.30 to 970.20 g (599.29 ± 172.47, mean ± SE) in weight were sampled by seine net on 1 occasion from Lake Mondsee (South Austria). After capture, the fish were brought alive to the laboratory and killed within 6 h by a sharp blow to the head. Gills were examined for ectoparasites, and gill filaments with attached *Ergasilus sieboldi* were removed and fixed in Bouin's fluid and then embedded in paraffin, sectioned at 7 µm and stained with Azan-Mallory. For electron microscope purposes, pieces of infected gills up to 7 mm in diameter were fixed in 2% glutaraldehyde in 0.1 M cacodylate buffer, post-fixed in 1% osmium tetroxide in the same buffer for 2 h, dehydrated in graded ethanol, transferred to propylene oxide and embedded in an Epon-Araldite mixture. Semithin sections were cut on a Reichert Om U2 ultramicrotome with glass knives and stained with Azure-A Methylene blue. Ultrathin sections were stained in a 50% alcohol uranyl acetate solution and lead citrate, and examined in a Hitachi H-800 electron microscope. Light photomicrographs were obtained with a Leitz photomicroscope. For comparative purposes, gills of 6 uninfected breams were processed by the same methods.

For immunohistochemical investigations, glass slides with tissue sections were immersed in 1:1 saturated potassium ethoxide and 100% ethanol for 30 min to remove the Epon-Araldite mixture from the sections (modified from the method of Xiang & Markel 1995). The slides were then rinsed in 3 changes of 100% ethanol for 7 min each to clean off potassium ethoxide, rehydrated in graded ethanol (100, 90, 80, 70%) and treated with 1% $NaIO_4$ in distilled water for 20 min. After some washes with physiological solution (phospate-buffered saline, PBS), sections were treated with 3% hydrogen peroxide for 30 min. After 2 washes with PBS, sections were incubated at room temperature in blocking buffer (10% normal goat serum in PBS) for 30 min. After 2 washes with PBS, the sections were incubated with a solution containing the primary antibody (3% BSA in PBS, 1:500 diluted rabbit anti-nitric oxide synthase I (NOS-I; bNOS, polyclonal antibody) (Chemicon) for 24 h at 4°C. As a negative control, adjacent sections were incubated in the same solution in the absence of the primary antibody. All the sections were washed 3 times in PBS. The antigen/antibody complex was revealed using Super Sensitive kit Multilink Immunodetection System (BioGenex). The sections were stained with chromogen-specific substrate for peroxidase and diaminobenzidine (DAB), washed in water, and then dehydrated, mounted and examined under bright-field illumination.

For counting the RCs, an average of 2642 ± 461 (mean ± SE) linear microns of gill lamellar tissue was screened from 10 different fish (5 uninfected and 5 infected), divided into 3 groups of sections: 5 sections of uninfected breams, 5 sections of lamellae at the point of parasite attachment and 5 sections of infected gills some distance away from the parasite attachment sites. The mean RC number per linear micron was calculated for each group and a 1-way analysis of variance (ANOVA) was used to detect differences between groups. Analyses of covariance (ANCOVA) were applied to the RC number covariate for the corresponding counted linear microns to ensure a correct statistical analysis.

RESULTS

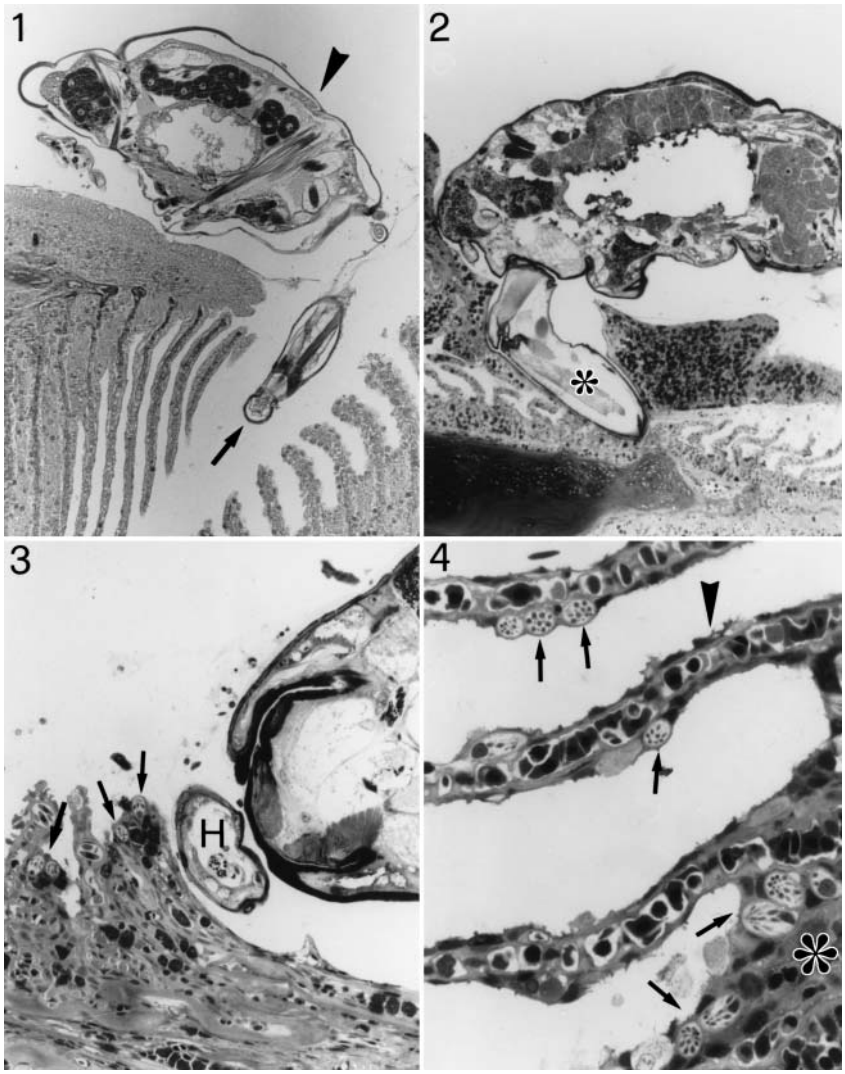
Gills of 7 out of 14 *Abramis brama* specimens were infected with *Ergasilus sieboldi*, the intensity of infection ranging from 1 to 23 parasites per host. Adult females attached to primary lamellae (gill filaments) with their claw-like second antennae (Figs. 1 & 2) close to the gill arch near the base of filaments (Fig. 3). The parasite's body lay between the hemibranchs with the axis parallel to the primary lamellae axis and with its cephalic extremity oriented towards the gill arch. In parasitized gills, enhanced mucous production, congestion, haemorrhages and primary lamellae erosions were encountered.

Histopathological and immunohistochemical observations

At the site of attachment, regressive phenomena prevailed (Figs. 1 & 2). Complete superficial tissue erosion with primary lamellae cartilage, blood vessel exposure and haemorrhages resulted from compression and mechanical action of the parasite mouth, swimming appendages and second antennae. Erosion, desquamation and necrosis of secondary lamellae occurred near the site of parasite attachment (Figs. 1 & 2).

At the distal part of the infected primary lamellae, proliferation of the interlamellar epithelium induced lamellar fusion. Moreover, massive mucous cell proliferation as well as EGC and RC infiltration were observed (Fig. 3), especially in the secondary lamellae (Fig. 4). A massive presence of EGCs was observed in the primary lamellae tissue beneath the covering epithelia, whereas RCs appeared in subepithelial and infraepithelial positions in linear clusters and arrays, and were consistently associated with EGCs. In parasitized gills, the cytoplasm of mucous cells reacted positively with the antibody against NOS (Figs. 5 & 6).

Differences of RCs/100 linear micron quotients (ANOVA, $p < 0.01$) were recorded between uninfected fish (mean ± SE: 0.29 ± 0.07) and parasitized conspecifics (2.43 ± 0.33); moreover, there was a difference among infected fish, namely between areas near an attached ectoparasite (3.05 ± 0.25) and areas some distance away (1.50 ± 0.24) (ANOVA, $p < 0.01$).



Figs. 1 to 4. Fig. 1. Attachment of adult female *Ergasilus sieboldi* (arrowhead) to gills of *Abramis brama*. Second antennae (arrow) of the copepod are visible between gill filaments (Azan-Mallory; ×140). Fig. 2. Parasite attached by clawed maxilliped (asterisk) deeply penetrating gill tissue (Azure-A Methylene blue; ×115). Fig. 3. Semithin section of gill with *E. sieboldi*, showing few rodlet cells (arrows) at depth of secondary lamellae near head of crustacean (H) (Azure-A Methylene blue; ×330). Fig. 4. Semithin section of infected gill, showing high number of rodlet cells (arrows) in primary (asterisk) and secondary (arrowhead) lamellae near the site of copepod attachment (Azure-A Methylene blue; ×800)

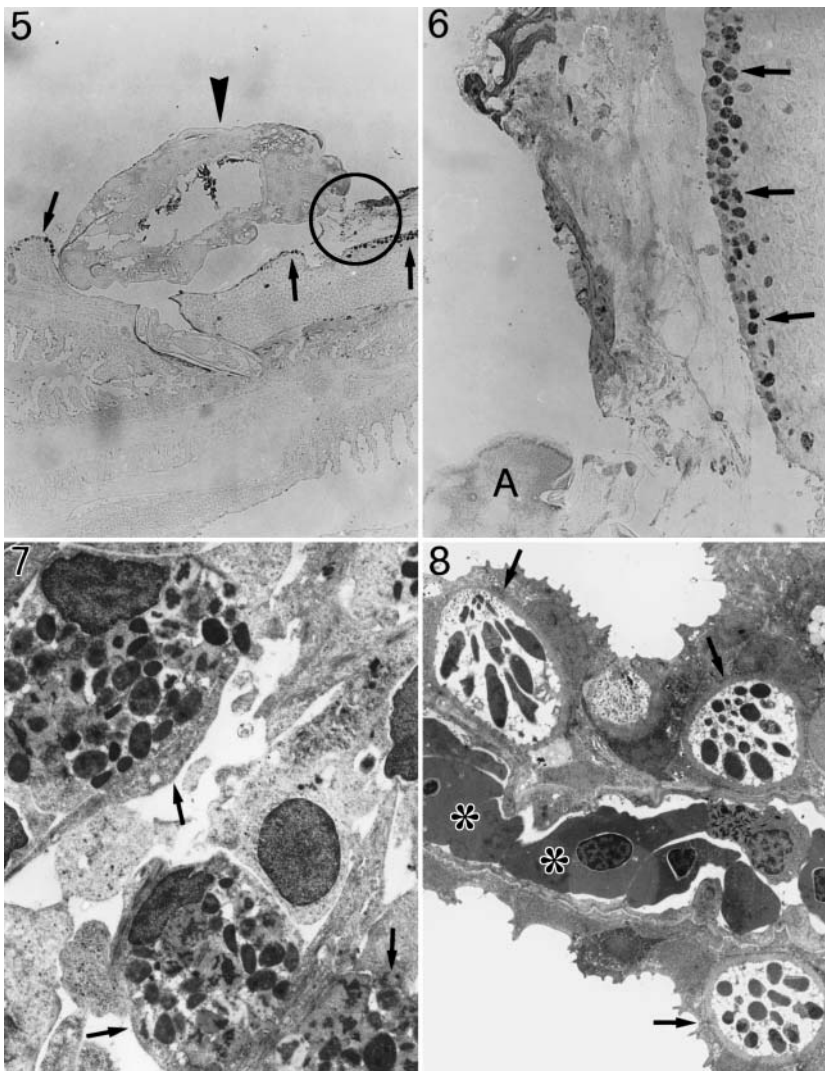
Ultrastructural observations

Primary lamellae of infected gills presented a high number of EGCs (Fig. 7). Such cells were voluminous, with their cytoplasm containing numerous round-to-ovoid granules. Some displayed a homogeneous, electron-dense content, but others had an outer clearer halo and an inner dense core. Granule rarefaction and dissolution were also encountered. In many instances, EGCs appeared in the blood vessels of the primary and secondary lamellae adherent to the lining endothelium.

RCs were numerous in primary lamellae as well as in the epithelium of both sides of the secondary lamellae; however, in the secondary site, they were often encountered between the respiratory epithelium lining and the pillar cells delimiting the blood vessels (Fig. 8). RCs displayed typical cytological features such as a subplasmalemmar fibrillar capsule, an eccentrically basally located nucleus (Fig. 9) and rodlets with their central dense core oriented to the apical part of the cells protruding in a crown-shaped configuration. No

evidence of rodlet extrusion was seen. Notwithstanding, rodlet dissolution and lateral extrusion in the interstitium were documented (Fig. 10). In such instances, the subplasmalemmar capsule disintegrated laterally, permitting the expulsion of the dissolving rodlets into the lateral interstitium (Fig. 10). RCs were contracted and an outer halo of amorphous substance appeared between them and the other cells (Fig. 11).

In the secondary lamellae, endothelial cells displayed villar protoplasmatic projection and increased their adhesiveness with respect to neutrophilic granulocytes, monocytes, lymphocytes and erythrocytes (namely endothelial cell 'activation'). Evidence of blood clotting was also encountered (Fig. 8). Neutrophilic granulocytes were particularly abundant in the blood vessels adhered to the activated endothelium and also in the interstitium around blood vessels (Fig. 12). They possessed an eccentrically located nucleus as well as elongated and flattened granules with a dense core (Fig. 12).



Figs. 5 to 8. *Abramis brama* infected by *Ergasilus sieboldi*. Fig. 5. Sagittal section of adult female *E. sieboldi* (arrowhead) attached to gill filament, immunoreactivity of cells (arrows) to nitric oxide synthase (NOS) is evident (×62). Fig. 6. High magnification of encircled area in Fig. 5; note presence of high number of mucous cells (arrows) positive to NOS; A = abdomen (×256). Fig. 7. Eosinophilic granular cells (arrows) within primary lamellae of infected gill (×5400). Fig. 8. Secondary lamellae, showing rodlet cells (arrows) and blood cells (asterisks) (×4200)

DISCUSSION

The copepods *Ergasilus briani* and *E. sieboldi* can co-occur on the same host. Apparently, *E. sieboldi* prefers smaller or younger fishes (Bauer et al. 1969, Alston & Lewis 1994) while, according to Dogiel et al. (1958) and Abdelhalim (1990), the intensity and prevalence of this copepod infection increases with increasing host size/age. During the present survey, bream smaller than 40 cm in total length harboured no more than 2 *E. sieboldi*, whereas, the highest intensity of infection was recorded in fish with a total length of over 41 cm.

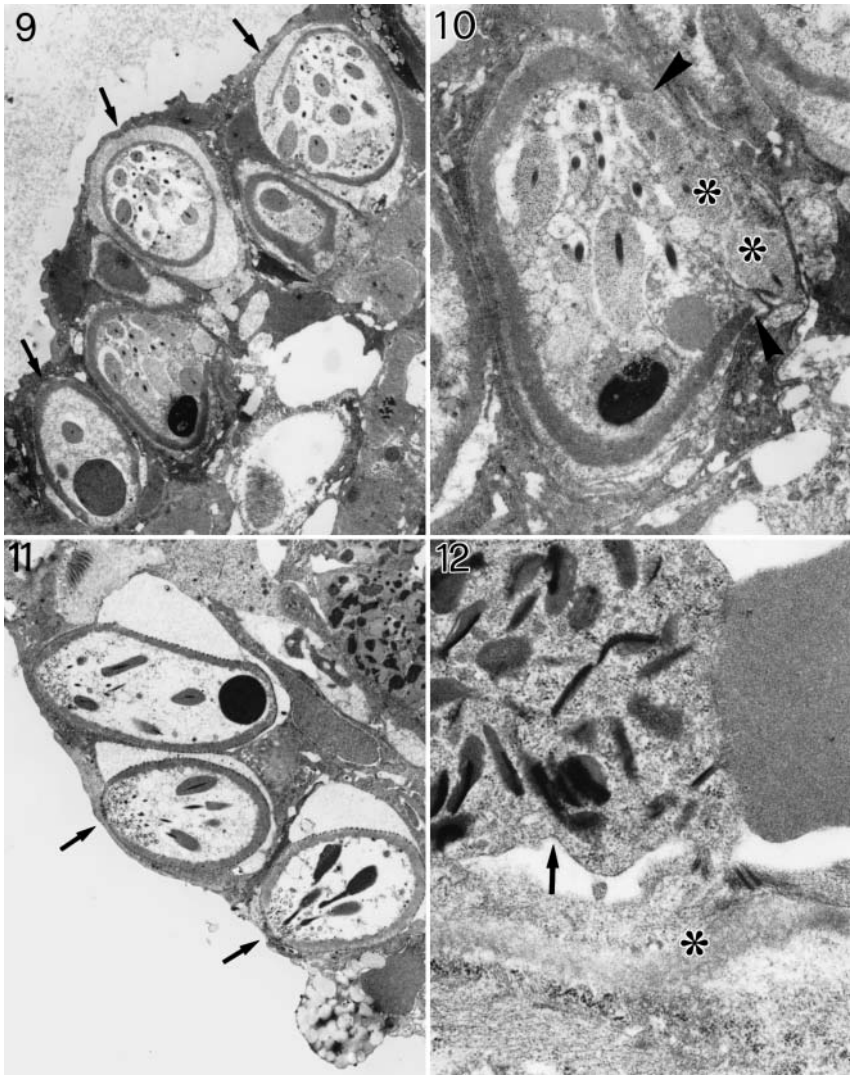
Some copepods (e.g. *Alella macrotrachelus*) feed by browsing on fish gill epithelium (Kabata & Cousens 1977, Roubal 1989) or by ingesting blood from ruptured blood vessels (Muraga et al. 1981). Copepods attach by using various appendages modified for grasping, and this ectoparasite activity can lead to secondary infection by pathogenic organisms (e.g. bacteria, fungi) and also to the development of adhesions between gill filaments (Nigrelli 1950, Reichenbach-Klinke & Elkan 1965). Consequently, fish respiration is impaired and reduced feeding, weight loss, and general deterioration of health can result (Reichenbach-Klinke & Elkan 1965). There are few quantitative reports concerning the extent of pathology caused by crustaceans in wild fish populations (Roubal 1999). Some data on damage to fish gills due to copepods appear in Kabata & Cousens (1977), Sutherland & Wittrock (1985) and Bennet & Bennet (2001).

The severity of damage caused by ergasilid infection of fish specimens is directly proportional to the number of copepods on the gills (Abdelhalim

1990). Accordingly, less than 20 *Ergasilus sieboldi* may have little effect, but, when their intensity exceeds 100, gill damage may be serious (Kabata 1970). Some pathological effects of ergasilids are decrease in body weight/condition factor, reduction in the osmoregulatory/respiratory function of the gills, blood cell changes and mortality. Regarding host mortality due to *E. sieboldi*, a high intensity of infection was reported to be the cause and details were provided by Dogiel et al. (1958), Fryer (1982) and Schäperclaus (1986).

As mentioned in the 'Results', a high number of rodlet cells was encountered, especially at the site of copepod attachment. Published records on the occurrence of these cells in relation to ectoparasites are very rare, with the available information referring only to a protozoan (Leino 1979, 1996); according to Leino, the RCs react to the presence of myxosporeans (Protozoa), and RC secretion has an antibiotic function. This author suggested that, in fish kidney and gill, parasite infection of the epithelium results in RC proliferation (Leino 1979, 1996). Similar findings were reported for the intestine of *Anguilla anguilla* and the liver and pancreas of *Phoxinus phoxinus* infected with endoparasitic helminths (Dezfuli et al. 1998, 2000, respectively). The present report is the first to document an increase in the number of RCs at the site of attachment of an ectoparasite, and may constitute an additional confirmation of the defensive role of these cells in the piscine inflammatory system against parasites (Dezfuli et al. 2000).

According to Campos-Perez et al. (2000), fish gills are an important site of NOS expression in rainbow trout, and these authors highlighted the importance of the gills as a tissue capable of initiating an immune response against infectious agents. The results of the present investigation provide further evidence in favour of the anti-parasitic, immune response of gill tissue.



Figs. 9 to 12. *Abramis brama* infected by *Ergasilus sieboldi*. Fig. 9. Few rodlet cells (arrows) near surface of primary lamellae (×3400). Fig. 10. High magnification of rodlet cell; note lateral opening of capsule (arrowheads) and extrusion of some rodlets (asterisks) (×8200). Fig. 11. Peculiarity of cytoplasm of 3 rodlet cells (arrows) beneath the covering epithelia of primary lamellae (×3000). Fig. 12. Secondary lamellae, a neutrophil granulocyte (arrow) is attached to endothelium (asterisk) (×27 000)

Acknowledgements. Thanks are due to William J. Poly of California Academy of Sciences, San Francisco, California, for the English revision of a draft of the manuscript. We are grateful to G. Bosi from the University of Milan for technical help. This investigation was supported by grants from the Italian Ministry of the University and Scientific Research and Technology.

LITERATURE CITED

Abdelhalim AI (1990) Morphology and epidemiology of some parasitic copepods (Poecilostomatoida: Ergasilidae) from British freshwater fish. PhD thesis, University of London

Alston S, Lewis JW (1994) The ergasilid parasites (Copepoda: Poecilostomatoida) of British freshwater fish. In: Pike AW, Lewis JW (eds) Parasitic diseases of fish. Samara Publishing, Tresaith, p 171–188

Bauer ON, Musselius VA, Strelkov YuA (1969) Diseases of pond fishes. Izdatel'stove Kolos, Moskva (in Russian; English translation: Israel Program for Scientific Translations, Jerusalem, 1973)

Bennet SM, Bennet MB (1994) Pathology of attachment and vascular damage associated with larval stages of *Dissonus manteri* Kabata, 1966 (Copepoda: Dissonidae) on the gills of coral trout, *Plectropomus leopardus* (Lacépède) (Serranidae). J Fish Dis 17:447–460

Bennet SM, Bennet MB (2001) Gill pathology caused by infestation of adult and preadult *Dissonus manteri* Kabata (Copepoda: Dissonidae) on coral trout, *Plectropomus leopardus* (Lacépède) (Serranidae). J Fish Dis 24:523–533

Brown JF, Tepperman BL, Hanson PJ, Whittle BJ, Moncada S (1992) Differential distribution of nitric oxide synthase between cell fractions isolated from the rat gastric mucosa. Biochem Biophy Res Comm 184:680–685

Brown JF, Keates AC, Hanson PJ, Whittle BJ (1993) Nitric oxide generators and cGMP stimulate mucus secretion by rat gastric mucosal cells. Am J Physiol 265:418–422

Campos-Perez JJ, Ward M, Grabowski PS, Ellis AE, Secombes CJ (2000) The gills are an important site of iNOS expression in rainbow trout *Onchorhynchus mykiss* after challenge with gram-positive pathogen *Renibacter salmoninarum*. Immunology 99:153–161

Coleman JW (2001) Nitric oxide in immunity and inflammation. Int Immunopharmacol 1:1397–1406

Dezfuli BS, Capuano S, Manera M (1998) A description of rodlet cell from the alimentary canal of *Anguilla anguilla* and their relationship with parasitic helminths. J Fish Biol 53:1084–1095

Dezfuli BS, Simoni E, Remigio R, Manera M (2000) Rodlet cells and other inflammatory cells of *Phoxinus phoxinus* infected with *Raphidascaris acus* (Nematoda). Dis Aquat Org 43:61–69

Dogiel VA, Petrushevskii GK, Polyanski YI (1958) Parasitology of fishes. Leningrad University Press (in Russian, English translation: Oliver & Boyd, London, 1961)

Einszporn-Orecka T (1973) Changes in the picture of peripheral blood of tench, *Tinca* (L.) under the influence of *Ergasilus sieboldi* Nordmann, II. Changes in the leuckocytic system. Acta Parasitol Pol 21:485–499

Fryer G (1982) The parasitic Copepoda and Branchiura of British freshwater fishes, a hand book and key. Freshw Biol Assoc Sci Publ 6:87

Kabata Z (1970) Crustacea as enemies of fishes. In: Snieszko SF, Axelrod HR (eds) Diseases of fishes, Book 1. TFH Publishers, Jersey City, PA

Kabata Z (1984) Diseases caused by metazoans: crustaceans. In: Kinne O (ed) Diseases of marine animals, Vol IV, Part 1. Introduction, Pisces. Biologische Anstalt Helgoland, Hamburg, p 321–399

Kabata Z, Cousens B (1977) Host-parasite relationship between sockeye salmon, *Oncorhynchus nerka* and *Salmincola californiensis* (Copepoda: Lernaeopodidae). J Fish Res Board Can 34:191–202

Kwon S, Newcomb RL, George S (2001) Mechanisms of synergistic cytochine-induced nitric oxide production in human alveolar epithelial cells. Nitric Oxide 1:1–13

Leino RL (1979) The effects of periodic acid-silver methenamine staining and protease digestion on the secretory granules of rodlet cells. In: Bailey GW (ed) Thirty-seventh Annual Meeting of the Electron Microscopy Society of America, Baton Rouge, LA, p 310–311

Leino RL (1996) Reaction of rodlet cells to a myxosporean infection in kidney of the bluegill, *Lepomis macrochirus*. Can J Zool 74:217–225

Manera M, Simoni E, Dezfuli BS (2001) The effect of dexametasone on the occurrence and ultrastructure of rodlet cells in goldfish. J Fish Biol 59:1239–1248

Motta Amado MAP, Rocha CEF (1995) Três novas espècies de copepodas parasitas do gênero *Ergasilus* (Poecilostomatoida, Ergasilidae), copepodas em filamentos branquiais de peixe mugilìdeos do Brasil. Nauplius 3:33–48

Muraga K, Kawatow K, Ichizono H (1981) Infestation by *Allela macrotrachelus* (Copepoda) of cultured black seabream. Fish Pathol 16:139–144

Nigrelli RF (1950) Lymphocystis disease and ergasilid parasites in fishes. J Parasitol 36:36

Reichenbach-Klinke H, Elkan E (1965) Part I. Fish. In: Reichenbach-Klinke H, Elkan E (eds) The principal diseases of lower vertebrate. Academic Press, London, p 108–109

Roubal FR (1989) Comparative pathology of some monogenean and copepod ectoparasites on the gills of *Acanthopagrus australis* (family Sparide). J Fish Biol 34:503–514

Roubal FR (1999) Extent of gill pathology in the toadfish *Tetractenos hamiltoni* by *Naobranchia variabilis* (Copepoda: Naobranchiidae). Dis Aquat Org 35:203–211

Schäperclaus W (1986) Fischkrankeiten Akademie-Verlag, Berlin (English translation: Oxonion Press PVT, New Delhi, 1997)

Sutherland DR, Wittrock DD (1985) The effects of *Salmincola californiensis* (Copepoda: Lernaeopodidae) on the gills of farm-raised rainbow trout, *Salmo gairdneri.* Can J Zool 63: 2893–2901

Xiang Z, Markel MD (1995) Bromodeoxyuridine immunohistochemistry of epon-embedded undecalcified bone in a canine fracture healing model. J Histochem Cytochem 43: 629–635

Editorial responsibility: Wolfgang Körting, Hannover, Germany

Submitted: May 8, 2002; Accepted: October 14, 2002
Proofs received from author(s): January 24, 2003