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Efficacy and Toxicity of Orally Administrated Anti-Coccidial Drug Treatment on *Enteromyxum leei* Infections in Sharpsnout Seabream (*Diplodus puntazzo* C.)

E. Golomazou[1], F. Athanassopoulou[1*], E. Karagouni[2], S. Vagianou[1], H. Tsantilas[1] and D. Karamanis[1]

[1] Laboratory of Ichthyology and Fish Pathology, School of Health Sciences, Faculty of Veterinary Medicine, University of Thessaly, 221 Trikalon Street, 43100 Karditsa, Greece

[2] Hellenic Pasteur Institute, 127 Vassilissis Sofias Avenue, 11521 Athens, Greece

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Abstract

Three drugs effective against myxosporeans and commonly used to treat spore-forming parasites were tested in sharpsnout sea bream (*Diplodus puntazzo*) infected by *Enteromyxum leei*. Two medicated diets were applied, one containing salinomycin and amprolium and the second containing fumagillin. Compared to untreated fish, both treatments resulted in significant reductions in prevalence, intensity of all developmental myxosporean stages, and mortality. The effect was more prominent with the salinomycin and amprolium combination, where the significant reductions in intensity, prevalence, and mortality were unaccompanied by any histopathological evidence of toxic side effects or growth reduction. Sporoblasts and mature spores with distorted structures were observed in both drug treatments, but were more prevalent in the salinomycin and amprolium treatment than in the fumagillin treatment, indicating direct effectiveness on the parasite. Salinomycin with amprolium is a promising treatment for myxosporean infections in intensively cultured warmwater fish, leading to parasite elimination.

Introduction

The recent growth of the aquaculture industry in Greece, together with the introduction of new fish species (*Diplodus puntazzo* C., *D. sargus*, *Dentex dentex*, and *Pagellus erythrinus*) in intensive rearing systems, has led to increased occurrence of pathogens that cause serious problems and limit development (Alvarez-Pellitero and Sitja-Bobadilla, 1993ab; Diamant et al., 1994; Diamant and Wajsbrot, 1997; Rigos et al., 1999; Golomazou et al., 2005). Myxosporeans, the most common parasites affecting intensively

* Corresponding author. E-mail: eathan@vet.uth.gr

reared fish in the Mediterranean, induce a broad spectrum of diseases depending on parasite species, host sensitivity, and environmental and feeding conditions (Sitja-Bobadilla et al., 1992; Alvarez-Pellitero and Sitja-Bobadilla 1993ab; Rigos et al., 1997). Recent losses in cultured sharpsnout seabream (*D. puntazzo*) due to the marine myxosporean *Enteromyxum leei* Diamant, Lom and Dykova, 1994 (formerly *Myxidium leei*) have raised questions about the viability of farming this species in Mediterranean mariculture (Rigos et al., 1999). *Enteromyxum leei* also infects *Sparus aurata* L. (Diamant 1992, 1997; Diamant et al., 1994), *Pagrus major* L. (Le Breton and Marques, 1995), and *P. pagrus* L. (Diamant, 1995).

High mortality rates are associated with the absence of adequate treatments for warmwater fish. In contrast to mammalian therapeutics, the use of pharmaceutical substances, particularly antiparasitic drugs, is limited in fish. Research on anti-myxosporean/microsporean treatments mainly concern salmonids and there is little research on Mediterranean fish. There are no licensed antiparasitic compounds or official minimal residue levels (MRL) for Mediterranean species; all information is extrapolated from coldwater species, especially salmonids. This can cause problems since treatments differ depending on environmental (temperature, pH, stability, toxicity to other aquatic animals) and biological factors (species, safety, metabolism, stress, residues).

The purpose of this study was to assess the anti-myxosporean effects of commercially available drugs used in other animals to treat spore-forming parasites on sharpsnout sea bream spontaneously infected with *E. leei.*

Materials and Methods

Fish. Three thousand infected *Diplodus puntazzo* (mean wt 15 g) were obtained from a sea cage on a commercial farm in northwestern Greece where *E. leei* infection was diagnosed by clinical signs (acute enteritis, anorexia, extensive emaciation) and high mortality. The fish were naturally infected with *E. leei* (prevalence 100%). The infection was confirmed in ten fish by parasitological examination. Trophozoites, sporoblasts, and mature parasite spores were found in fresh scrapings of the gut mucosa of the posterior intestine.

The fish were separated into three equal groups. Each group was placed in an 8-ton concrete raceway tank with a flow-through supply of pathogen-free bore-hole water at a temperature of 21±1°C, salinity 30‰, and pH 7. Fish were acclimatized for 7-10 days. Before the start of the experiment, 30 fish (10 from each group) were sampled for bacteriology and parasitology.

Diets. Fish in tank A were fed a medicated diet containing salinomycin (70 mg/kg biomass) and amprolium (100 mg/kg biomass), two anti-coccidial drugs commonly used to treat spore-forming parasites, especially in poultry, for 56 days. Fish in tank B were fed a medicated diet containing bicyclohexylammonium fumagillin (6 mg/kg biomass), a commonly used anti-myxosporean drug, for 56 days. Fish in tank C (control) were fed a regular non-medicated diet. The drug compounds were of commercial grade (Table 1), diluted in cod liver oil and coated onto commercial feed pellets with a mechanical mixer. Control fish were fed the same diet mixed with the same quantity of cod liver oil. The diets were freshly prepared before feeding and fed to the fish by hand.

Sampling. Mortality and the presence of clinical signs were recorded daily throughout the experiment. In eight weekly samplings, ten fish from each tank were sampled, weighed, killed by an overdose of anesthetic, and examined as follows.

Microbiological and parasitological examination. Kidney and spleen samples were inoculated onto tryptone soy agar (TAS) supplemented with NaCl 2% and thiosulphate citrate bile salt agar (TCBS) for bacteriological examination according to methods described by Roberts and Shepherd (1997). Fresh scrapings and smears of internal organs (posterior intestine, gall bladder, kidney, liver, spleen, heart) and gills were examined according to methods described by Roberts (1989). Infection prevalence was assessed in ten random samples from each tank at each sam-

Table 1. Drugs used in experimental treatments.

Drug	*Commercial form (composition)*	*Trade name*	*Company*
Amprolium	Medicated premix (50%)	Amprolium	Veterin
Fumagillin (dicyclohexylamine)	Powder oral solution (20 mg/g)	Fumidil®	CEVA
Salinomycine (salinomycine sodium)	Medicated premix (12%)	Salinomycin	Haechst

pling in ten random viewing fields per slide (magnification x 400).

Infection intensity was estimated by counting the spores in each myxosporean stage per viewing field in fresh scrapings of the posterior intestine of ten fish from each group at each sampling. Spores were counted in ten random viewing fields per slide (magnification x 400). Intensity was arbitrarily classified into four levels: A = 1-5, B = 6-10, C = 11-15, and D >15 spores/field. Dry smears of organs were stained with Giemsa (Drury and Wallington 1980).

Histological examination. Gill, kidney, digestive tract (esophagus, stomach, and anterior, middle, and posterior parts of the intestine), liver, spleen, gall bladder, and heart tissues of five fish from each group in each sampling were processed histologically. The tissues were fixed in 10% buffered formalin, processed, and stained with hematoxylin and eosin (H&E; Merck, Darmstadt, Germany) and Giemsa-Von Kossa (Merck) according to standard methods (Drury and Wallington, 1980).

Statistical analysis. Analysis of variance (ANOVA) and non-parametric tests (NPT) were used to test for statistically significant changes in measured data over time and between groups. The effects of treatment on the prevalence of parasites was studied using the General Lineal Model (GLM) of repeated measures (GLM-RM) or GLM univariate analysis (two-way ANOVA). Where appropriate, post hoc Games-Howell tests were performed to reveal statistically significant differences between groups. One-way ANOVA with the Welch statistic due to unequal variances was used to test for the mortality equality of group means for the 56 days. The NPT Kruskal-Wallis χ^2 test was used to confirm differences in group mortality and compare body weights between groups. Analyses were performed with the Statistical Package for the Social Sciences (SPSS Inc., Chicago, Illinois, Windows ver. 12.0) and differences were considered statistically significant when $p<0.05$.

Results

Prevalence. At the beginning of the experiment, the prevalence of *E. leei* infection in all groups was 100%, based on the presence of all developmental myxosporean stages in fresh scrapings of the gut mucosa of the posterior intestine. By the end of the experiment, the prevalence dropped in groups A and B but remained high in the control (Fig. 1). There were sporoblasts and mature spores with distorted structures in the treated groups but not in the control (Figs. 2-4).

Intensity. The intensity of the infection was estimated by observing the frequency of the developmental myxosporean stages in fresh scrapings of the gut mucosa of the posterior intestine (Figs. 5-7).

Growth and mortality rates. Extensive emaciation and hemorrhage in the intestinal mucosa were observed in all dead fish. The cumulative mortality rate was 18.82% for group A, 25.04% for group B, and 39.7% in the control (Fig. 8). Body weight, an indication of the general well-being of the fish, did not significantly differ between groups.

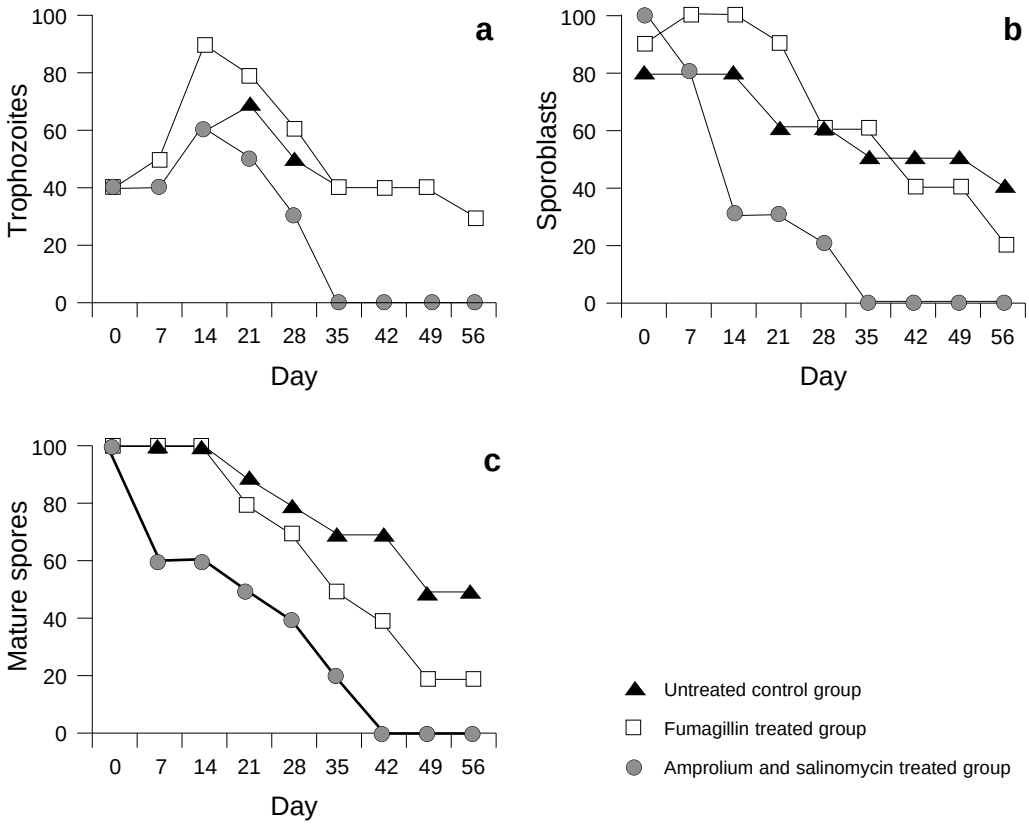

Fig. 1. Prevalence of *Enteromyxum leei* (a) trophozoites, (b) sporoblasts, and (c) mature spores in sharpsnout seabream treated with diets containing amprolium/salinomycin, fumagillin, or no anti-coccidial drug (control).

Statistical analysis. There were significant effects (GLM-RM) of drug treatment on total mature spores in terms of both treatment ($F_{2,27}$ = 749.12; p = 0.000) and time ($F_{8,216}$ = 928.73; p = 0.000). The post hoc test revealed a significant difference in total mature spores between the control group and groups B (13.3±1.1; p = 0.000) and A (42.2±1.1; p = 0.000). Significant interactions between treatment and time were observed ($F_{8,216}$ = 372.35; p = 0.000). The differences between the three groups were significant except for weeks 4 and 5. For the total 56 days, the mean percent of mature spores was 78.89±0.85 in the control group, 65.56±0.85 in group B, and 36.67±0.85 in group A.

The same results were obtained with two-way ANOVA. The effects on total mature spores were significant for both treatment ($F_{2,243}$ = 653.078; p = 0.000) and time ($F_{8,243}$ = 378.32; p = 0.000) and the total mature spores of the control group significantly differed from those of groups B (13.3±1.12; p = 0.000) and A (42.2±1.12; p = 0.000). The interaction between treatment and time was also statistically significant ($F_{16,243}$ = 18.32; p = 0.000).

The effects on total trophozoites were significant for both treatment ($F_{2,243}$ = 228.34; p = 0.000) and time ($F_{8,243}$ = 118.34; p = 0.000)

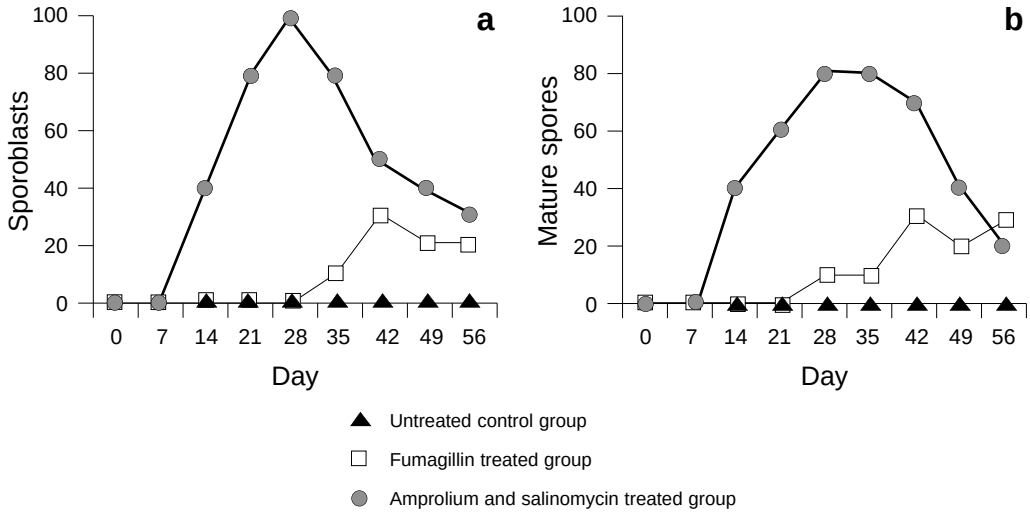

Fig. 2. Prevalence of distorted *Enteromyxum leei* (a) sporoblasts and (b) mature spores in sharpsnout seabream treated with diets containing amprolium/salinomycin, fumagillin, or no anti-coccidial drug (control).

with a significant difference between total trophozoites in the control group and in group A (21.0±3.1; p = 0.000). The opposite was observed between the control group and group B (-6.8±2.7; p = 0.038, Games-Howell).

Effects on total sporoblasts were likewise significant for both treatment ($F_{2,243}$ = 630.58; p = 0.000) and time ($F_{8,243}$ = 306.92; p = 0.000) with a significant difference between total sporoblasts of the control group and group A (21.0±3.1;,p = 0.000). There was a significant interaction between treatment and time ($F_{8,243}$ = 41.90; p = 0.000). The post hoc test revealed a significant difference between total sporoblasts in the control group and in group A (32.2±4.2; p = 0.000) although the difference between the control group and group B was not significant (-7.8±3.6; p = 0.081, Games-Howell) except during the last week (one-way ANOVA, -20.0±6.1; p = 0.017, Games-Howell).

Mortality significantly differed between groups (χ^2 = 44.093, d.f. 2, p = 0.000; $F_{2,107}$ = 30.66, p = 0.000). Mean fish death per day was 5.875±0.225 in the control, 3.964±0.262 in group B, and 2.982±0.332 in group A. The differences were 1.91±0.34 between the control and group B (p = 0.000) and 2.89±0.40 between the control and group A (p = 0.000).

There were no statistically significant differences in body weight between the control and the treated groups (χ^2 = 1.191, d.f. 2, p = 0.551).

Histopathology of E. leei. The fish in all groups were intensely infected before treatment, as were the fish in the control at the end of the experiment. Pathology included degeneration, apoptosis, and necrosis in the epithelial layer of the intestine and hemorrhage in the mucosal layer (Fig. 9). There was an inflammatory reaction near early trophozoites in the mucosal layer that remained until the parasite reached the sporoblast stage. The epithelium of the gall bladder was degenerated and all stages of the parasite were present in the lumen.

Histological sections of all the fish were compared with sections of uninfected healthy

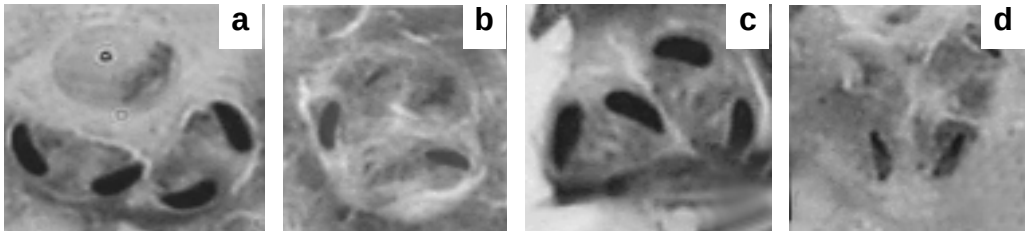

Fig. 3. (a) Normal sporoblasts, (b) distorted sporoblasts, (c) normal mature spores, and (d) distorted mature spores of *Enteromyxum leei* in fresh scrapings of gut mucosa of the posterior intestine of sharpsnout seabream treated with a diet containing amprolium and salinomycin; samples stained with Giemsa (x 400).

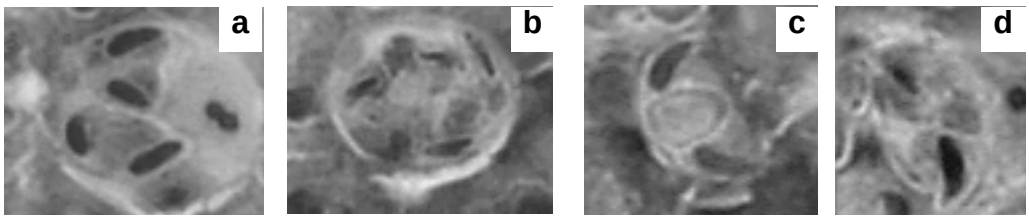

Fig. 4. (a) Normal sporoblasts, (b) distorted sporoblasts, (c) normal mature spores, and (d) distorted mature spores of *Enteromyxum leei* in fresh scrapings of gut mucosa of the posterior intestine of sharpsnout seabream treated with a diet containing bicyclohexyl-ammonium fumagillin; samples stained with Giemsa (x 400).

fish. In contrast to the control fish, fish in group A had few visible spores in the intestinal tract after treatment and no pathological lesions in any of the organs. Renal lesions were observed only in fish from group B. There was slight inflammation in the interstitial renal tissue in these fish and, in a few cases, hemorrhage and congestion in the liver. The glomerular capsule was thickened in all group B fish.

Discussion

Treatment of fish myxosporidioses is a problem (Molnar, 1993). This study compares the therapeutic outcomes of two antiparasitic medicated feeds. Treatment success is measured by changes in prevalence and intensity of each developmental myxosporean stage and the mortality rate of the fish. Both treatments resulted in a significant reduction of the prevalence and frequency of developmental myxosporean stages and fish mortality when compared to the untreated control. Further, sporoblasts and mature spores with distorted structures were observed only in the treated fish and not in the untreated control.

The combination of salinomycin and amprolium (group A) was the more effective treatment. Salinomycin is an antimicrobial agent belonging to the ionophores. It is a highly lipophilic polyether that accumulates in cell membranes and catalyzes rapid potassium movement, disturbing its intracellular balance and killing sensitive microorganisms (Russell and Houlihal, 2003). Oral administration of salinomycin to tapir fish (*Gnathonemus petersii*) naturally infected with the gill parasite, *Henneguya* sp., caused irreversible damage to the plasmodial developmental stages of the parasite (Dohle et al., 2002). Amprolium is a structural analogue of thiamine (vitamin B1) that causes competitive inhibition of thiamine utilization by parasites. It acts upon the first

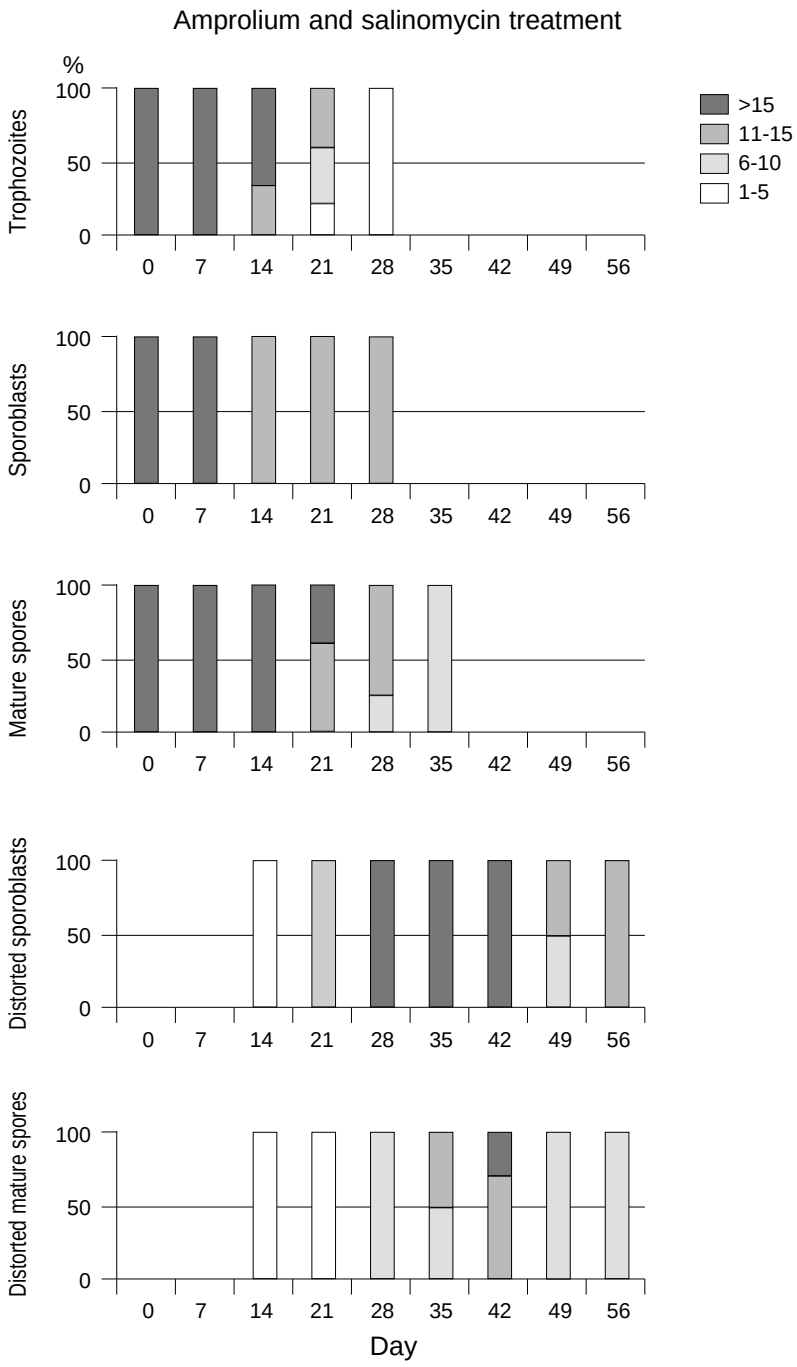

Fig. 5. Number of *Enteromyxum leei* spores per viewing field in fresh scrapings of gut mucosa of the posterior intestine of sharpsnout seabream treated with a diet containing amprolium and salinomycin; mean of 10 random viewing fields per slide (x 400).

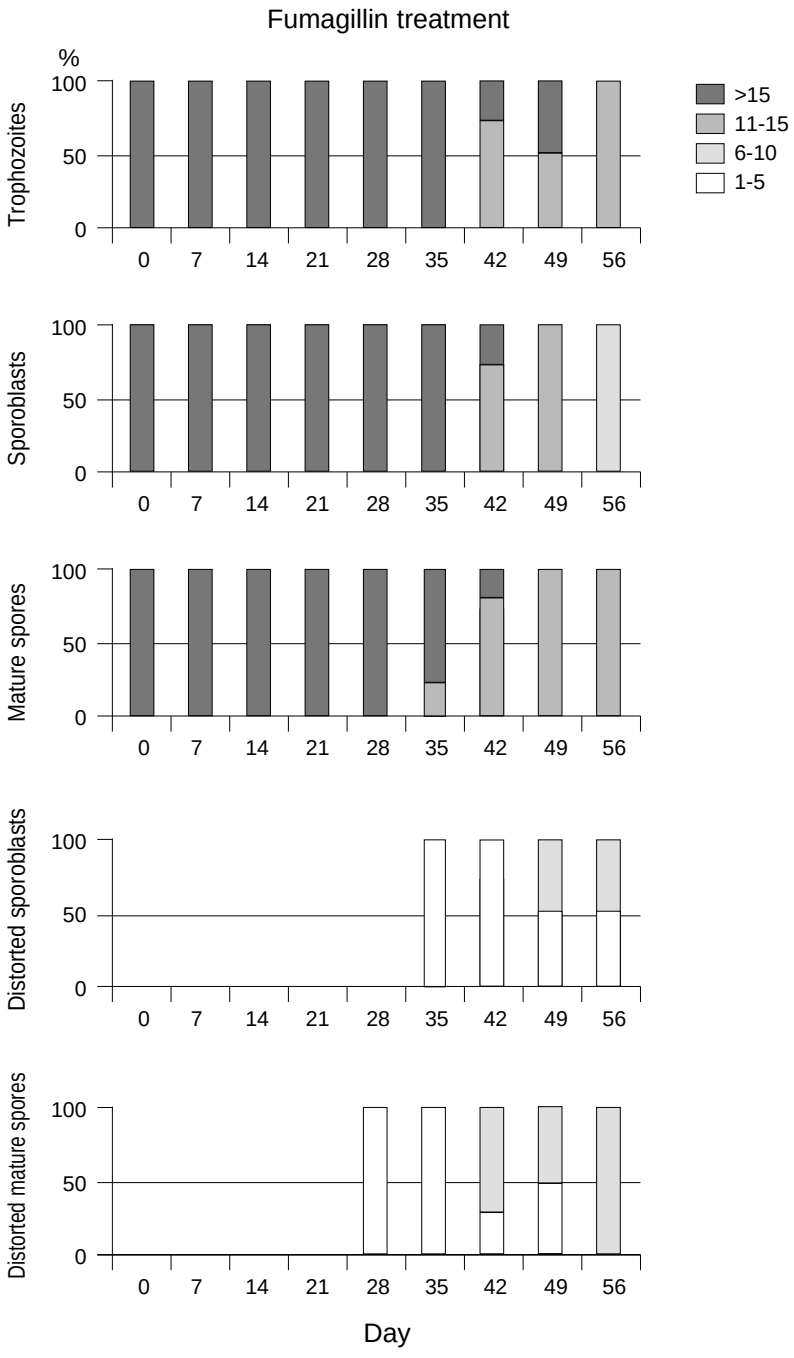

Fig. 6. Number of *Enteromyxum leei* per viewing field in fresh scrapings of gut mucosa of the posterior intestine in sharpsnout seabream treated with a diet containing fumagillin; mean of 10 random viewing fields per slide (x 400).

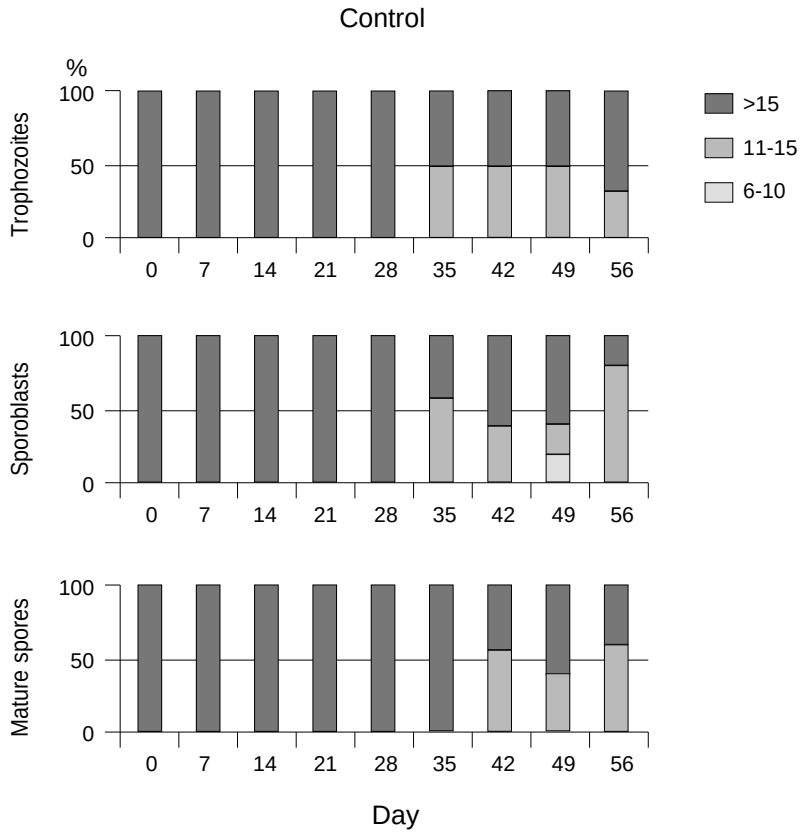

Fig. 7. Number of myxosporean stages per viewing field in fresh scrapings of gut mucosa of the posterior intestine among untreated (control) fish; mean of 10 random viewing fields per slide (x 400).

coccidian generation, preventing differentiation of merozoites. It may also suppress sexual stages and sporulation of oocysts. Amprolium is one of the safest anti-coccidian drugs and is used extensively in the poultry industry (Hamamoto et al., 2000). However, amprolium was ineffective as a monotherapy against many parasitic diseases in rainbow trout (Tojo and Santamarina, 1998abc; Speare et al., 1999). In the present study, the combination of salinomycin and amprolium resulted in a greater reduction of prevalence and maintained the intensity of infection at a lower level than fumagillin.

Combination treatments are well known for several pathogens (Haberkorn, 1996; Croft, 1997). Salinomycin/amprolium treatment proved successful in myxosporean infections of *Myxobolus* sp. in *D. puntazzo* (Athanassopoulou et al., 2004a) and *Polysporoplasma sparis* in *Sparus aurata* (Athanassopoulou et al., 2004b; Karagouni et al., 2005). At the doses used in the experiment, the combination caused no histopathological lesions in the organs of the treated fish. The effects of the combination may be attributed to direct cytotoxic action on the parasite and influence on the host's innate immunity enabling effective defense mechanisms to eliminate the parasite (Karagouni et al., 2005).

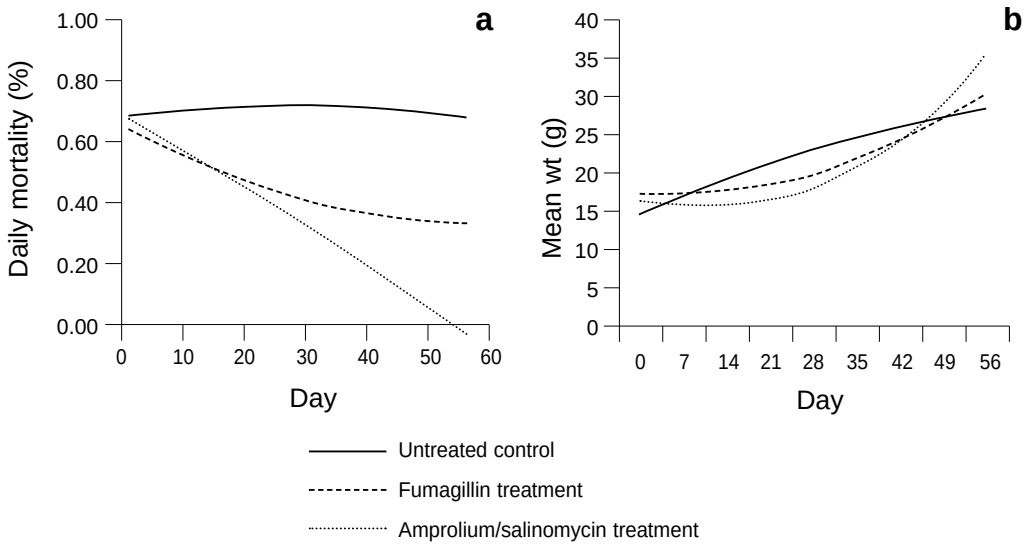

Fig. 8. (a) Mortality rate and (b) mean weight of sharpsnout seabream infected by *Enteromyxum leei* in treated and untreated groups.

Although fumagillin and its analogue TNP-470 have produced promising results in the control of Microsporea and Myxosporea parasites in several fish species (Molnar et al., 1987; Hedrick et al., 1988, 1991; Higgins and Kent, 1988; Kent and Dawe, 1994; Speare et al., 1999), it proved less effective than salinomycin/amprolium in this study. Nevertheless, fumagillin reduced the intensity of the infection compared to the control. Fumagillin is effective against *Myxidium giardi* (Szekely et al., 1988) and *Thelohanellus hovorkai* and *Sphaerospora renicola* in common carp when administrated during the infective period (Molnar et al., 1987; Yokoyama et al., 1999) or when early intracellular trophozoites and more developed plasmodia of *Hoferellus carassi* exist (Yokoyama et al., 1990). It has been used against *Myxobolus cerebralis* and PKX in rainbow trout (El-Matbouli and Hoffman, 1991) and the myxosporean *Sphaerospora testicularis* in sea bass (Sitja-Bobadilla and Alvarez-Pellitero, 1992).

Fumagillin is an expensive drug and toxic in high doses (Athanassopoulou et al., 2004 ab). Side effects range from inappetence to mortality (Sitja-Bobadilla and Alvarez-Pellitero, 1992). The most commonly reported side effects are moderate and include growth reduction in rainbow trout during treatment (Kent and Dawe, 1994) and depletion of the renal interstitium and vacuolation in the epithelium of the renal tubules in chinook salmon (Hedrick et al., 1988).

In conclusion, this is the first time that the combination of salinomycin and amprolium proved to be a safe and effective treatment against *E. leei* infections in *D. puntazzo*. This combination is a promising drug treatment for myxosporean infections in intensively cultured Mediterranean fish. The combination caused no histopathological lesions in the organs of treated fish for the doses used in the experiment. Its effects may be attributed to both a direct cytotoxic action of the drug on the parasite and an immunostimulatory effect on the host's innate immunity resulting in parasite elimination via effective defense mechanisms (Karagouni et al., 2005).

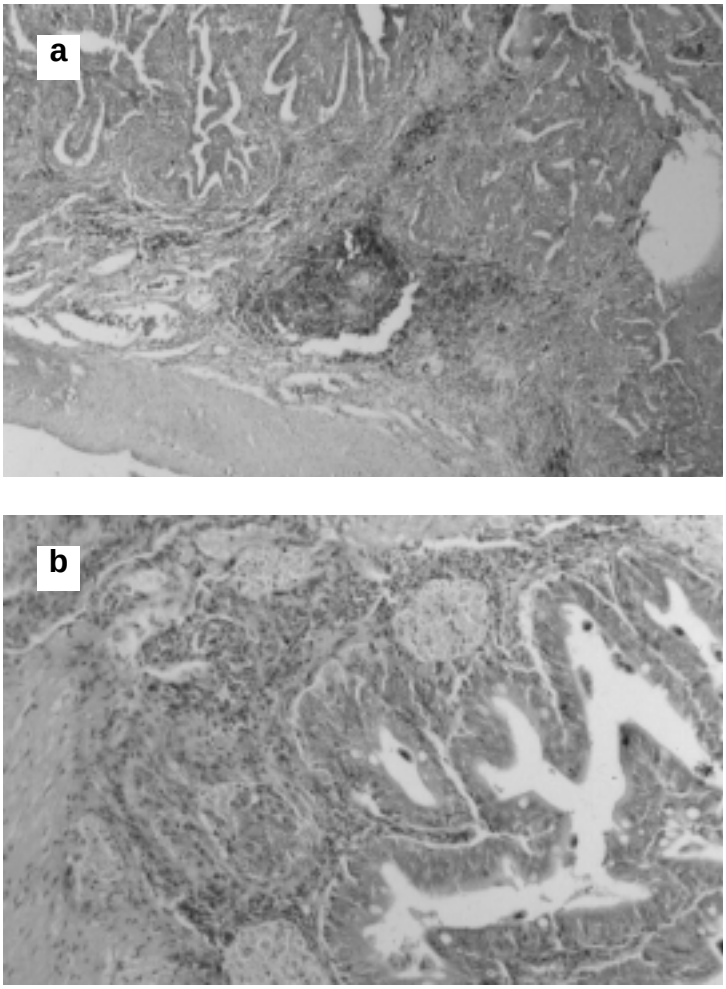

Fig. 9. Histological section of intestine of sharpsnout seabream infected with *Enteromyxum leei* showing (a) degeneration, apoptosis, and hemorrhage in the epithelial layer, and (b) degeneration, apoptosis, and granulomata formation hemorrhage in the mucosal layer. H&E, x 400.

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