Available online at www.sciencedirect.com
ScienceDirect
Aquaculture 261 (2006) 856–864
Aquaculture
www.elsevier.com/locate/aqua-online

In vivo and *in vitro* treatments against *Sparicotyle chrysophrii* (Monogenea: Microcotylidae) parasitizing the gills of gilthead sea bream (*Sparus aurata* L.)

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Received 19 May 2006; received in revised form 6 September 2006; accepted 10 September 2006

Abstract

The effect of *in vitro* and *in vivo* treatments against *Sparicotyle chrysophrii*, a microcotylid parasite of gilthead sea bream (*Sparus aurata* L.), was studied. *In vitro* chemical treatments were targeted to eggs, oncomiracidia and adults, and were tested both as disinfectants and therapeutics for infected animals. The compounds were: distilled water, formalin, limoseptic ®, hydrogen peroxide, chlorine, and praziquantel (PZQ). Larvae were sensitive to all the treatments, but adults were more resistant, as chlorine (60 ppm – 1 h), hydrogen peroxide (100 ppm – 30 min) and PZQ (50 ppm – 30 min) produced only 10% mortality. All adults were killed only with distilled water, limoseptic (0.1% – 5 min), formalin (300 ppm – 30 min), or hydrogen peroxide (200 ppm – 30 min). Eggs were the most resistant stage, as only 30 min in limoseptic (0.1% in distilled water) or in formalin (300 ppm) prevented hatching. PZQ was used *in vivo* either as a curative or preventive treatment. The highest dose tested (400 mg kg^{-1} BW; effective dose 116.3 mg kg^{-1} BW due to palatability problems leading to 45% reduction in host food intake) did not significantly decrease prevalence of infection when given for 6 consecutive days. A lower dose (200 mg kg^{-1} BW) (effective dose 158.1 mg kg^{-1} BW) was rejected to a lower degree and decreased the prevalence of infection from 90% to 40%. When a lower dose (40 mg kg^{-1} BW) was administered for longer periods (20 days), food intake was reduced slightly, but the infection did not decrease significantly. The oral intubation with PZQ (200 mg kg^{-1} BW) once a week for 4 weeks did not prevent the infection of fish by cohabitation. However, a significant reduction in the abundance of the parasite was registered. In view of the results, recommendations for fish treatment and disinfection of aquaculture facilities are discussed.

Keywords: Ectoparasites; Praziquantel; Chemotherapeutics; Oral treatment; Sparidae; Polyopisthocotylea

1. Introduction

Gilthead sea bream (*Sparus aurata* L.) is the main aquacultured fish species in the Mediterranean basin, with more than 70,000 Mt in 2003 (APROMAR, 2004). The exponential tonnage increase of the last years has been supported by the massive establishment of sea cages, though it has also contributed to the extension and dispersal of parasitic diseases (Sitjà-Bobadilla, 2004). One of them is the monogenosis produced by the polyopisthocotylean *Sparicotyle chrysophrii.* This ectoparasite has also been found in *Diplodus puntazzo* (see Di Cave et al., 2003), though with lower infection levels than in gilthead sea bream (Athanassopoulou et al., 2005). Mortalities due to polyopisthocotyleans

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doi:10.1016/j.aquaculture.2006.09.012

have been described in several cultured fish species (Kubota and Takakuwa, 1963; Silan et al., 1985; Ogawa, 2002; Hayward, 2004, Sonia Ternengo, personal communication, Univ. Corsica, France), and some of them are responsible for 22% of total production cost (see Whittington et al., 2002). *S. chrysophrii* has caused increased mortalities mainly in sea cages during spring and summer (Faisal and Iman, 1990; Sanz, 1992; Alvarez-Pellitero, 2004; Planas, 2006), but no data are available on its economic impact. This parasite is favoured by high densities and net biofouling (authors' unpublished observations) and disease signs include lethargy due to hypoxia, emaciation, histopathological damage, severe anaemia. Mixed infections with other parasites and secondary bacterial infections are common (Cruz e Silva et al., 1997).

Several treatments with various chemotherapeutants against monogeneans have been reported (Schmahl, 1991, 1993), with certain species showing low susceptibility to chemicals. However, fewer reports on experimental treatments against microcotilids are available (Kim and Choi, 1998; Kim et al., 1998; Katharios et al., 2006), and none deal with *S. chrysophrii*. Although some reports had shown that monogenean eggs are resistant to chemical treatments (Diggles et al., 1993; Yoshinaga et al., 2000), most studies have concentrated on the efficacy of chemotherapeutants for removal of adult monogeneans from the host. On-site, cage treatments for *S. chrysophrii* include formalin baths, net removal and cleaning. In addition, different chemicals are used in routine disinfections in fish farms. However, the real efficacy of both chemotherapeutants and disinfectants has not been monitored. Because of the drawbacks of bathing infected fish with certain compounds, especially in sea cages, the possibility of administering parasiticides in the feed would be more convenient. Therefore, the aim of the present work was the assessment of the efficacy of different chemical compounds against *S. chrysophrii* to be used both as disinfectants and/or treatment of infected animals, using a panel of *in vivo* and *in vitro* assays.

2. Materials and methods

2.1. Fish

Naturally infected gilthead sea bream were obtained from a commercial fish farm and maintained in the facilities of the Instituto de Acuicultura de Torre de la Sal (IATS) in 500-L fiber-glass tanks receiving sand-filtered sea water (open system, 37.5‰ salinity, flow=20 L min^{-1}), with an oxygen content higher than 85% saturation. Upon arrival, some fish were killed and checked as explained below for the presence of the parasite. They were used both as a source of the different parasitic stages tested in the *in vitro* treatments, and as infected fish in the *in vivo* trials. In the latter, fish were transferred to 250-L fiber-glass tanks and water was mesh-filtered (10 μm), photoperiod followed the natural cycle (40° 5′ N; 0° 10′ E) between November and January, and water was heated (18–20 °C) to favour parasite transmission.

For preventive *in vivo* treatments, non-parasitized fish were also obtained from commercial farms, and maintained under the same conditions as parasitized fish. The absence of the monogenean was checked upon arrival.

2.2. In vitro treatments

Parasitized fish were killed by a blow on the head, bled to diminish blood content in gills, their gills arches excised and the adult monogeneans gently removed and immersed in Petri dishes containing sterilized sea water (SW) with an antibiotic antimicotic mixture (100 U mL^{-1} penicillin, 100 μg mL^{-1} streptomycin, and 0.25 μg mL^{-1} amphotericin B) (SW-PSA) to prevent bacteria proliferation. Only active worms were transferred to 6 well plates (Costar) with 5 mL of SW-PSA, each well holding 40 μm-meshed cell strainers (Falcon).

Table 1
Effects of various *in vitro* treatments on the different *Sparicotyle chrysophrii* stages

Treatment		Egg hatching*	Larval mortality (%)	Adult mortality (%)
Reactive (dose)	Time			
Sea water (SW)	1 h	yes	0	0
Distilled water (DW)	5 min	yes	100	100
	30 min	yes	100	100
Limoseptic ®	5 min	yes	100	100
(0.1% in DW)	30 min	no	100	100
Limoseptic ®	5 min	yes	100	0
(0.1% in SW)	30 min	yes	100	100
Formalin (300 ppm)	30 min	no	100	100
	1 h	no	100	100
Chlorine (12 ppm)	1 h	yes	100	0
Chlorine (60 ppm)	1 h	yes	100	10
H_2O_2 (50 ppm)	30 min	nd	80	0
H_2O_2 (100 ppm)	30 min	nd	90	10
H_2O_2 (200 ppm)	30 min	nd	100	100
PZQ (25 ppm in SW)	30 min	yes	nd	20
PZQ (50 ppm in SW)	30 min	yes	nd	10
PZQ (100 ppm in SW)	50 min	yes	nd	10

*The efficacy against eggs is indicated as hatching occurring (yes) or not (no). nd=not determined.

Table 2
Results of the oral treatment with praziquantel in curative (CUR) and preventive (PREV) trials of gilthead sea bream

Praziquantel treatment				Fish before treatment					Fish after treatment					
Type	Dose (mg kg BW^{-1})		Duration (days)	Initial weight (g)	*N*	Infection			Sampling			Infection		
	I	A				*P* (%)	AB	MI	Days	*N*	Group	*P* (%)	AB	MI
CUR-1	200	158.1	6	76.8	30	100	13.6	13.6	7	10	T	40	4	10
										10	NT	90	5.8	6.4
	400	116.3	6						7	10	T	70	3.3	4.7
										10	NT	90	5.8	6.4
CUR-2	40	24.7	20	90.1	20	100	52.4	52.4	21	7	T	42.8	9.1	15
										7	NT	71.4	10.7	16
CUR-3	40	37.1	20	31.3	13	85.7	8	9.1	20	7	T	71.4	1.8	2
										7	NT	85.7	4.7	5
PREV	200		1*	30.9	20	n.a.	n.a	n.a.	35	7	T	85.7	3.8[a]	4.5
										7	NT	100	8[b]	8

The infection levels of *Sparicotyle chrysophrii* before and after the treatments are expressed as mean prevalence (*P*), abundance (AB) and mean intensity (MI). Different superscript letters indicate statistically significant differences between the values of treated (T) and non-treated (NT) fish. In CUR trials, the actual (*A*) dose was lower than the intended (*I*) one. n.a.=not applicable; *One day per week, during 4 weeks.

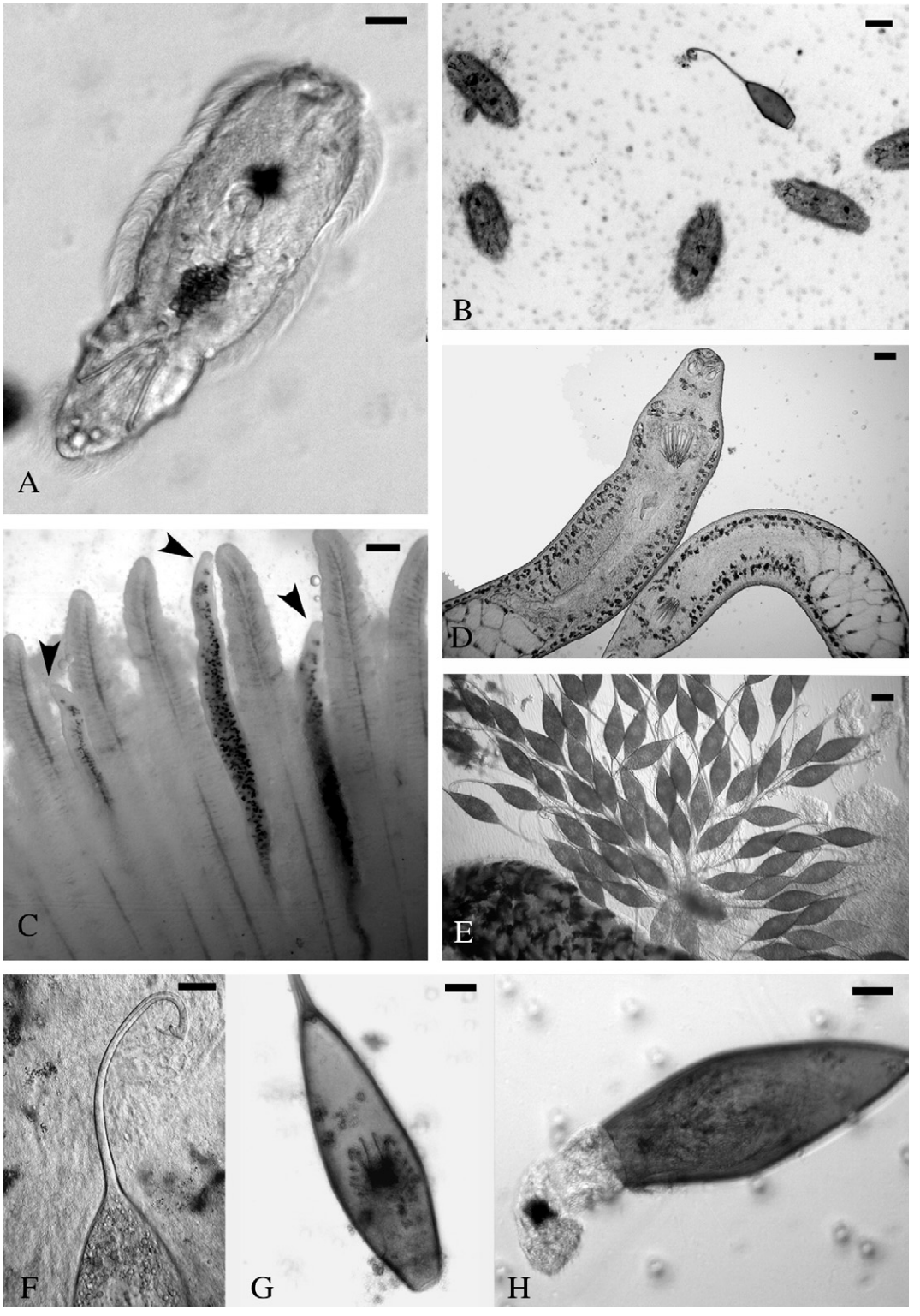
Eggs were harvested from tanks holding parasitized fish. For this purpose, a PVC ring provided with a 250 μm-mesh was left for several days floating on the water on the upper layer of the tank. After washing the mesh with SW, the resulting volume was left to settle down for 1 h in a 1-L graded cylinder, and the obtained sediment was screened under an stereomicroscope. The collected eggs were immersed in Petri dishes with SW-PSA. Some eggs were incubated in 24-well culture plates (Costar), each well holding 8 μm-meshed cell culture inserts (Falcon) for several weeks until hatching occurred (20 °C, 12 h light/12 h darkness). Egg shells were removed, and the inserts containing the newly hatched oncomiracidia (1 day old) were transferred to other plates containing the different compounds to be tested. Another batch of eggs was directly used for the treatments without incubation. In the latter case, eggs in different stages were included. The number of eggs in each well was different and estimated separately because it was impossible to disentangle them from the egg filaments.

Batches of eggs (at least 30 eggs), freshly hatched oncomiracidia (10) and adults (10) were separately allocated in each of the insert/strainer-holding wells containing the different compounds. Details on the concentrations of the compounds and timing can be found in Table 1. Limoseptic® (a commercial mixture of 15% formaldehyde, 5% glyoxal, 15% glutaraldehyde and 17% benzalkonium chloride) and sodium hypochlorite were chosen to be tested as disinfectants, whereas hydrogen peroxide, formalin and praziquantel (PZQ) for treatment of infected fish. Control wells contained SW-PSA and wells with distilled water (DW) were used as control of compounds dissolved in DW. To prevent solubilization problems, PZQ working doses were obtained from a concentrated solution (1000 ppm) in 100% ethanol. To discard the possible effect of ethanol on the parasite, other control wells with the corresponding percentage of ethanol were also included.

After the different treatments, the parasite stages were washed three times by transferring the strains to clean wells with SW-PSA. The same procedure was applied to CTRL wells. Adult and larvae survival was assessed by observation of all the specimens contained in each well with an inverted microscope (Olympus, IX71) during the treatment, just after washing and up to 48 h (every 12 h) after the treatment. Larvae were considered dead when appeared lysed or their motility was lost. Egg survival was assessed similarly, on a daily basis, but the observation lasted for 2 weeks, which is the average time for egg hatching under laboratory conditions (20 °C, 12 h ligh/12 h darkness) (authors'

Fig. 1. A. Freshly hatched oncomiracidium of *S. chrysophrii* before being used in *in vitro* treatments. Notice the dense ciliature covering the body surface. B. Immobilized oncomiracidia after formalin treatment (300 ppm – 30 min).C. Adults attached to the gills of infected gilthead sea bream. Arrowheads point to the anterior ends. D. Fresh smear of adults isolated from the gills of *in vivo* treated fish. E. Intermingled group of eggs before *in vitro* treatments. F. Detail of an egg showing the typical hooked posterior end. G. Egg treated with formalin (300 ppm – 30 min) with a dead larva inside. H. Egg treated with limoseptic (0.1% DW-30 min) with a dead larva stuck at the opening end. Bar scales: 20 μm for A, F–H; 60 μm for B, D, E; 200 μm for C.

unpublished observations). Sea water was changed every 48 h during egg incubation. The efficacy of the treatment was considered null when egg hatching was not completely prevented. Most treatments were run twice with different batches of the parasite. As the first run did not include all the compounds and the results



obtained were very similar to the second one, only data of the second run are shown.

2.3. In vivo treatments

Several curative and preventive oral treatments were performed with praziquantel (PZQ, Nanjing Pharmaceutical Co. Ltd., China). Three different curative (CUR-1, CUR-2 and CUR-3) trials were performed with naturally infected gilthead sea bream, by feeding with a commercial pelleted food (Proaqua, Spain) adsorbed with different doses of PZQ. In CUR-2 and CUR-3 the same PZQ dose was administered, but the initial intensity of infection of the fish was higher in the former. In all the CUR trials a parallel group remained untreated (NT) and was fed at 1% body weight (BW). Details on the fish, doses and timing can be found in Table 2. The actual food intake was registered for all the groups.

In the preventive (PREV) treatment, 20 non-parasitized fish were orally intubated with PZQ under slight anaesthesia (T group), whereas other 20 fish remained untreated (NT group). T fish were starved 1 day prior to intubation and received 0.2 mL of a 2.5% w/v PZQ solution in ethanol:olive oil (5:95 parts), which resulted in a dose of 200 mg kg^{-1} BW. Animals were observed until recovery from anaesthesia and placed again in their tanks. One day after the first oral intubation, the cohabitation of both T and NT fish with 10 naturally infected fish each was initiated in two separate tanks. Subsequently, T fish received repeated doses of PZQ by the same procedure every week during 1 month. A group of non-parasitized fish was kept untreated and without contact with parasitized fish in another tank, as control (CTRL).

The efficacy of the treatments was evaluated by examination of seven (CUR-2, CUR-3, PREV-1) or ten (CUR-1) randomly sampled fish from each group (Table 2). Fish were killed by a blow on the head, bled, the gill arches excised, and the number and type of stages of the monogenean were recorded. The prevalence, intensity and abundance (*sensu* Bush et al., 1997) of the parasite were calculated.

2.4. Statistics

The influence of the *in vivo* treatments on prevalence of infection was analyzed statistically using a chi-squared test of independence (Sokal and Rohlf, 1981), with Yates correction for continuity when necessary. The Fischer exact test was run when the expected values of the contingency table were very low. A student *t*-test or a Mann-Whitney rank sum test was used to compare the abundance of parasites in treated and untreated fish in the different *in vivo* trials. The statistical analyses were performed with Sigma Stat (SPSS Science, Chicago, IL), and the minimum significance was set at $P<0.05$.

3. Results

3.1. In vitro treatments

Table 1 shows the results of the *in vitro* treatments with the different compounds. Oncomiracidia (Fig. 1A) were sensitive to all the treatments, as they died immediately after exposure (Fig. 1B). Distilled water lysed, as expected, the larvae. Adults (Fig. 1C–D) were killed by all treatments, except chlorine (even at the highest tested dose) and PZQ (mortality was only 20% with the highest dose). No mortality was observed among adults incubated with the corresponding amount of ethanol, except in those with 10% ethanol (highest PZQ dose), which had 16.7% mortality. Hydrogen peroxide was completely effective against adults only at the highest dose tested. Limoseptic was totally effective when prepared with DW, whereas egg hatching was not prevented when prepared with SW. Eggs (Fig. 1E–F) were the most resistant stage, as 30 min in limoseptic (0.1% DW) or in formalin (300 ppm) were necessary to prevent hatching completely. In some eggs, the oncomiracidium appeared stuck at the tip of the egg or dead inside the egg shell (Fig. 1G–H).

3.2. In vivo treatment with praziquantel

Table 2 shows the results of the oral treatment with praziquantel. When high doses of PZQ were fed to the fish for a short period of time (CUR-1), food intake was reduced in T fish up to 45% in comparison to CTRL animals. Thus, the intended doses of 200 and 400 mg kg^{-1} BW were reduced to 158.1 mg kg^{-1} BW and 116.3 mg kg^{-1} BW, respectively. When lower doses at longer times were fed, food intake was slightly reduced: 24.3% and 7.3% for CUR-2 and CUR-3, respectively. Thus, palatability problems seem to be dose dependant. In CUR-2, the actual PZQ dose was reduced further due to the presence of the parasite, as non-treated parasitized fish (NT) also had a reduced food intake (0.8% BW). In none of the CUR trials the infection was completely eliminated. The highest decrease of the infection (both in terms of prevalence and intensity) was achieved with the highest actual dose for a short time, but the chi-squared test revealed no relationship between the parasitic status and the treatment, and differences in parasite abundance between T and NT fish were not statistically significant.

In the preventive trial, both T and NT fish became infected with no significant difference in prevalence (chi-squared test). However, parasite abundance was significantly lower in T than in NT fish (*t*-test, $P=0.043$). CTRL fish were not infected.

4. Discussion

One of the refrains of the fight against fish parasites is the scarcity of registered antiparasitic preparations. In Europe they have been progressively reduced and are practically non-existent, whereas the efficacy and doses of many drugs have been determined only for cold-water species (Burka et al., 1997). In addition, the application of some treatments to high numbers of fish is not feasible or very expensive. Effective disinfection methods for all the equipment used in aquaculture practice (nets, air tubes and stones, tanks, containers, etc.) are important to prevent horizontal transmission and parasite geographical dispersal (reviewed in Torgersen and Hastein, 1995). In the present study, the efficacy of several compounds (both disinfectants and therapeutics) against *S. chrysophrii* was demonstrated.

Formalin has been used as an effective therapeutic compound not only in aquaculture, but also in zoo medicine, aquarium industry, and to fight aquatic animal diseases (see Wooster et al., 2005 and www.oie.int). It is commonly used to treat ectoparasites and fungi both in fresh and marine aquacultured fish. In the current study, formalin was indeed effective *in vitro* against all the stages of *S. chrysophrii*. However, different works have shown the constrains in the use of formalin to control monogenosis in live fish, partly due to the resistance of parasite stages, and also to side effects on hosts (Yildiz and Pulatsu, 1999). A 200 ppm 1 h-bath removed all *Microcotyle* sp. from the sparid *Pagrus pagrus* (Katharios et al., 2006), but it was ineffective against *M. hiatulae* (Thoney and Hagris, 1991) and *Entobdella hippoglossi* in *Hippoglossus hippoglossus* (Svendsen and Haug, 1991). A 1 h-bath with 250 ppm formalin reduced the occurrence of *Gyrodactylus* in Nile tilapia, but not that of *Dactylogyrus* sp., and a 1 h-bath of 50 ppm was not effective at all (Vargas et al., 2003). Similarly, a 1 h-bath with 400 ppm formalin was moderately effective for removing *Benedenia seriolae* and *Zeuxapta seriolae* from *Seriola lalandi* gills, but a large proportion of *B. seriolae* eggs and *Z. seriolae* adults exposed to the treatment remained viable or deposited viable eggs, respectively (Sharp et al., 2004). A 400 ppm 25 min-bath was necessary for the removal of adult and juvenile *Polylabroides multispinosus* from the sparid *Acanthopagrus australis* (Diggles et al., 1993). However, human health risks associated with formalin treatments in aquaculture have been pointed out (Wooster et al., 2005). Furthermore, some countries, such as Japan, have forbidden its use in off-shore cultures due to the harmful effect on the environment, and others have restricted its use to a maximum dose of 170 ppm when used at temperatures greater than 10 °C (FDA, USA).

Due to these security hazards and environmental issues, alternative treatments are urgently needed. Therefore, we monitored the possible efficacy of other compounds, such as hydrogen peroxide. It was completely effective against *S. chrysophrii* adults at the highest dose tested (200 ppm), which is within the recommended range (Rach et al., 1995). This strong oxidizing agent has been previously used against bacteria (Rach et al., 2003), amoeba (Powell and Clark, 2004), fungi (Marking et al., 1994), protozoans (Montgomery-Brock et al., 2001), crustaceans (Treasurer and Grant, 1997) and monogeneans (Rach et al., 2000; Ogawa, 2002; Mansell et al., 2005). However, some reports have pointed out the limited effect when mature parasites are embedded in the branchial cavity wall, and the side effects on fish when water temperature is high (Ogawa and Yokoyama, 1998; Burka et al., 1997).

The possible efficacy of the anthelmintic praziquantel (PZQ) was another alternative approach to treat *S. chrysophrii* infected fish. PZQ is a synthetic pyrazinoisoquinoline, which was chosen for its known successful use against digenean and cestode infections in mammals (reviewed by Martin et al., 1997). The drug induces a Ca^{2+} influx across the worm tegument, causing an immediate muscular contraction, rapid vacuolisation, disruption of the tegument, and impairment of sucker function (Redman et al., 1996). Although the target site of the drug is probably a calcium permeable membrane channel, the molecular mechanisms involved are still unknown (Köhler, 2001). In the present study, we investigated the efficacy of PZQ both in *in vivo* and *in vitro* experiments. The results showed that PZQ is not effective against *S. chrysophrii* adults and eggs *in vitro*, even at the highest tested dose (100 ppm–1 h). When orally administered with food, PZQ was most effective at the highest ingested dose (158 mg kg^{-1} BW), but it did not completely eliminate the infection in fish. PZQ preventive oral treatment did not prevent the infection either, but reduced parasite abundance significantly. The decrease in the infection levels observed in NT fish was due to the experimental conditions, which do not favour parasite transmission as much as farm conditions. In fish, PZQ has been used both in oral and bath treatments against monogeneans, including microcotylids, with

variable effectiveness depending on the parasite, stage, host, dose, etc. (Buchmann, 1987; Schmahl and Mehlhorn, 1985; Thoney, 1990; Silan et al., 1996; Kim and Choi, 1998; Kim et al., 1998, 2001b; Tojo and Santamarina, 1998; Kim and Cho, 2000; Janse and Borgsteede, 2003). Furthermore, some authors have pointed out the toxicity of PZQ (Onaka et al., 2003). In most studies, total removal of the parasites was not achieved, and thus the progressive re-infection of the hosts and the continuous horizontal infection in the facilities could not be avoided. A total efficacy of PZQ has only been reported for very long-duration baths (Chisholm and Whittington, 2002), and for high oral doses (Kim et al., 1998; Rubio-Godoy and Tinsley, 2004). When *Takifugu rubripes* was fed a diet containing praziquantel at 40 mg kg^{-1} BW, horizontal infection was prevented, but *Heterobothrium okamotoi* was not completely eliminated from infected fish (Hirazawa et al., 2000). These results are probably due to the fact that PZQ is rapidly eliminated from the fish, and the plasma levels decrease sharply between 24 and 48 h after oral treatment depending on the dose and host species (Kim et al., 2001a; Tubbs and Tingle, 2006). In our case, the efficacy of the highest intended dose (400 mg kg^{-1} BW), which is also the highest one tested thus far in fish, could not be evaluated due to palatability problems. Furthermore, when the intensity of infection was very high, treatment with food was very difficult due to fish anorexia, as food intake was naturally reduced. The low palatability is most probably due to the bitter taste of PZQ, when used adsorbed to the pellets. Thus, if higher doses of PZQ have to be used, the compound should be mixed with the commercial food previously to pelleting and certain additives should be included to mask the bitterness.

Chlorine and limoseptic were tested as disinfectants of water, surfaces and utensils. Chlorine treatments are often used to kill pathogens on rearing equipment and in wastewater of fish rearing and fish slaughtering facilities (Jacobsen et al., 1989; Liltved and Landfald, 1995). In the current work, chlorine was not completely effective against *S. chrysophrii*, as 12 ppm for 1 h killed oncomiracidia but not adults, and even a dose of 60 ppm for 1 h was not capable to kill adults or egg hatching. The effectiveness of chlorine against monogeneans in *in vitro* trials is variable. For *Pseudodactylogyrus* spp. a 3-h treatment of 24-60 mg L^{-1} was necessary for the complete eradication of oncomiracidia, and eggs were only partially killed by exposing them to 12 and 36 mg L^{-1} chlorine for 1 and 24 h (Umeda et al., 2006). For *H. okamotoi*, 2.4 ppm for 1 h killed the oncomiracidia, but a 24 h exposure to more than 60 ppm was needed to prevent egg hatching completely (Hirazawa et al., 2003). The hardness of the egg shell has led some authors to consider monogenean eggs more resistant to chlorine than fish viruses and bacteria (Hirazawa et al., 2003).

The commercial disinfectant limoseptic was effective against all the parasite stages only when prepared with DW and applied for 30 min. When prepared with SW, it did not prevent egg hatching even at the longest exposure time. These differences among the two preparations cannot be completely attributed to the effect of DW itself, since DW for 30 min did not prevent egg hatching. Nevertheless, DW could contribute to permeate limoseptic into the egg shell, preventing hatching. Limoseptic is used as virucide, bactericide and fungicide to disinfect surfaces in agropecuarian and hospital facilities. The efficacy of the working solution against fungi when used to dip utensils decreases with time, and with the presence of organic material (Plaza et al., 2006). Therefore, complete eradication of *S. chrysophrii* eggs attached to the surfaces, requires disinfection of aquaculture utensils for long times (>30 min), especially in the presence of biofouling. Bathing solutions should be renewed weekly.

In conclusion, in the absence of effective parasiticides to be delivered with food, the application of long disinfection times for utensils and containers with limoseptic or similar biodegradable compounds should be combined with removal of nets that contained the infected animals, and baths-treatments of infected fish. Considering the *in vitro* results, formalin and hydrogen peroxide could be adequate for such purposes, but more *in vivo* experiments are needed to determine the best doses and timing. If this 3-step procedure is not applied at the same time, re-infection can occur easily, as eggs can resist attached to not properly disinfected nets and surfaces.

Acknowledgements

Funding for this study was provided by the Spanish Ministerio de Ciencia y Tecnología (project no. AGL-2002-0475-C02-C01). The authors are grateful to M. Alonso and P. Cabrera for excellent assistance in experimental infections, and to Ramon Soriano (Andrés Pintaluba S.A.) for providing the medicated food.

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