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ORIGINAL PAPER

Gill histopathology of cultured European sea bass, *Dicentrarchus labrax* (L.), infected with *Diplectanum aequans* (Wagener 1857) Diesing 1958 (Diplectanidae: Monogenea)

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Abstract The mortality of juvenile European sea bass, *Dicentrarchus labrax* (L.), in the spring of the last 5 years in the northern coast of the Adriatic Sea has been attributed to heavy infections of the gill monogenean *Diplectanum aequans* (Wagener 1857) Diesing 1858. The histopathological examination of 38 sets of gills from hosts measuring 16.46±0.26 cm in total length (mean±S.E.) and weighing 45.98±2.37 g (mean±S.E.) were conducted using light and transmission electron microscopy. Twenty-eight (73.6%) *D. labrax* specimens were infected (34.61±4.42, mean intensity±S.E.; 5–100, range) with the majority of *D. aequans* attaching to the median and apical portions of the primary gill filaments. The sites of attachment were marked by the common presence of haemorrhages and a white mucoid exudate. In histological sections, the opisthaptors of the parasites were observed to penetrate deeply, lying in close proximity to the basal membrane of primary lamella where they induced a hyperplastic response. Disruption and fusion of the secondary lamellae were common in all infected specimens with several individuals also exhibiting a marked erosion and inflammation of the epithelium of the primary and secondary lamellae. In infected fish, cellular changes in the epithelium underlying the bodies of worms were noted typified by an elevation in the number of mucous and rodlet cells and a reduction in the number of chloride cells.

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Introduction

Dicentrarchus labrax (L.) is the second most important cultured marine species in Italy with 9,600 tonnes being produced in 2003, worth an estimated *$7,058,690 (FAO 2003). Production, however, is marred by the annual loss of ~5–10% of the juvenile stock in the spring of each year which, have been attributed to heavy infections of *Diplectanum aequans* (Wagener 1857) Diesing 1858 (Monogenea, Diplectanidae). The genus *Diplectanum* Diesing 1858 is the largest in the family Diplectanidae (Hayward 1996) and one species member, *D. aequans* is a common parasite of both cultured and wild *D. labrax*. Monogeneans are common in the Mediterranean and their presence on a range of cultured species have been responsible for mortalities or a reduction in the health of stocks including *Microcotyle* van Beneden and Hesse 1863 on *Sparus aurata* L. (Sanz 1992; Alvarez-Pellitero and Crespo 1995; Padrós and Crespo 1995) and *Zeuxapta seriolae* (Meserve 1938) on *Seriola dumerili* Risso (Grau et al. 2003; Montero et al. 2004).

Heavy monogenean infections through their attachment and feeding can induce a range of histopathological changes to the epithelium, which can facilitate the invasion and establishment of a range of secondary fungal, bacterial and/or viral infections (Stoskopf 1993; Cone 1995). Literature on the histopathological impacts inflicted by

various monogeneans is extensive, e.g. Hayward et al. (2001), Padrós et al. (2001), Ogawa (2002), Montero et al. (2004), Mansell et al. (2005) *et inter alia* including a synopsis provided in recent review by Buchmann et al. (2004). Among those listed in this latter account, the work of Oliver (1977) and González-Lanza et al. (1991) dealt specifically with *D. aequans* infections on European sea bass but did not provide details on the host cellular reaction.

This investigation, therefore, was undertaken to obtain information on the host's cellular responses as determined by information gathered from light and transmission electron microscopy. An additional aim of the current study is to provide further evidence on role of rodlet cells (RCs) and other piscine inflammatory cells in infected fish. Moreover, a comparison between the gill reactions exhibited by *Abramis brama* (L.) to an infection of *Ergasilus sieboldi* Nordmann 1832 (Crustacea) and by *D. labrax* infected with *D. aequans* will be presented and discussed.

Materials and methods

In June and July 2004, 38 specimens of *D. labrax* measuring 16.46±0.26 cm in total length (mean±S.E.) and weighing 45.98±2.37 g (mean±S.E.), were obtained from a local supplier in Rovigo, Italy. After capture, the fish were brought alive to the laboratory and killed within 2 h and then examined for the presence of parasites.

The gills were examined for ectoparasites and gill filaments with attached monogeneans were removed and fixed in Bouin's fluid and then embedded in paraffin wax. The blocks were then sectioned at 5 μm and stained with haematoxylin-eosin and with Alcian blue-PAS reaction to observe the mucous and cellular granular elements within each section. For light and electron microscopy, infected gill filaments, which measured up to 7 mm in length, were fixed in 2% glutaraldehyde in 0.1 M cacodylate buffer for 4 h at 4°C before post-fixing them in 1% osmium tetroxide in the same buffer for 2 h.

The specimens were then dehydrated through a graded ethanol series before transferring them into propylene oxide and embedding them in an Epoxy-Araldite® mixture. Semi-thin sections (1.5 μm) were cut on a Reichert Om U 2 ultramicrotome using glass knives and then stained with toluidine blue. Ultra-thin sections (90 nm) were stained with a 4% uranyl acetate solution in 50% alcohol and Reynold's lead citrate and examined using a Hitachi H-800 electron microscope. For comparative purposes, the gills of ten uninfected sea bass were also processed.

Infected gill filaments collected from several sea bass were prepared for scanning electron microscopy (Cambridge Instruments Steroscan 360) following the methods detailed in Amin and Dezfuli (1995). Light photomicrographs were taken using a Nikon microscope ECLIPSE 80i.

The number of rodlet cells (RCs), chloride cells (CCs) and mucous cells (MCs) in the gills were determined with the aid of a light microscope and computerised image analysis software (Lucia G 4.8, Laboratory Imaging, Praha, Czech Republic). Twenty randomly selected tissue areas, measuring 25,000 μm^2 in sections, were prepared from both infected and uninfected fish. The total number of RCs, CCs and MCs in each section were then counted. An ANOVA, on the square root transformed data, was used to detect significant differences in the number of each cell type between the two groups of fish using the commercial statistical package SPSS (ver. 13.0.1; SPSS, Chicago, IL, USA).

Results

Twenty-eight (73.6%) of the 38 specimens of *D. labrax* that were studied were infected with *D. aequans*. Externally, all the *D. labrax* used for the study appeared to be in good health, in body condition and in coloration. Three of the fish, however, before being killed appeared to swim a little slower than the others but did not exhibit any other noticeable deviations in normal behaviour. Examination of the gills on anaesthetised fish, however, revealed extensive mucus hypersecretion, haemorrhagia and swelling especially on the apical portions of the secondary gill lamellae. Macroscopically, the condition of the viscera appeared to be normal except for a paler tone to the liver and, in certain instances, also to the spleen, which was attributed to the anaemic condition of the host.

The intensity of *D. aequans* infection ranged from 5 to 100 parasites per host (34.61±4.42, mean intensity±S.E.). Worms were commonly encountered on the median (Fig. 1a) and apical portions (Fig. 2a-c) on adjacent lamellae in small aggregations (Figs. 1a and 2a).

The examination of histological material revealed that the opisthaptor of *D. aequans* penetrates deep into the basement membrane (Fig. 1c,d) and the connective tissue of the primary lamella (Fig. 2b,c) causing destruction to the secondary lamellae (Figs. 1a,b and 2c). The worm's attachment to the host's gill epithelium is ensured by the dorsal and ventral spines of the opisthaptoral squamodiscs (Figs. 1d and 2b,d) and by the hamuli, which penetrate deep into the epithelial cells (Fig. 1c). From the histological sections, it would appear that it is the activity of the hamuli that cause erosion and are responsible for the detachment of gill cells.

This was confirmed by the common observation of cellular residue in close proximity to the body of the parasite (Fig. 1a). In addition, a marked reaction typified by

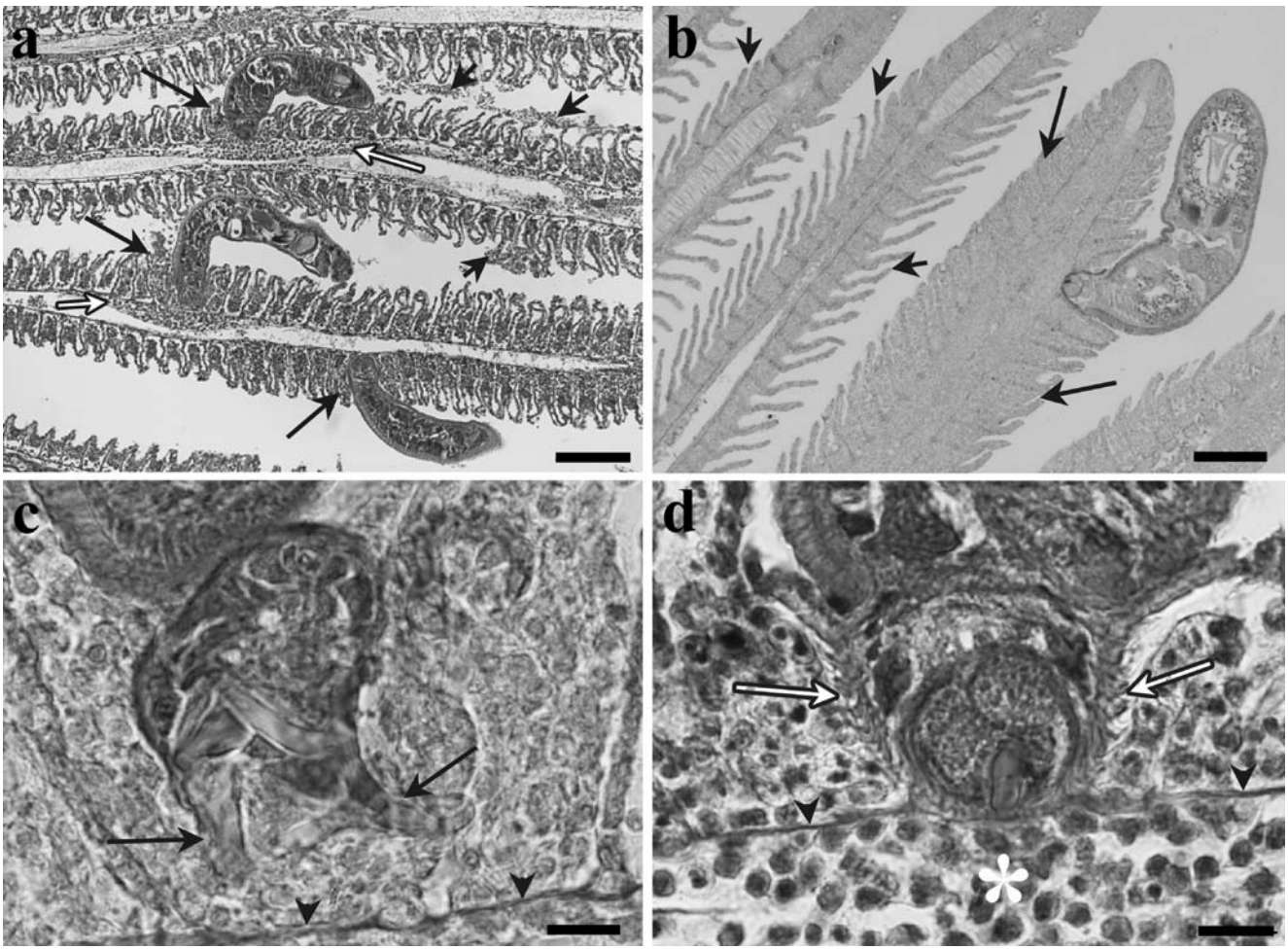

Fig. 1 **a** *Dicentrarchus labrax* gills infected with *Diplectanum aequans* and stained with Alcian blue-PAS. Monogeneans can be seen attached with their opisthaptors to the adjacent lamellae but note the interruption (*arrows*) to the secondary lamellae, the lamellar oedema (*empty arrows*) and the presence of epithelial residue in close proximity to the bodies of worms (*short arrows*). **b** An Alcian blue-PAS stained histological section of tissue showing the attachment of *D. aequans* to the apices of primary gill filaments. The *arrows* denote areas where there is fusion of the secondary lamellae, while the *short arrows* indicate areas of normal, uninfected gill tissue. **c** The force of diplectanid attachment brings the opisthaptor in close proximity to the basement membrane (*arrow heads*), with the hamuli points (*arrows*) penetrating gill cells (Alcian blue-PAS). **d** The opisthaptor of *Diplectanum* in intimate contact with the basement membrane (*arrow heads*). Note the close association of the squamodisc spines (*arrows*) with the gill epithelium. It can also be seen that the secondary lamellae have lost their integrity and that the presence of lamellar oedema (*asterisk*) is evident (Alcian blue-PAS)

a proliferation of epithelial tissue commonly resulting in the fusion of the secondary lamellae was noted in the vicinity of the opisthaptor (Figs. 1a,b and 2c). In some gills, this latter pathology was accompanied by the presence of blood vessel aneurysms (telangiectasis).

Observations with the transmission electron microscope to investigate the cellular response of the host revealed there to be a marked reduction in the recruitment of eosinophilic granular cells and activated neutrophilic granulocytes at the site of attachment. Lamellar oedema (Fig. 1a,d) and contraction of pillar cells (Fig. 2d) were also evident. Contraction of the latter, produced ischemia at the base of the lamellae and, consequently, an enlargement of the marginal (inner and outer) blood channels of the gills. In addition to this, necrosis of host tissue, vacuolization of fish cells and the presence of macrophages with electron dense inclusions within them, were noted in the region where each spine of the squamodisc makes intimate contact with the gill lamellae (Figs. 2a-c and 3a).

In comparison to uninfected fish, in parasitized gills, higher numbers of mucous cells (MCs) and rodlet cells (RCs) were observed (Table 1); beneath the body of each worm attaching to the primary lamellae these cells were positioned such that they were close to the lamella surface where they could discharge their contents (Fig. 3b,c). It is interesting to note, however, that the number of chloride cells (CCs) in all the infected gills, whether it be at the site of parasite attachment or in adjacent areas, were lower than the counts determined in the uninfected tissue sections (Table 1).

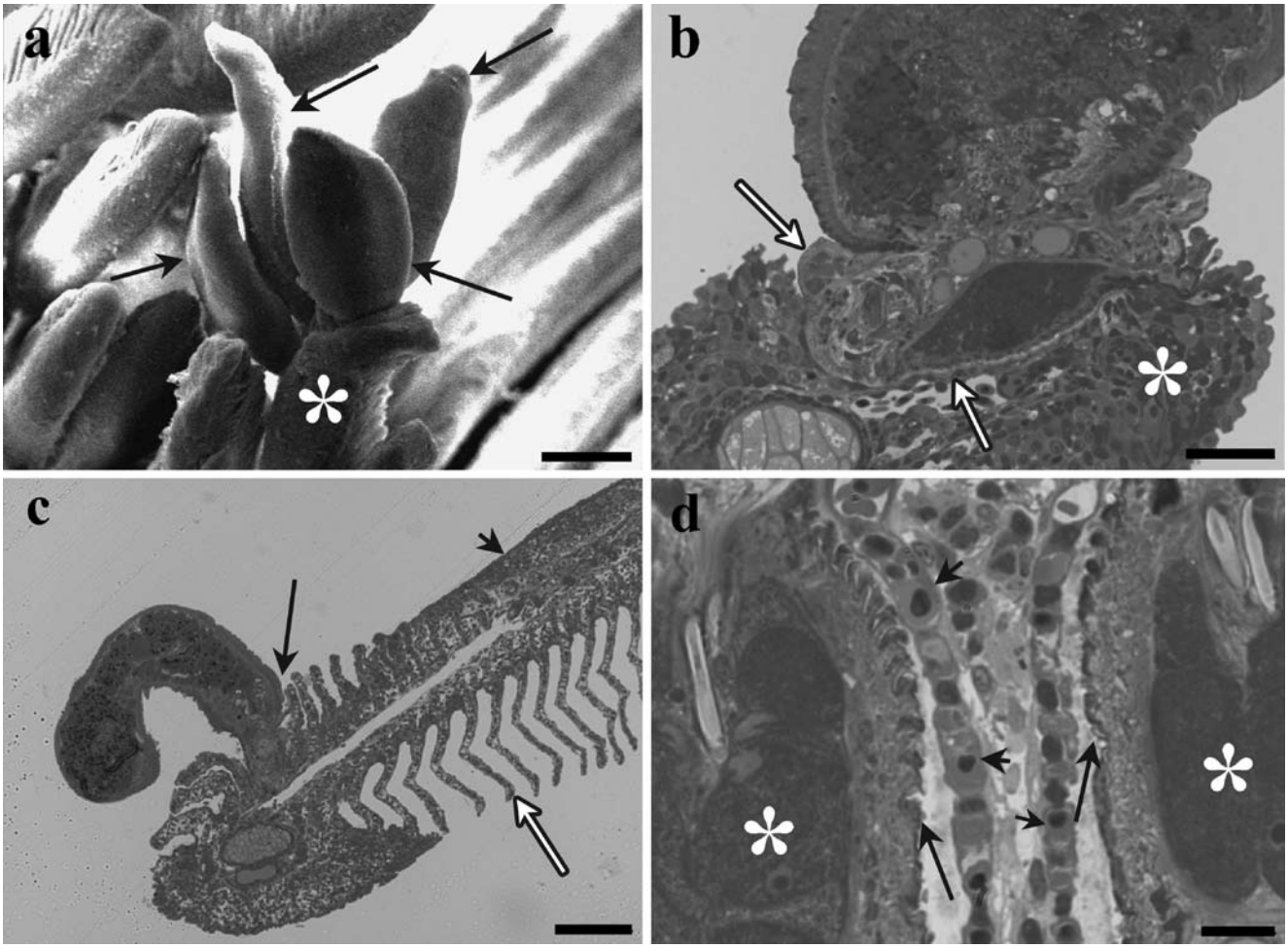

Fig. 2 **a** A scanning electron micrograph showing four specimens of *D. aequans* (*arrows*) attached to the apices of gill lamellae (*asterisk*). **b** A sagittal semi-thin section stained with toluidine blue through the posterior end of a *Diplectanum* specimen. The opisthaptors (*arrows*) of the monogeneans can be seen to penetrate deep into the gill lamellae (*asterisk*). **c** A semi-thin section of *D. aequans* stained with toluidine blue attached to the apex of the gill. It can be seen that there is an interruption to the continuity of the secondary lamella (*arrow*), a fusion of the lamellae (*short arrow*) at the site of attachment compared to the normal condition of the secondary lamellae (*empty arrow*) positioned well away from attaching fluke. **d** A toluidine-blue-stained semi-thin section showing the intimate contact between the squamodiscs (*arrows*) of two flukes (*asterisks*) and the secondary lamellae of its host. Note the absence of gill epithelium and the contraction of the pillar cells (*short arrows*)

Discussion

Diplectanum aequans is a common parasite of *D. labrax* and has a wide geographic distribution (Paling 1966; Oliver 1977; González-Lanza et al. 1991; Sterud 2002; Cecchini and Cognetti-Varriale 2003; Colorni and Diamant 2005; Mladineo 2005), with numerous prophylactic and chemotherapeutic approaches having been tested in trials to control infections (Giavenni 1983; Cognetti-Varriale et al. 1992; Silan et al. 1996). Such studies include the effects of temperature on the dynamics of infection and on the impact of dehydration on the hatching success of *D. aequans* (Cecchini 1994; Cecchini et al. 1998; Cecchini et al. 2001; Cecchini and Cognetti-Varriale 2003).

The occurrence of *Diplectanum* spp. is widespread throughout the Mediterranean and Atlantic areas coinciding closely with the distribution of its host. Although this study reports mortalities of juvenile *D. labrax* attributed to *D. aequans*, infections of *D. laubieri* Lambert and Maillard 1974, are considered as more pathogenic (Alvarez-Pellitero 2004).

During the present survey, particular attention was paid to the cellular responses exhibited by the host. At the site of parasite attachment and the immediate surrounding vicinity, there was a marked absence in the number of eosinophilic granular cells (EGCs) and activated neutrophilic granulocytes. This observation, however, is the opposite to the host reaction seen on the gills of *Abramis brama* infected with the crustacean *Ergasilus sieboldi*. In the latter case, the parasite elicited an intense host cellular reaction at the site of parasite attachment with a high number of inflammatory cells, EGCs and neutrophilic granular cells being recorded

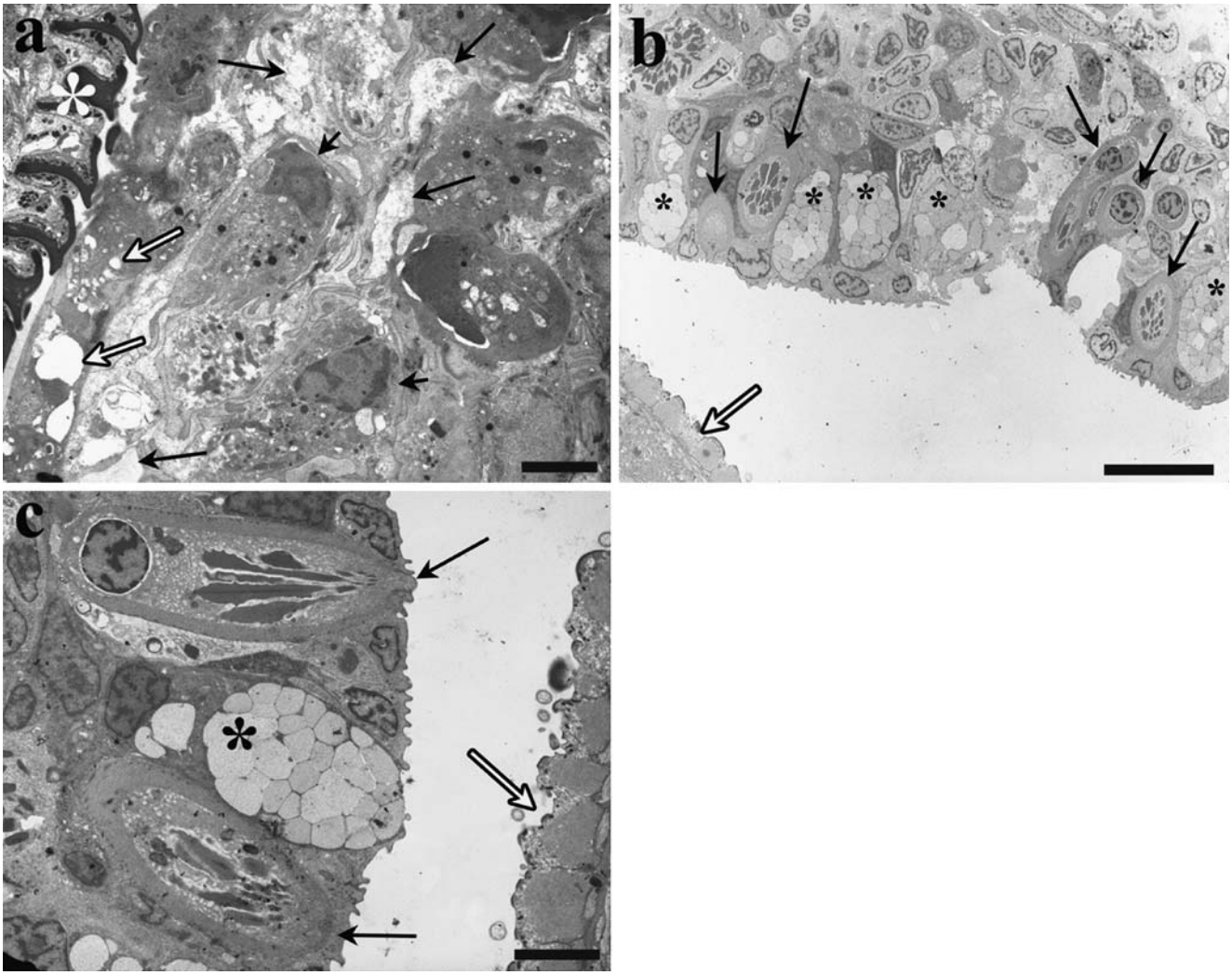

Fig. 3 **a** A transmission electron micrograph showing the interface between the diplectanid's opisthaptor (*asterisk*) and host tissue. Vacuolization of host cells (*empty arrows*) and interstitial oedema (*arrows*) are evident, as are macrophages (*short arrows*) with villous projections and slightly electron dense inclusions. **b** In close proximity to the body of the diplectanid (*empty arrow*), a high number of mucous cells (*asterisk*) and several rodlet cells (*arrows*) can be seen. **c** Rodlet cells (*arrows*) and mucous cells (*asterisk*) on the apex of the lamella in proximity to the site of *Diplectanum* attachment (*empty arrow*)

Table 1 The number of rodlet, chloride and mucous cells in a sample of gills taken from uninfected *Dicentrarchus labrax* and also in a sample of fish infected with *Diplectanum aequans*

	Uninfected (n=20)	Infected (n=20)
Rodlet cells	0.883 ± 0.156^{A}	1.475 ± 0.143^{B}
Chloride cells	4.744 ± 0.186^{A}	3.245 ± 0.215^{B}
Mucous cells	2.305 ± 0.156^{A}	2.798 ± 0.102^{B}

^{A,B} ANOVA, $p<0.01$

Data represent the mean±standard error number of cells in an area measuring 25000 μm^2 derived from 20 individual counts. The data have been transformed using their square root and then subjected to statistical analysis (ANOVA, $p<0.01$) to detect differences between the two groups of fish.

(Dezfuli et al. 2003). When the attachment mechanisms between the two parasite species are compared, it would appear that the comparatively small opisthaptor of *D. aequans*, and at the levels observed, does not impact sufficiently to induce a host cellular reaction.

There are two types of cells exclusive to fish, chloride cells (CCs) and rodlet cells (RCs). Chloride cells are located only in the gills and are the main site of ion absorption and secretion (Katoh and Kaneko 2003; Pritchard 2003). The infected gills of *D. labrax* in the current study were found to have approximately 30% fewer CCs (3.245±0.215 cells in *D. aequans* parasitised *D. labrax* and 4.744±0.186 cells in uninfected *D. labrax*) (see Table 1). While a reduction in the number of CCs will have an impact on the host's ability to regulate its ion balance (Pritchard 2003; Evans et al. 2005), the consequences of this are unknown.

Rodlet cells are known from a range of fish tissues, notably the epithelia of many organs (Morrison and Odense 1978; Manera and Dezfuli 2004). A high number of RCs have been recorded in hosts responding to a wide range of parasite infections including protozoans (Leino 1996; Dezfuli et al. 2004), digeneans (Reite 1997; Dezfuli et al. 1998), acanthocephalans (Dezfuli et al. 1998), nematodes (Dezfuli et al. 2000), cestodes (Reite 1997; Bosi et al. 2005) and crustaceans (Dezfuli et al. 2003). The current study, however, is the first to document the relationship between a monogenean and host RCs.

Unlike the observations made in other host-parasite systems, the number of RCs recorded in this study were distributed in locations far from the site of parasite attachment. Despite the moderate parasite burdens, it could be argued that the numbers of *D. aequans* were not heavy enough to elicit a complete inflammatory response and that the distribution of RCs that were found play an inflammatory role akin and in synergy to that of EGCs (Manera and Dezfuli 2004).

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