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Changes in the neuromodulators of the diffuse endocrine system of the alimentary canal of farmed rainbow trout, *Oncorhynchus mykiss* (Walbaum), naturally infected with *Eubothrium crassum* (Cestoda)

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Abstract

A histopathological and immunohistochemical study on the intestines of 45 specimens of farmed rainbow trout, *Oncorhynchus mykiss* (Walbaum), from Loch Awe, Scotland, revealed a number of cellular deviations in individuals naturally infected with the pseudophyllidean cestode *Eubothrium crassum* (Bloch, 1779). Twenty-five individuals (55.5%) were infected with an average worm burden of 18.84 ± 4.06 (mean ± SE) cestodes per host (range, 2–80 worms; total 471 worms). The cestodes, measuring an average 8.23 ± 1.10 cm (mean ± SE; range, 5.3–13.0 cm) in length, were found attached by their scolices to the mucosal lining of the distal portion of the pyloric caeca. Within the caeca, the strobila evoked a mild catarrhal enteritis, namely an enhanced mucus production with epithelial cellular desquamation, a leucocytic infiltration of the lamina propria-submucosa and vacuolization of the intestinal epithelial cells. Eosinophilic granular cells of the stratum granulosum exhibited granular depletion, while within the catarrh, the presence of a high number of rodlet cells was noticed. Immunohistochemically, the occurrence of *E. crassum* caused a significant reduction in the number of bombesin-, gastrin-releasing peptide and glucagon-like immunoreactive endocrine cells, but an increase in the relative densities of endocrine cells containing cholecystokinin-8- and gastrin-like substances. There were, however, no significant differences in the number of endocrine cells that were immunoreactive to secretin, neuropeptide Y and peptide histidine–isoleucine antisera in the digestive tracts of either the infected or non-infected *O. mykiss*.

Keywords: alimentary canal, *Eubothrium crassum*, immunohistochemistry, neuroendocrine system, neuromodulators, *Oncorhynchus mykiss*.

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Introduction

The cestode genus *Eubothrium* Nybelin, 1922 (Pseudophyllidea) is atypical in that it possesses species that are exclusive to marine hosts, species that only infect freshwater hosts and species like *E. crassum* (Bloch, 1779) that can infect hosts that occupy both environments (Kennedy 1978; Andersen & Kennedy 1983). *Eubothrium crassum* is a common cestode of Atlantic salmon, *Salmo salar* L., and a wide range of other salmonid fish (see Scholz, Kuchta, Shinn, Šnábel & Hanzelová 2003).

In the 1990s, *Eubothrium* infections of farmed marine *S. salar* in Norway and Scotland were common, with fish with a severe infection typically harbouring up to 500 individuals, and in one case as many as 1700 worms in a single host (Mitchell 1993).

There is a considerable body of information regarding the effects of helminth infection in animals and several well-documented cases on the influence of enteric worms on the host-gut neuroendocrine system (Fairweather 1997; Dezfuli,

Arrighi, Domeneghini & Bosi 2000; Dezfuli, Pironi, Giari, Domeneghini & Bosi 2002; Dezfuli, Giari, Arrighi, Domeneghini & Bosi 2003; Dezfuli, Giari, Simoni, Shinn & Bosi 2004; Bosi, Domeneghini, Arrighi, Giari, Simoni & Dezfuli 2005). Intestinal helminths often in turn induce changes in the morphology of the host tissues, which can in turn induce structural and functional changes in the digestive physiology of the host (Castro 1992; Fairweather 1997; Hoste 2001).

Earlier studies of our group have looked at the effect of two intestinal helminths, the acanthocephalan *Pomphorhynchus laevis* (Müller, 1776) and the cestode *Cyathocephalus truncatus* (Pallas, 1781) on the neuroendocrine system of brown trout, *Salmo trutta* L. (Dezfuli *et al.* 2000, 2002, 2003). These worms often influence the number of neuroendocrine cells of the host alimentary canal. The aim of the current study was to assess the impact of a single intestinal parasite, *E. crassum*, on the presence, distribution and role of specific neuromodulators of the food intake of *O. mykiss* and to compare the profiles obtained with an uninfected group of rainbow trout. A further aim of the project was to study the distribution and density of rodlet cells in infected tissues and their role in the host inflammatory response.

Materials and methods

In September 2002, 45 specimens of rainbow trout, *Oncorhynchus mykiss* (Walbaum), measuring 28.08 ± 0.47 cm (mean ± SE; range, 17.5–33 cm) in fork length were obtained from a commercial farm in Loch Awe, Scotland (56°14.0′N, 5°17.2′W). Fish were transported to the Institute of Aquaculture, University of Stirling, Scotland in aerated tanks and then given a lethal dose of the anaesthetic MS222 (Sandoz, Basel, Switzerland), and weighed and measured before severing the spinal cord. The fish were dissected ventrally, sexed and pieces of infected pyloric caeca and proximal intestine, measuring up to 15 × 15 mm in size, were excised and fixed in chilled (4 °C) Bouin's fluid for 7 h. The samples were then transferred to 70% alcohol and dehydrated through a graded alcohol series and prepared for paraffin embedding. Cut sections (7-μm thick) were stained with either haematoxylin–eosin, periodic acid-Schiff (PAS) or alcian blue/PAS or used for immunohistochemical analysis as follows: 7-μm tissue sections were dewaxed and immersed in a freshly prepared 1% H_2O_2 solution in absolute methanol for 15 min to block the endogenous peroxidase activity. Sections were then incubated in 1:20 normal goat serum (DakoCytomation; DAKO, Milan, Italy) in Tris-buffered saline (TBS: 0.05 M Tris-HCl, 0.15 M NaCl) for 30 min to prevent background prior to incubation with the primary antisera in a humidity chamber. The antisera used, the working dilution and the incubation time used for each of the neuropeptides are detailed in Table 1. The sections were then incubated for 30 min with ENVISION + ™, peroxidase, rabbit (DakoCytomation)

Table 1 Primary antisera used in this study

Antisera raised in rabbit	Code	Source	Dilution	Incubation
Bombesin	IHC 7113	Peninsula Lab., Inc., Belmont, CA, USA	1:500	Overnight at 4 °C
Bombesin	1400-0004	Biogenesis Ltd, Poole, UK	1:200	Overnight at 4 °C
CCK-8	IHC 7181	Peninsula Lab.	1:500	2 h at RT
Gastrin	AB 930	Chemicon Int., Temecula, CA, USA	1:200	2 h at RT
Gastrin	IHC 7186	Peninsula Lab.	1:400	2 h at RT
GRP	4620-3104	Biogenesis Ltd	1:250	Overnight at 4 °C
Glucagon	4660-0904	Biogenesis Ltd	1:50	24 h at 4 °C
Glucagon	T-4359 (IHC 7165)	Peninsula Lab.	1:500	Overnight at 4 °C
NPY	6730-0004	Biogenesis Ltd	1:50	Overnight at 4 °C
PHI	7260-0004	Biogenesis Ltd	1:100	Overnight at 4 °C
Secretin	8240-0004	Biogenesis Ltd	1:50	24 h at RT
Secretin	IHC 7162	Peninsula Lab.	1:500	Overnight at 4 °C

CCK, cholecystokinin; GRP, gastrin-releasing peptide; NPY, neuropeptide Y; PHI, peptide histidine isoleucine; RT, room temperature.

and goat anti-rabbit immunoglobulins conjugated to a peroxidase labelled polymer. Immunoreactive sites were visualized using a freshly prepared DAB solution (0.04% w/v 3–3′ diaminobenzidine tetrahydrochloride and 0.005% H_2O_2 in Tris-HCl 0.05 M, pH 7.4). Sections were then counterstained with Mayer's haematoxylin, dehydrated and mounted using Eukitt (O. Kindler & Co., Freiburg, Germany).

The controls for the specificity of the immunohistochemical reactions were performed by the pre-absorption of each antiserum with the corresponding antigen (Table 2). As positive controls, pig and rat tissue samples were tested in the same way.

For comparison of the number of endocrine cells of intestinal folds between healthy and infected *O. mykiss*, specimens of fish with 8–32 *E. crassum* were chosen. The intensities of infection selected were based on our previous study (Dezfuli *et al.* 2003) in which there was no significant difference in the number of endocrine cells of fish with less than eight parasites as well as in those with above 32 helminths per host. Ten intestinal folds in two sections from seven healthy trout and from nine infected conspecifics were examined (140 and 180 intestinal folds respectively). Comparable intestinal regions were examined from healthy and parasitized rainbow trout. The mean number of endocrine cells per intestinal fold that were immunoreactive to bombesin, gastrin-releasing peptide, cholecystokinin-8, gastrin, glucagons, secretin, neuropeptide Y and peptide histidine–isoleucine antisera in uninfected (control) and parasitized groups of trout were compared using the Student's *t*-test. The level of significance was set at $P = 0.05$.

Stained sections were examined by light microscopy using a standard Olympus BX51 microscope and digital images were obtained using the program DP-Soft (Olympus, Tokyo, Japan).

Table 2 Peptides used for absorption controls

Peptide	Code	Source	Antigen concentration (mg mL^{-1})
Bombesin	B 4272	Sigma Chemicals, St Louis, MO, USA	80
CCK-8	H 2085	Bachem AG, Bubendorf, Switzerland	50
Gastrin	G 3131	Sigma Chemicals	50
GRP	H 3120	Bachem AG	10
Glucagon	H 6790	Bachem AG	100
NPY	H 6375	Bachem AG	100
PHI (PHM-27)	H 6355	Bachem AG	70
Secretin	S 7147	Sigma Chemicals	30

CCK, cholecystokinin; GRP, gastrin-releasing peptide; NPY, neuropeptide Y; PHI, peptide histidine isoleucine.

Results

Twenty-five (55.5%) of the 45 *O. mykiss* were infected with *E. crassum*. The intensity of infection ranged from 2 to 80 worms per host with an average worm burden of 18.84 ± 4.06 (average worm length 8.23 ± 1.10 cm; range, 5.3–13.0 cm). The pyloric caeca and the proximal intestine bore the heaviest infections with the vast majority of tapeworms being embedded within the distal ends of the pyloric caeca (Fig. 1a) with the strobila extending posteriorly into the stomach and, in larger specimens, into the fore gut. Observations of histological material showed that many *E. crassum* were free in the lumen of the caeca (Fig. 1a); nevertheless, in several instances, the cestode was found attached to the epithelium of the caecum by means of the scolex bothria (Fig. 1b). The strobila of the cestode evoked a mild catarrhal enteritis (Fig. 1a), namely an enhanced mucus production coupled with epithelial cellular desquamation and an infiltration of leucocytes into the lamina propria-submucosa. In addition, cellular desquamation at the apices of intestinal ridges and a vacuolization of intestinal epithelial cells was also evident. In contrast to this, intestinal ridges that were not in contact with the tapeworm strobila did not show any signs of cellular degeneration. Eosinophilic granular cells (EGCs) of the stratum granulosum exhibited granular depletion suggestive of a massive degranulation. At the base of intestinal ridges, the mitotic index in epithelial cells appeared enhanced with up to three mitotic figures per field when observed at high magnification. Rodlet cells (RCs) were observed in the mucosal epithelium (Fig. 1c,d) and also among the epithelial cells of the catarrh (i.e. mucus plus cellular debris), which itself was in close proximity to the tegument of the parasite.

In the present study, eight neuropeptides were recognized following the use of 12 different antisera applied to the intestinal tissue sections taken from infected and non-infected fish (Table 1). Analysis of the immunohistochemical staining revealed a number of different populations of endocrine cells belonging to the diffuse endocrine system (DES) within the intestinal mucosa of both infected and uninfected fish. In the proximal intestine of infected *O. mykiss*, a statistically significant lower mean number of endocrine cells that were immunoreac-

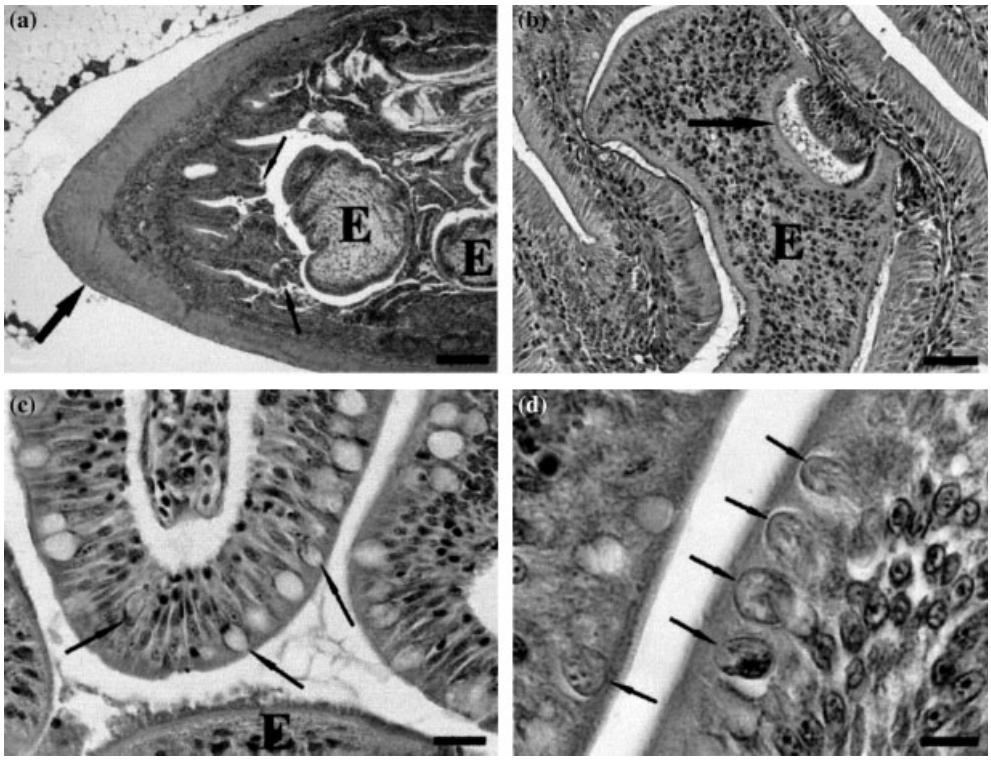

Figure 1 (a) A cross-section through a caecum of *Oncorhynchus mykiss* infected with *Eubothrium crassum* that are free in the lumen. The thick arrow indicates the end of the caecum, whilst the thin arrows highlight catarrh in close proximity to the cestode strobila (bar = 200 μm). (b) Attachment of the tapeworm scolex by its bothria (arrow) to the epithelium of the caecum (bar = 50 μm). (c) The occurrence of rodlet cells (arrows) in close proximity to the strobila of *E. crassum* (bar = 20 μm). (d) High magnification of the rodlet cells (arrows) observed in the epithelia of the *E. crassum*-infected caecum (bar = 10 μm). E, *E. crassum*.

tive to the bombesin and gastrin-releasing peptide antisera were observed when compared with the number of positive cells in uninfected specimens (Table 3, Fig. 2a,b).

In parasitized *O. mykiss*, the mean number of endocrine cells per intestinal fold that were immunoreactive to the anti-cholecystokinin-8 antisera were significantly higher than those in uninfected fish (Table 3, Fig. 2c). In contrast to this, uninfected fish had a significantly higher number of endocrine cells containing a glucagon-like peptide (Table 3, Fig. 3a,b). *Oncorhynchus mykiss* infected with *E. crassum* were also found to possess a high density of endocrine cells that were immunoreactive to the anti-gastrin serum (Table 3, Fig. 3c,d).

In the proximal intestine of infected and uninfected *O. mykiss*, however, there were no significant differences in the number of endocrine cells positive to the anti-secretin, neuropeptide Y or the peptide histidine–isoleucine sera (Fig. 4a,b, see Table 3). The positive control sections prepared from pigs and rats gave the expected immunoreactivities and no immunoreactive signals were detected in the sections treated with the pre-absorbed antisera.

Table 3 Mean number of endocrine cells per intestinal fold immunoreactive to the tested antisera in the intestine of *Oncorhynchus mykiss* parasitized with *Eubothrium crassum* (140 intestinal folds from seven uninfected and 180 intestinal folds from nine infected fish were counted)

Antiserum	Uninfected trout	Infected trout	*t*-value	*P*-value
Bombesin	0.11 ± 0.03	0.04 ± 0.02	2.192	0.029*
GRP	0.09 ± 0.02	0.02 ± 0.02	2.553	0.011*
CCK-8	0.26 ± 0.05	1.53 ± 0.12	−8.766	0.000**
Gastrin	0.02 ± 0.01	1.90 ± 0.13	−13.063	0.000**
Glucagon	1.77 ± 0.07	0.83 ± 0.07	9.092	0.000**
Secretin	0.33 ± 0.37	0.43 ± 0.63	−1.667	0.096
NPY	0.97 ± 0.07	1.21 ± 0.07	−1.392	0.165
PHI	0.06 ± 0.01	0.09 ± 0.02	−1.383	0.0168

Values are given as mean ± SE. The Student's *t*-test is performed by the SAS program.

CCK, cholecystokinin; GRP, gastrin-releasing peptide; NPY, neuropeptide Y; PHI, peptide histidine isoleucine.

Differences between mean numbers of endocrine cells from uninfected and parasitized rainbow trout are significant at $^{**}P < 0.001$, and $^{*}P < 0.005$.

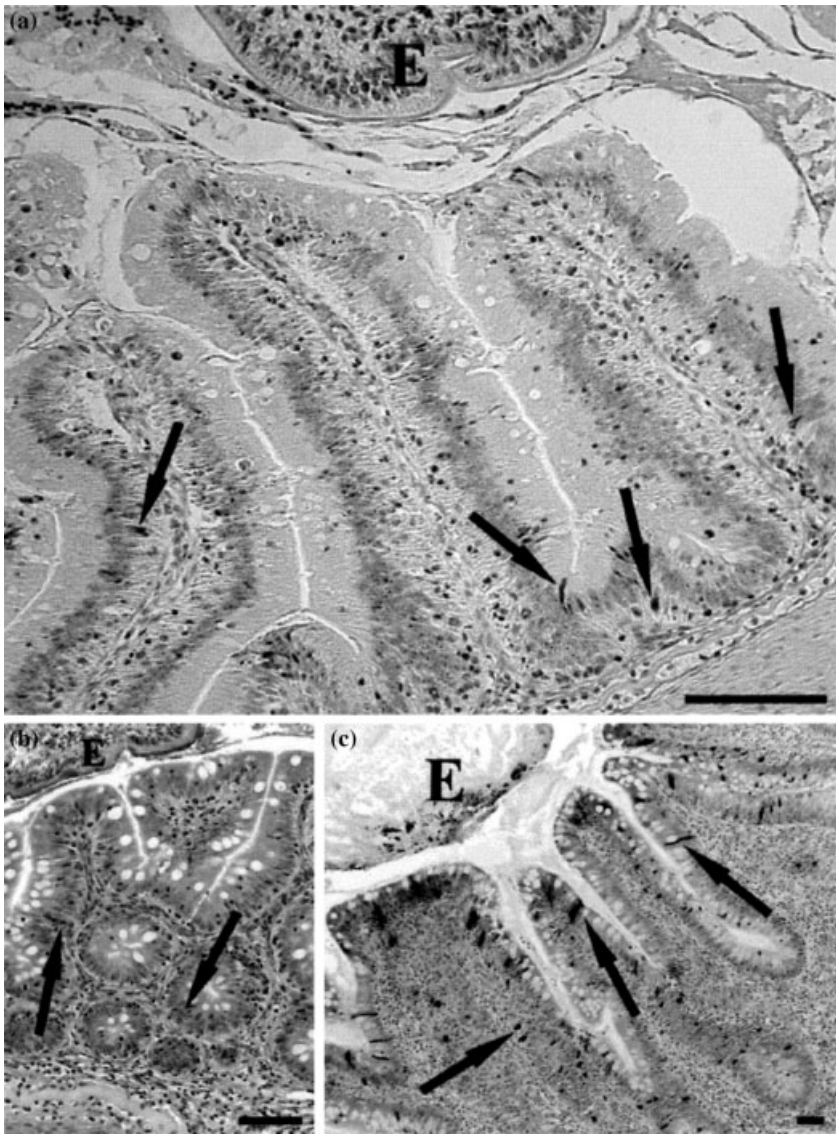

Figure 2 (a) Bombesin-like immunoreactive endocrine cells (arrows) in the intestinal folds of a *Eubothrium crassum*-infected *Onchorhynchus mykiss*. (b) Endocrine cells (arrows) containing a gastrin-releasing peptide-like substance in the proximal intestine of a fish infected with *E. crassum*. (c) A high number of cholecystokinin-8-like immunoreactive endocrine cells (arrows) in cestode-infected *O. mykiss*. E, *E. crassum* (bars = 100 μm).

Discussion

In tissue sections of *O. mykiss* infected with the pseudophyllidean cestode *E. crassum*, a high number of rodlet cells were observed in comparison with uninfected fish. The nature of RCs in response to parasitic infections remains controversial and the structure and distribution of these cells has led to speculation regarding their function (Leino 1996). RCs represent inflammatory cells that have a similar role to eosinophilic granule cells, epithelioid cells and mesothelial cells (Manera & Dezfuli 2004). Interestingly, there are several records of an increase in the number of RCs at the sites of protozoan infection (Leino 1996; Dezfuli *et al.* 2004) and in tissues surrounding a range of metazoan parasites (Dezfuli, Capuano & Manera 1998; Reite 1998; Dezfuli *et al.* 2000, 2003).

In Mitchell's (1993) assessment of *E. crassum* in aquaculture stock, he states that low chronic infections could account for a potential 10–20% loss in growth. With reference to the impact of enteric

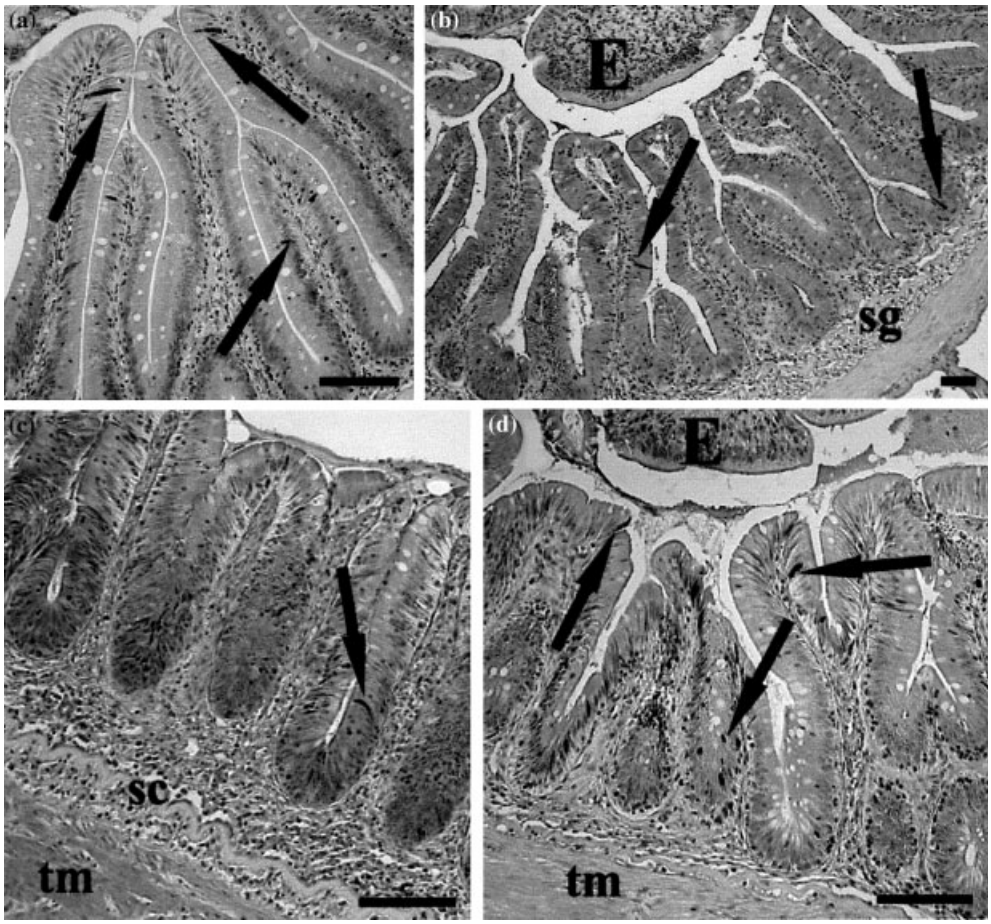

Figure 3 (a) Several endocrine cells (arrows) containing a glucagon-like substance are evident in uninfected *Onchorhynchus mykiss*. (b) In contrast to the observations made in uninfected *O. mykiss*, parasitized individuals possess a very low number of glucagon-like immunoreactive endocrine cells (arrows). (c) A positive immunoreaction to the anti-gastrin serum within the endocrine cell (arrow) of healthy *O. mykiss*. (d) An increased number of endocrine cells (arrows) immunoreactive to the gastrin-like serum in infected *O.mykiss*. E, *Eubothrium crassum*; sc, stratum compactum; sg, stratum granulosum; tm, tunica muscularis (bars = 100 μm).

helminths on host nutrition, however, there are a number of contradictory reports. Rees (1967) commented that intestinal cestodes of fish do not influence the host if the food supply is adequate. In support of this, Ingham & Arme (1973) found no evidence of adverse effects of *E. crassum* and *Proteocephalus* sp. infection on the nutritional status of infected *O. mykiss*. Smith (1973) and Hofmann, Kennedy & Meder (1986) observing salmonids infected with *E. salvelini* (Schrank, 1790) suggested that competition for limited food resources between the parasite and host results in reduced condition in fish. A number of studies have shown that parasitized hosts compensate for this increased demand for energy by increasing their feeding activity.

The neuroendocrine system of vertebrates includes the enteric nervous and the diffuse endocrine systems (DES), both of which play important roles in co-ordinating several intestinal processes (Hansen & Skadhauge 1995; Larsson 2000; Palmer & Greenwood-Van Meerveld 2001). A component of the DES is the endocrine cells of the gut, which represent a highly specialized mucosal sub-population of cells (Rindi, Leiter, Kopin, Bordi & Solcia 2004). Gut endocrine cells are recognized by the expression of several regulatory molecules. The regulatory peptides produced are involved in the modulation of digestive functions such as enzyme secretion, nutrient uptake and peristalsis (Hansen & Skadhauge 1995).

Several studies on the effects of intestinal parasites have shown that the main detrimental consequences for the host are localized at the site of infection (Hoste 2001). For example, the occurrence of worms

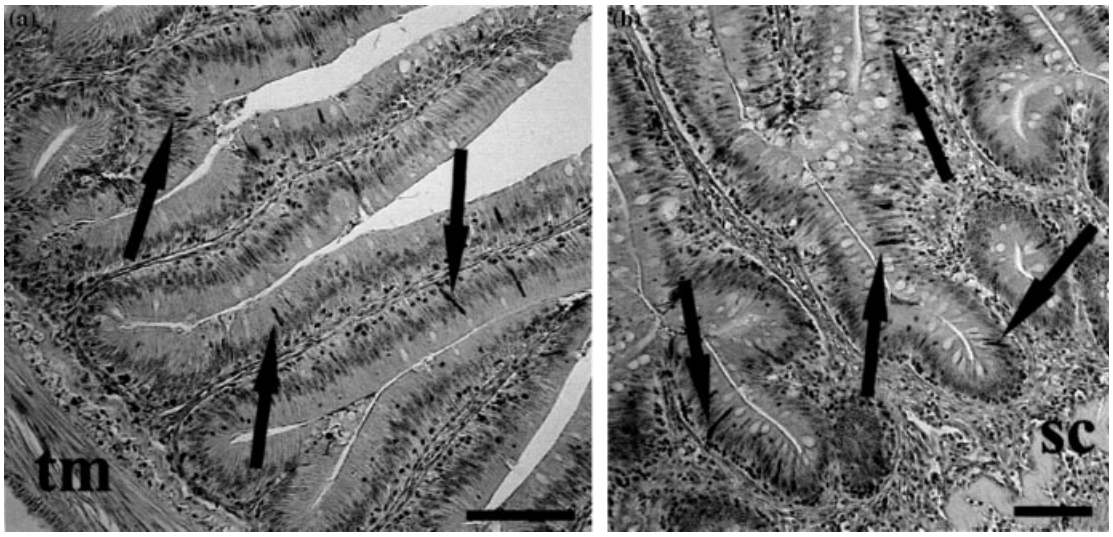

Figure 4 (a) A neuropeptide Y-like substance can clearly be seen in the endocrine cells (arrows) of uninfected *Oncorhynchus mykiss*. (b) Several endocrine cells (arrows) reacting to the anti-secretin serum in the proximal intestine of infected *O. mykiss*. sc, stratum compactum; tm, tunica muscularis (bars = 100 μm).

induces structural changes to the digestive system, which impact on the diffuse endocrine system resulting in alterations to the functioning of the gastrointestinal tract (Castro 1992; Fairweather 1997; Fox 1997; Palmer & Greenwood-Van Meerveld 2001). Although most investigations have focused on parasitic infections in mammals (Fox 1997; Roberts, Hardie, Chappell & Mercer 1999; Eysker & Ploeger 2000; Mercer, Mitchell, Moar, Bissett, Geissler, Bruce & Chappell 2000), there are few fish parasite-based studies. Of the studies that do exist for fish, the majority have been published by the current authors (see Dezfuli *et al.* 2000, 2002, 2003, 2004; Bosi, Di Giancamillo, Arrighi & Domeneghini 2004; Bosi *et al.* 2005).

Immunohistochemical analysis of intestinal sections taken from *O. mykiss* infected with *E. crassum* revealed significant increases in the mean number of endocrine cells positive for a gastrin and a cholecystokinin-8-like substance. It is well known that in vertebrates, gastrin primarily regulates gastric acid secretion (Larsson 2000), while cholecystokinin-8, besides other functions, stimulates pancreatic secretion, gallbladder contraction and regulates gastrointestinal motility (Jönsson, Holmgren & Holstein 1987). In teleosts, several studies support the essential role of a cholecystokinin-8-like substance regulating the food intake stimulus (Himick & Peter 1994a; Le Bail & Boeuf 1997). Experimentally, Gélineau & Boujard (2001) demonstrated that the oral administration of cholecystokinin antagonists resulted in an increase in food consumption in *O. mykiss*. Considering this latter study and the current findings for gastrin and cholecystokinin-8, it is suggested that the occurrence of *E. crassum* in *O. mykiss* could affect host nutrient uptake by inducing the host to ingest less food.

Bombesin and gastrin-releasing peptide belong to the same peptide family (Jensen 2001), and are found in all the major vertebrate groups (Holmgren & Jensen 1994). The ability of bombesin/gastrin-releasing peptide to suppress food intake after peripheral injection has been demonstrated in several mammalian species (Jensen 2001), as well as in goldfish, *Carassius auratus* (L.), and in carp, *Cyprinus carpio* L. (Beach, McVean, Roberts & Thorndyke 1988; Himick & Peter 1994b). In the current study, a significant decrease in the relative densities of endocrine cells that were immunoreactive to both antisera were found in *O. mykiss* infected with *E. crassum*. The results obtained with the anti-bombesin serum are in agreement with those found in *S. trutta* infected with *C. truncatus* (Dezfuli *et al.* 2003).

Glucagon is a peptide formed by 29 amino acids whose sequence appears to be well conserved among vertebrates. In fish, glucagon is a hyperglycaemic and lipolytic substance (Moon 1998) and glucagon-like immunoreactivities have been observed in endocrine cells of the intestine in both *O. mykiss* (Beorlegui, Martìnez & Sesma 1992) and *S. trutta* (Bosi *et al.* 2004). In fish, this peptide is reputed to

be a potential anorexigenic factor, i.e. a compound suppressing the host's appetite (Navarro, Carneiro, Parrizas, Maestro, Planas & Gutierrez 1993; Le Bail & Boeuf 1997). The occurrence of *E. crassum* in the intestine of *O. mykiss* induced a significant decrease in the mean number of glucagon-like immunoreactive endocrine cells. A similar decrease was also observed in *S. trutta* infected with *C. truncatus* (Dezfuli *et al.* 2003). It is likely that a host with a reduced number of glucagon secreting cells would have an increased appetite.

All peptides studied in this investigation are involved in the transmission of peripheral satiety signals to the central feeding system (Jensen 2001; Ritter 2004). In mammals, intestinal nutrients trigger the secretion of neuroactive substances from the intestinal epithelium, and these substances activate vagal sensory neurons that effect changes in the food intake stimulus (Ritter 2004). The integrity of the intestinal structure is a prerequisite for the normal control of food intake (Gay, Ressayre, Garcia-Villar, Bueno & Fioramonti 2003). It is interesting, therefore, that infectious agents, including worm parasites, appear to be capable of modifying their host's neuroendocrine system, and consequently, the host's appetite to meet their own requirements (Fox 1997; Mercer & Chappell 2000). In this study, we have demonstrated increases in some neuromodulators (i.e. an increase in positive cells) and decreases in others but it is the overall net effect on the gastrointestine of the host that is important. What we do not know at the moment is how each of these neuromodulators interact with one another and whether a rise in one suppresses the production of another.

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