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Efficacy and toxicity of orally administrated anti-coccidial drugs for innovative treatments of *Myxobolus* sp. infection in *Puntazzo puntazzo*

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ABSTRACT: This study tested drugs and therapeutic compounds to determine effective commercial treatment for fishes infected with myxosporeans. Two series of shore-based experiments and 1 field trial were performed. For the shore-based experiments we used *Puntazzo puntazzo* (ca. 20 g weight) with kidneys infected with *Myxobolus* sp. Initially, 6 different doses of Fumagillin, 2 doses of Toltrazuril, and 1 dose of Amprolium, ESB3 and Salinomycin were tested. In the second shore-based experiment, infected fish were treated with Origanum essential oils, Toltrazuril with propylene glycol, Amprolium, and a combination of Salinomycin 12% + Amprolium (SA). In the field trial, *P. puntazzo* (ca. 165 g) infected with the parasite were treated with SA, Origanum essential oils and Fumagillin. In all trials, the drugs were added to the feed and administered according to the selected regimen. Their efficacy was evaluated in terms of mortality (acceptable level was <3%), pathology and prevalence rate of *Myxobolus* sp. Lesions were observed only in fish treated with Fumagillin and Toltrazuril. Pathology due to treatment with Fumagillin was observed only at doses > 6 mg kg^{-1} body wt for 6 wk in the interstitial renal tissue, where slight inflammation arose. The highest dose tested (25 mg kg^{-1}) also produced necrosis in the interstitial tissue, degeneration of the epithelial cells of the tubules and a reduction in melanomacrophage centre numbers. The SA combination proved the most effective treatment for *Myxobolus* sp. infection of *P. puntazzo* as (1) the therapeutic regimen and commercial product was not toxic and (2) a significant reduction occurred in the prevalence rate.

KEY WORDS: *Puntazzo puntazzo* · Anti-myxosporean treatment · Histopathology

INTRODUCTION

The most common parasites affecting Mediterranean fishes are the myxosporeans (especially *Myxidium leei*), often implicated in serious losses in cultured sharpsnout sea bream *Puntazzo puntazzo* and sea bream *Sparus aurata* (Diamant et al. 1994). *M. leei* is very pathogenic to *P. puntazzo* and recent outbreaks have questioned the viability of farming (Rigos et al. 1999). Occasional heavy and prolonged mortalities and the absence of an adequate treatment comprise a cost-ineffecient operation. In contrast to mammalian therapeutics, the use of pharmaceutical substances, particularly antiparasitic drugs, in fishes, is limited. Anti-myxosporean/microsporean treatments are generally reported for salmonids (Hedrick et al. 1988, 1991, Kent & Dawe 1994, Speare et al. 1999) and few for Mediterranean fishes (Sitja-Bobadilla & Alvarez-Pellitero 1992,

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Athanassopoulou 1998a, Rigos et al. 2000, Athanassopoulou et al. 2003). Although researchers have made major advances in regard to antibiotic treatments, sensitivity and drug residues in tissues, progress in the field of antihelminthic treatment of warmwater fishes (such as sea bream and bass) is very limited. There are no licensed antiparasitic compounds for Mediterranean species or official minimal residue limits (MRLs) currently available and all information is extrapolated from coldwater species, especially salmonids. This can cause problems, as treatment conditions differ greatly in terms of environmental (temperature, pH, stability, toxicity to other aquatic animals) and individual (safety, metabolism, stress, residues) factors.

The taxonomy, epidemiology and pathology of myxosporean parasites other than *Myxidium leei* infecting *Puntazzo puntazzo* have not been well studied, and few reports exist (Athanassopoulou et al. 1998, 1999, Mladineo 2003) making the identification very difficult. An unidentified *Myxobolus* species has been reported from the small intestine of annular sea bream *Diplodus annularis* in Croatia (Mladineo 2003) in low numbers and prevalence (10%), with a spore size larger than that of the parasite found in the kidneys of *P. puntazzo* in the present work. The latter histozoic parasite is commonly found in the kidney of cultured *P. puntazzo* and *Sparus aurata* from farms all over Greece at a high prevalence and intensity in the summer and is currently under identification (Athanassopoulou 2000, Nengas et al. 2000, F. Athanassopoulou unpubl. data).

The purpose of this study was to assess different commercially available drugs commonly used for the treatment of spore-forming parasites in other animals to determine if they have an anti-myxosporean effect in naturally infected sharpsnout sea bream *Puntazzo puntazzo.* The fish used were obtained from commercial farms in southern Greece with a history of recurrent myxosporean infections such as *Myxidium leei* in the gall bladder and intestine of *P. puntazzo* and *Myxobolus* sp. cysts in the kidney. These farms have been studied in detail over the past 3 yr (Nengas et al. 2000, F. Athanassopoulou unpubl. data). During the present study, *M. leei* infections were very low (<3% only in summer months); therefore, *Myxobolus* sp. was selected as a convenient model for assessing the efficacy of pharmaceutical treatments. The efficacy of each selected drug scheme was evaluated in terms of mortality, pathology and prevalence rate of *Myxobolus* sp. cysts in the kidney.

MATERIALS AND METHODS

Experimental fish. *Puntazzo puntazzo* used in all experiments of the present study were obtained from 3 cage farms in southern Greece that were monitored at monthly intervals over the past few years and had a history of recurrent myxosporean infections (*Myxobolus* sp. and *M. leei*). *Myxobolus* sp. was used to test the efficacy of the treatments in the experiments (Figs. 1 & 2). Prevalence of infection from previous monitoring experience was taken into account in the design of 3 experimental trials. Fish for the first shore-based experiment were selected from a farm with a high starting prevalence of *Myxobolus* sp. infection (120 fish infected out of 150 examined = 80%) in the kidney, and the experiment was carried out during the summer when infection was expected to remain stable or be slightly reduced in untreated control fish at the end of the experiment (Expt 1: treatment during high prevalence and intensity). Fish for the second shore-based

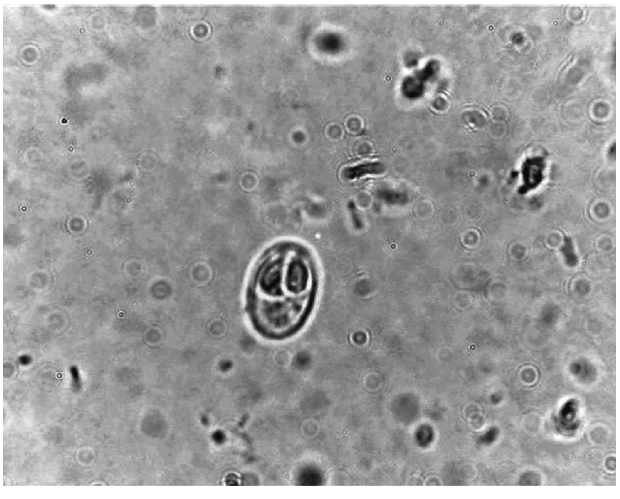

Fig. 1. Mature *Myxobolus* sp. from kidney. Fresh preparation, ×300

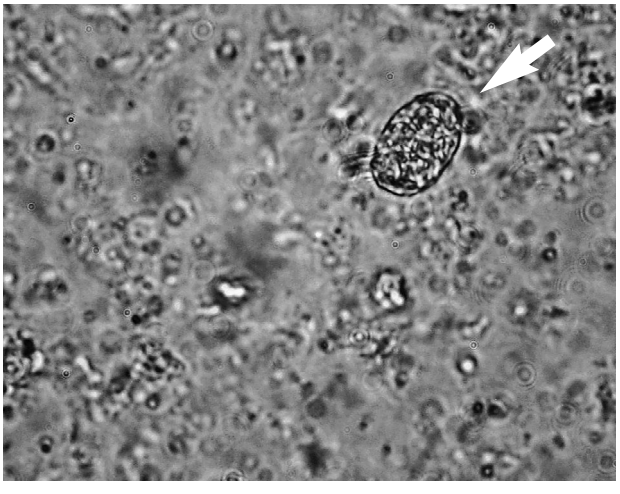

Fig. 2. Trophozoite of *Myxobolus* sp. from kidney (arrow). Fresh preparation, ×300

experiment were selected from a farm with low starting prevalence of *Myxobolus* sp. infection (15 fish infected out of 100 examined = 15%), and the experiment was carried out during the autumn when infection was expected to remain stable or increase slightly (Expt 2: preventive treatment). Fish for the small-scale field experiment (Expt 3) were selected from a farm with low starting prevalence of *Myxobolus* sp. infection (15%), and the experiment was carried out during spring and the beginning of summer when infection was expected to increase considerably. This last experiment resembled the commercial situation (commercial fish farms) when treatment is likely to prevent infection. The drugs were evaluated in terms of prevalence reduction, mortality level and pathology. The intensity of *Myxobolus* sp. infection was taken into consideration when evaluating the histopathology results. Fish were tested for myxosporean prevalence and other microbial diseases before transfer to shore-based and caged experimental facilities, as described below.

After transfer to experimental tanks or cages, fish were acclimatised for 7 to 10 d in 1 large holding tank. We sampled 30 fish from the holding tank 4 d before the start of each experiment, before allocation to different treatment tanks/cages for bacteriological and parasitological examination. Mortality was recorded daily for all experiments. Kidney and spleen samples were inoculated onto tryptone soy agar (TSA) and thiosulphate citrate bile salt agar (TCBS) for bacteriological tests (Roberts & Shepherd 1997). After anaesthesia, squash imprints of gill, skin, gall bladder, liver, spleen, kidney, muscle, brain and gut tissue were made from freshly killed fish by severing the spine, and the imprints were examined for the presence of parasites according to the methods described by Roberts (1989). After allocating fish to the various tanks/cages, samples were taken for parasitological examination at weekly intervals (see Tables 1 to 3); all daily moribund fish were examined. For histopathological examination, data from normal fish were also used for comparison (i.e. healthy cultured fish under the same holding conditions, sampled when infection and mortality was not present in the farm) (adjacent and distant sites from those used for the field trials). We also used data from healthy cultured fish (same holding conditions) from other farms for comparison. These fish are hereafter referred to as 'normal fish'. Untreated control fish infected with *Myxobolus* sp. are referred to as 'untreated control fish'.

Expt 1. First shore-based experiment. This experiment assessed the efficacy of treatment with a broad spectrum of commercially available drugs known to be effective against spore-forming parasites. Doses and regimens were extrapolated from those used for salmonids or other fish species for which data were

Table 1. Experimental conditions, protocols, efficacy and resultant mortality of *Puntazzo puntazzo* administered anti-myxosporean drugs. Fish averaged 20 g weight (Expt 1). C: control; F1 to F6: Fumagillin; T1 and T2: Toltrazuril; Amp: Amprolium 50%; ESB-3 (30%); A+ESB3 = Amprolium 50%+ ESB-3 (30%); SA: Salinomycin 12% + Amprolium 50%; To: tone of biomass; rep: repeat whole scheme

	C	F1	F2	F3	F4	F5	F6	T1	T2	Amp	ESB-3 (30%)	A+ESB-3 (30%)	SA
Regimen	0	2 mg kg^{-1} ×6 wk	6 mg kg^{-1} ×6 wk	6 mg kg^{-1} ×3 wk	15 mg kg^{-1} ×3 wk	15 mg kg^{-1} ×6 wk	25 mg kg^{-1} ×3 wk	600 ml To^{-1} ×2 d/ rep 5 d	600 ml To^{-1} ×2 d/3 d-off rep 5 d	190 g To^{-1} ×30 d	200 g To^{-1} ×3 d rep 15 d	100 g To^{-1}+ 100 g To^{-1} ×30 d	100g To^{-1}+ 100 g To^{-1} ×30 d
Sampling interval	Weekly	Weekly	Weekly	Weekly	Weekly	Weekly	Weekly	Weekly	Weekly	Weekly	Weekly	Weekly	Weekly
Expt duration (d)	60	60	60	60	60	60	60	60	60	60	60	60	60
Prevalence (%) Initial	80	80	80	80	80	80	80	80	80	80	80	80	80 80
Final	65	54	22	20	22	19	19	33	45	26	66	60	27
Cumulative morality (%)	32.5	2	4	4	8	16	32.2	2	6	2	12	8	2

Table 2. Experimental conditions, protocols, efficacy and resultant mortality of *Puntazzo puntazzo* administered anti-myxosporean drugs. Fish averaged 20 g weight (Expt 2). C: control; F1 and F2: Fumagillin; S: Salinomycin; SA: Salinomycin 12% + Amprolium 50%; R1 and R2: Oregano oils; Tp1 and Tp2: Toltrazuril + Propylene glycol; *: minimal concentration of Propylene glycol to improve taste; To: tone of biomass; rep: repeat whole scheme

	C	F1	F2	S	SA	R1	R2	Tp1*	Tp2*
Regimen	–	6 mg kg^{-1} ×6 wk	6 mg kg^{-1} ×3 wk	70 To^{-1}×30 d rep 5 d	60 g To^{-1}+ 100 g To^{-1} ×30 d	8 ml kg^{-1} BW	12 ml 5 kg^{-1} BW	600 ml To^{-1} ×2 d rep 15 d	600 ml To^{-1} ×2 d/ 3 d-off/ 2 d-on/ rep 15 d
Sampling interval (d)	Weekly	Weekly	Weekly	Weekly	Weekly	Weekly	Weekly	Weekly	Weekly
Expt duration (d)	70	70	70	70	70	70	70	70	70
Prevalence (%)									
Initial	15	15	15	15	15	15	15	15	15
Final	33	11	12	14	5	9	8	10	9
Cumulative mortality (%)	10	1	2	0	1	5	3	3	0

available. For new drugs, this information was extrapolated from poultry data and adjusted for fish.

Puntazzo puntazzo, each weighing ca. 20 g, and with kidney naturally infected with *Myxobolus* sp. (80% initial prevalence) were obtained from a single farm in July 2000. No other infective microorganism was identified. The fish were divided into equal groups of 100 fish each; 2 replicate tanks were used for each drug. A group of 300 fish was used as control (untreated control fish). Each test group was placed into a separate experimental tank equipped with an open-system, borehole-water intake at one of the dose rates given in Table 1. Fish were acclimatised for 7 d before the start of the experiment and starved for 3 d before feeding the medicated diets to ensure maximum uptake of the drugs. Mortalities were recorded daily for a period of 70 d. The trial was carried out at a water temperature of 22°C, salinity of 30 and pH of 7. Doses, regimens, feeding rates and sampling procedures are given in Table 1.

Expt 2. Second shore-based experiment. This experiment estimated the treatment impact using the most promising drugs from the previous experiment with refined dosage and application regimens. In addition, Origanum essential oils were used and propylene glycol was included to increase the palatability of Toltrazuril-medicated food. Experimental protocols and samplings are shown in Table 2.

Puntazzzo puntazzo weighing approximately 20 g and with kidney naturally infected with *Myxobolus* sp. (15% initial infection) were obtained from a single farm in September 2000. No other infective microorganism was identified. The fish were divided into groups of 250 fish each. 2 replicate tanks were used for each drug. A group of 500 fish were used as control. The doses, regimens, feeding rates and sampling procedures are given in Table 2. All the other parameters were as in Expt 1.

Table 3. Experimental conditions, protocols, efficacy and resultant mortality of *Puntazzo puntazzo* administered anti-myxosporean drugs. Fish averaged 165 g weight (Expt 3). SA: Salinomycin 12% + Amprolium 50%; R: Oregano oils; F: Fumagillin; To: tone of biomass

	C	SA	R	F
Regimen	–	60 g To^{-1} + 100 g To^{-1} ×30 d	8 ml 5 kg^{-1} BW ×30 d	6 mg kg^{-1} BW ×6 wk
Sampling	Weekly	Weekly	Weekly	Weekly
Expt duration (d)	73	73	73	73
Prevalence (%)				
Initial	15.6	15.6	15.6	15.6
Final	95.2	9.5	50	50
Cumulative mortality (%)	18.7	10.5	15.5	19.6

Expt 3. Field experiment. This experiment assessed, under a small-scale field situation, the efficacy of treatment with those drugs that had achieved optimum results in Expt 2. Experimental protocols and sampling procedure are shown in Table 3. *Puntazzo puntazzo*, each weighing ca. 1.5 g, were introduced into commercial cages, each containing 1000 fish, where they remained until they weighed 165 g. When prevalence of *Myxobolus* sp. had attained ca. 15%, the

fish were placed in 4 smaller experimental cages, each containing 250 fish. We sampled 30 fish from each cage for microbiological and parasitological examination as described in 'Experimental fish' above to establish the level of infection before the start of the experiment. Fish were acclimatised for 7 d before the initiation of the experiment and starved for 3 d before being fed the medicated diets to ensure maximum drug uptake; 1 cage was used as an untreated control group. Mortalities were recorded daily for a period of 70 d. The trial was carried out at a temperature of 20 (initial water temperature) to 26°C, salinity 38, and pH 7.

Pharmaceutical diets. The following drugs were selected to treat *Myxobolus* sp. infection: Fumagillin, an anti-myxosporean drug, the anti-coccidial drugs Toltrazuril, ESB-3, Salinomycin and Amprolium, commonly used for the treatment of spore-forming parasites, especially in poultry, and Origanum essential oils that have been found to have inhibitory effects on microorganisms (Athanassopoulou et al. 2000) and spore-forming organisms (Sivropoulou et al. 1996, Mejiholm & Dalgaard 2002). The compounds used in all experiments were of commercial grade, as shown in Table 4. These were diluted in cod liver oil and top-coated onto commercial pellets with a mechanical mixer. Untreated control fish were fed the same diet mixed with the same quantity of cod liver oil. The medicated diets were freshly prepared and were hand-fed ad libitum to fish by hand.

Histology and parasitology. Tissues of gills, kidney, intestine, liver, spleen, stomach, swim bladder and brain of 10% of the fish in each sample in each experimental tank were processed histologically. The tissues were fixed in 10% buffered formalin, processed and stained with haematoxylin and eosin (H&E) and Giemsa and Von Kossa stains according to the methods of Drury & Wallington (1980). Parasitological examination was performed according to the methods of Roberts (1989) and Athanassopoulou (1990).

Statistical analysis. The results were evaluated in terms of prevalence (reduction of *Myxobolus* sp. cysts) and mortality levels. Unacceptable mortality was set at 3%. Prevalence and cumulative mortality were cross-tabulated and compared at the 95% confidence level by Pearson chi-square tests.

RESULTS

Expt 1. First shore experiment

Initial prevalence of *Myxobolus* sp. infection in the fish was 80%; this remained high in the untreated control group until the end of the experimental period (65%); cumulative mortality was 33%. No other parasites or microbial pathogens were present during the experiment. In terms of prevalence reduction, the most efficient drugs were Fumagillin 2, 3, 4, 5, 6 (F1–6) and Salinomycin 12% + Amprolium (SA) (treatments statistically different from the control group at $p < 0.05$; no statistical difference between treatments). These drugs reduced prevalence from 80 to 20–26% compared to a final prevalence of 65% in untreated controls. In terms of reduction in mortality, the best results were obtained with F2, F3, Amprolium (Amp) and SA (treatments statistically different from control group at $p < 0.05$; no statistical difference between treatments) (Table 5). Therefore, the drugs selected for use were: F2, F3, SA and Amp.

Expt 2. Second shore experiment

Initial prevalence of *Myxobolus* sp. infection in the fish was 15%, and this had increased to 33% in the untreated control group by the end of the experimental period; cumulative mortality was 10%. No other parasites or microbial pathogens were present during

Table 4. *Myxobolus* sp. infecting *Puntazzo puntazzo*. Drugs used in shore and field treatments (Expts 1 to 3)

Drug	Abbreviation	Commercial form Composition	Trade name	Company
Fumagillin (dicyclohexylamine)	F (F1–F6)	Powder oral solution (20 mg g^{-1})	Fumidil®	CEVA
Salinomycin (salinomycin sodium)	S	Medicated premix (12%)	Salinomycin	Haechst
Amprolium	Amp	Medicated premix (50%)	Amprolium	Veterin
Origanum essential oils	R (R1–R2)	Oil solution (5%)	Orego-Stim® (Ecodiar Liquid)	Ecofarm Hellas
Toltrazuril	T (T1–T2)	Oral solution (25 mg ml^{-1})	Baycox®	Bayer
ESB-3	E3	Powder (30%)	ESB-3	Ciba Geigy

Table 5. *Myxobolus* sp. infecting *Puntazzo puntazzo.* Mortality (%) and prevalence (%) during shore treatment (Expt 1). x: statistically significant ($p < 0.05$) from untreated control fish; o: not statistically significant from untreated control fish; C: untreated control fish; F1 to F6: Fumagillin; T1 and T2: Toltrazuril: E3: ESB-3; AE: Amprolium 50% + ESB3 30%; SA: Salinomycin 12% + Amprolium 50%

Mortality	Prevalence		Pharmaceutical treatment												
			C	F1	F2	F3	F4	F5	F6	T1	T2	Amp	E3	AE	SA
32.3	66.7	C	x	o	x	x	x	x	x	o	x	o	x	o	x	o	x	o	x	x
2.0	52.9	F1			o	o	o	x	o	x	x	x	o	o	x	o	o	o	x	o	x	o	x	x
4.0	22.2	F2					o	o	o	o	x	o	x	o	o	o	o	o	o	o	x	o	o	x	o	o
4.0	20.0	F3							o	o	x	o	x	o	o	o	o	o	o	o	x	x	o	x	o	o
8.0	22.2	F4									x	o	x	o	x	o	o	x	o	o	x	o	x	o	x	o
16.0	18.8	F5											x	o	x	o	x	o	x	o	o	x	o	x	o	x	o
32.2	18.8	F6													x	o	x	o	x	o	x	x	x	x	x	x
2.0	33.3	T1															x	o	o	o	o	o	o	o	x	o
6.0	44.4	T2																	x	o	x	o	o	o	x	o
2.0	26.3	Amp																			x	x	x	x	o	o
12.0	6.7	E3																					o	o	x	x
8.0	58.8	AE																							x	x
2.0	26.3	SA																								

the experiment. Infection prevalence and mortality of treated fish were statistically significantly different from those of untreated controls. The most effective drug was SA. All drugs except Origanum essential oils (R1, R2) resulted in low levels of mortality, but SA was selected since this resulted in lowest prevalence; however, prevalence in SA did not differ significantly from that in F1 and F2 (Table 6).

Expt 3. Field experiment

Initial prevalence of *Myxobolus* sp. infection in the fish was 15%, and this had increased to 95.2% in the untreated control group by the end of the experimental period; cumulative mortality was 18.7%. Mortality in all cages was high compared to that in the shore-based experiments. As no other infection or disease was detected, this was attributed to the restricted space in these cages (each fish weighed ca. 165 g). In the field, cumulative mortality due to infection is normally low (<10%). As there was no statistically significant difference in mortality between the different treatments, the results of this experiment were evaluated only in terms of reduction in infection prevalence. The best results (statistically significant) were observed with the SA combination (Table 7), which reduced prevalence to 9.5%. Fish treated with this drug also had the lowest mortality (10.5%).

Table 6. *Myxobolus* sp. infection of *Puntazzo puntazzo.* Mortality (%) and prevalence (%) during shore treatment (Expt 2). x: statistically significant ($p < 0.05$) from untreated control fish; o: not statistically significant from untreated control fish; C: untreated control fish; F1 and F2: Fumagillin; S: Salinomycin; SA: Salinomycin 12% + Amprolium 50%; R1 and R2: Origanum essential oils; T1 and T2: Toltrazuril

Mortality	Prevalence		Pharmaceutical treatment								
			C	F1	F2	S	SA	R1	R2	T1	T2
10.0	33.0	C	x	x	x	x	x	x	x	x	x	x	x	x
1.0	11.0	F1			o	o	x	o	o	o	x	o	x	o	x	o
2.0	12.0	F2					x	o	o	o	o	x	o	o	x	o
0.0	14.0	S						x	x	o	o	o	o	o	x	o
1.0	5.0	SA								o	o	o	o	o	x	o
5.0	9.0	R1										o	o	o	x	o
3.0	8.0	R2												o	x	o
3.0	10.0	T1													x	o
0.0	9.0	T2														

Histology

In all experiments, infected fish before treatment, as well as untreated controls at the end of the experiments, were intensively infected with *Myxobolus* sp. cysts in the renal interstitial tissue (mean number of cysts per viewing field at 100× = 5–8). These were demarcated by connective tissue showing no intense reaction. Some of the cysts contained amorphous material that was very often calcified (2 of 8 cysts observed showed a positive Von Kossa reaction), and demarcation of melanomacrophage centres (MMC) with no apparent spores was also present (2 of 8 cysts). Histological sections of all fish were also compared with sections from non-infected normal fish. In contrast to untreated control fish, SA-treated fish had either no cysts, or a few spores that were free in MMC. Some fish showed MMC demarcation (1 cyst per viewing field at 100×) after SA treatment, and no pathological lesions were found in any organ of SA-treated fish.

Lesions were observed only in fish treated with Fumagillin and Toltrazuril. Pathology due to treatment with Fumagillin was observed in the interstitial renal tissue, where slight inflammation was apparent, only after >6 mg kg^{-1} body wt for 6 wk. The highest dose tested (25 mg kg^{-1} body wt) also induced necrosis of the interstitial tissue and degeneration of the epithelial cells of the tubules and a reduction in MMC numbers. In a few cases, haemorrhage

Table 7. *Myxobolus* sp. infecting *Puntazzo puntazzo.* Mortality (%) and prevalence (%) during field treatment (Expt 3). x: statistically significant ($p < 0.05$) from untreated control fish; o: not statistically significant from untreated control fish; C: untreated control fish; SA: Salinomycin 12% + Amprolium 50%; R: Origanum essential oils; F: Fumagillin

Mortality	Prevalence		Pharmaceutical treatment			
			C	SA	R	F
18.7	95.2	C		o x	o x	o x
10.5	9.5	SA			o x	o x
15.5	50.0	R				o o
19.6	50.0	F				

and congestion of the liver also occurred. In all Fumagillin-treated fish, thickening of the glomerular capsule occurred; this was more marked at higher doses. In comparison to untreated control fish, MMC size and numbers were not affected in fish treated with doses up to 15 mg kg^{-1} body wt. Fish treated with Toltrazuril, even 7 d post-treatment, displayed intense oedema in the enteric epithelium. When Toltrazuril was combined with propylene glycol, extensive inflammation, necrosis and haemorrhage in the renal interstitial tissue also occurred. MMC were fewer at all Toltrazuril doses tested compared to untreated controls or normal fish; MMC decreased even further when propylene glycol was used.

DISCUSSION

No therapy for fish myxosporosis has been found (Molnar 1993). Fumagillin (and its more recent analogs) are the only proven drugs for treating Microsporea and Myxosporea infections (Molnar et al. 1987, Hedrick et al. 1988, 1991, Higgings & Kent 1988, Kent & Dawe 1994, Speare et al. 1999). This drug has proved effective against *Myxidium giardi* (Szekely et al. 1988), *Thelohanellus hovorkai* and *Sphaerospora renicola* in common carp when administrated during the infective period (Molnár et al. 1987, Yokoyama et al. 1999) and (when early intracellular trophozoites and more developed plasmodia of *Hoferellus carassi* exist; Yokoyama et al. 1990) against *Myxobolus cerebralis* and *Tetracapsuloides bryosalmona* in rainbow trout *Oncorhynchus mykiss* (El-Matbouli & Hoffman 1991) and against the myxosporean *Sphaerospora testicularis* in sea bass *Dicentrarchus labrax* (Sitja-Bobadilla & Alvarez-Pellitero 1992). However, this drug has some toxic effects. Side effects can range from inappetence to mortality (Sitja-Bobadilla & Alvarez-Pellitero 1992); however, moderate side effects are most commonly reported, such as reduced growth in rainbow trout *O. mykiss* during treatment (Kent & Dawe 1994) and depletion of the renal interstitium and vacuolation in the epithelium of the renal tubules in Chinook salmon *Oncorhynchus tshawytscha* (Hedrick et al. 1988). Sitja-Bobadilla & Alvarez-Pellitero (1992) found toxic side effects in sea bass, mainly consisting of a decrease in haemoglobin, haematocrit and red blood cell counts; however, these changes were not accompanied by histological anomalies and are considered reversible (Lauren et al. 1989, Wishkovsky et al. 1990, Hedrick et al. 1991, Sovenyi 1992). An important comprehensive study on the toxicity and pharmakokinetics of Fumagillin in rainbow trout was performed by Lauren et al. (1989), who reported that high doses of the drug are lethal, causing haemorrhage and congestion of the liver and tubular degeneration of the kidney, whereas low doses produce reversible changes in haematopoietic tissue only in the kidneys (aplastic anaemia). The use of Fumagillin involves prolonged treatment periods and is consequently expensive; however, in Greece, it is the only prescription drug available for treatment of *Myxidium leei* and other microsporean infections, and is generally believed to be safe for the Sparidae (Athanassopoulou 1998, F. Athanassopoulou unpubl. data) when used at doses less than 10 mg kg^{-1} body wt. Rigos et al. (2000) reported only insignificant differences in blood parameters in sea bream treated with the drug for 30 d and no conclusive evidence of toxicity at 22°C at doses of 0.33 to 0.66 mg kg^{-1} feed d^{-1}. In our study, Fumagillin was also safe at doses of <6 mg kg^{-1} body wt, but was less effective than other drugs (such as SA) for treatment of *Myxobolus* sp. infections in *Puntazzo puntazzo.* Nevertheless, the controversy regarding immunosupression by this drug has resulted in new drugs being tested experimentally to treat myxosporean infections in fish species such as *P. puntazzo* (Higgins & Kent 1988, Schmahl et al. 1991, Dohle et al. 2002).

Toltrazuril is another drug used in the treatment of spore-producing parasites and fish-infecting members of the Coccidia, Microsporea and Myxozoa (Mehlhorn et al. 1988). It has proved active against pre-spore stages of *Myxobolus* sp. in the gills of bream *Abramis brama*, as well as against developmental stages of *Henneguya* sp. parasitic in the gills of *Gnathonemus petersi* (Schmahl et al. 1989, 1991). Toltrazuril affects the spores as well as the xenoma wall (Schmahl et al. 1989). In contrast to these observations, Molnar (1993) reported that Toltrazuril was ineffective in the treatment of *Sphaerospora renicola* in common carp. Nevertheless, the drug has been suggested to be effective in the treatment of Myxosporea infection (including *Myxidium leei*) of Mediterranean fishes (Lytra 1997). Our present study has shown that Toltrazuril is ineffective in the treatment of and toxic to sharpsnout sea

bream. Its toxicity and pathology increases when propylene glycol is added (as taste enhancer). ESB-3, the other drug used in Expt 1 was both ineffective and toxic to this species.

Amprolium, ESB-3 and Salinomycin have also been used for anticoccidial control in animals (Coombs & Muller 2002), but there is little information available on the toxicity and immunological response of these drugs in fishes, particularly in Mediterranean species.

According to the results of Expts 1 and 2 of the present study, the best drugs are SA and Fumagillin at low doses. Fumagillin, as noted above, is a well known anti-myxosporean and therefore some action against *Myxobolus* sp. in *Puntazzo puntazzo* was anticipated. Hedrick et al. (1988) suggested that it can be prophylactically used during periods of high infectivity to prevent serious losses. Molnar et al. (1987) suggested that feeding with Fumagillin prior to myxosporean infection in carp was required to ensure efficacy of the drug. Our study examined the preventive effect of the drug at high and low initial prevalence when prevalence was expected to be stable, and low doses satisfactorily lowered the prevalence without any serious side effects. Toltrazuril is often used in combination with sulphonamides to treat coccidial infections in animals. It has been shown to be more active than sulphlorpyrazine when treatment is begun in the early stages of the coccidian life cycle, but sulphonamide is more effective during the later stages of the parasite's development (Laczay et al. 1995). This may be the reason why Toltrazuril was not effective in Expt 1 (in which infection had already progressed to a later stage), and was more useful in combination with sulphonamide in Expts 2 and 3.

Amprolium is a structural analogue of thiamine (Vitamin B1) and causes a competitive inhibition of thiamine utilisation by the parasite. It acts upon the first generation in the cells of the intestinal wall, preventing differentiation of the merozoites. It may also suppress the sexual stages and sporulation of the oocysts. Amprolium has been proved ineffective against many protozoans (Tojo & Santamarina 1998a,b) and for *Loma salmonae* infection in trout *Oncorhynchus mykiss* (Speare et al. 1999). In all these studies, however, the drug was used by itself, and the successful results achieved in our study may have been due to the combined effect of this drug with Salinomycin. Combination therapies are well known, particularly in poultry (Coombs & Muller 2002). For many years, the standard treatment of coccidiosis in animals and humans comprised synergistically acting folic acid antagonists and a sulphonamide, but the observed toxicity was attributed mainly to the sulphonamide component (Haberkorn 1996). However, sulphonamides do not have a negative effect on the immune system of fishes (Lunden & Bylund 2002). In particular, Salinomycin is an antibiotic belonging to ionophorous polyethers and acts as a chelator with monovalent cations; it shows a strong activity against many microorganisms, including coccidia. It causes shrinking of the pellicula, vacuolisation of the cytoplasm and destruction of mitochondria (Kinashi et al. 1973). Salinomycin administrated orally has deleterious effects on the trophozoite cytoplasm and on the presporogonic and pansporoblastic stages of *Henneguya* sp. parasitising the gills of the tapir fish *Gnathonemus petersii*, in which a severe shrinking of the plasmodia and an enlargement of the sutural ridges in the pansporoplasts and malformation of the polar capsules was observed by Dohle et al. (2002). Origanum essential oils have been found to have inhibitory effects on microorganisms (Athanassopoulou et al. 2000) and spore-forming organisms (Sivropoulou et al. 1996, Mejiholm & Dalgaard 2002). Our study is the first to test them against myxosporean infections in fish. In both Expts 2 and 3, these drugs significantly reduced prevalence (especially Expt 3). However, more experimental data are required in order to assess these drugs.

An interesting histopathological result was the MMC changes observed during treatment with Fumagillin and Toltrazuril. The MMC have several functions, including roles in innate and adaptive immunity (Wolke 1992). A change in 1 or more MMC parameter (size, shape and pigmentation) has been considered a biomarker of exposure to polluted water and of fish health (Blazer et al. 1987). In our study there was a distinct reduction in the numbers of MMC in Toltrazuril-treated fish (even more when given in combination with propylene glycol) and during treatment with Fumagillin in high doses, whereas, in SA-treated fish, MMC remained stable, similar to untreated control or to normal uninfected fish (comparisons were made with previous samples from the same farm). This may indicate that MMC can be used as indicators of drug toxicity.

In our study, the combination of Salinomycin and Amprolium (SA) proved the most successful and safe treatment of *Myxobolus* sp. infection in sharpsnout sea bream. This combination successfully lowered prevalence in fish with initially high infections of Myxosporea, even when parasite prevalence was expected to increase (i.e. in summer months: Expts 2 and 3). This drug combination caused no histopathological lesions in the organs of treated fish at the doses used in all 3 experiments, and the intensity of the *Myxobolus* sp. cysts was significantly and steadily reduced. Amprolium is considered to be low to moderately toxic to different aquatic organisms (Canton & von Esch 1976) and is a cheap compound widely used in poultry farming as an anti-coccidial drug. This is the first time that

this combination has proved a successful and safe treatment of myxosporean infections in a fish, and it would seem to be a promising drug for treatment of cultured *Puntazzo puntazzo*. However, more information is required to assess its efficacy and toxicity for other myxosporean species as well as its effect on the hosts' immune system.

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