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A *Saprolegnia parasitica* challenge system for rainbow trout: assessment of Pyceze as an anti-fungal agent for both fish and ova

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ABSTRACT: A reproducible *Saprolegnia parasitica* spore delivery system was developed and demonstrated to be effective in providing a sustained spore challenge for up to 10 d. Treatment of rainbow trout with slow-release intraperitoneal implants containing cortisol resulted in chronically elevated blood cortisol levels and rendered the fish susceptible to infection by *S. parasitica* when exposed to the spore challenge. Sham-implanted fish were not susceptible to infection. Bronopol (2-bromo-2-nitropropane-1,3-diol), formulated as Pyceze, was effective in protecting predisposed fish from infection by *S. parasitica* when administered as a daily bath/flush treatment at concentrations of 15 mg l^{-1} and greater. Pyceze was also demonstrated to protect fertilised rainbow trout ova from *S. parasitica* challenge when administered as a daily bath/flush treatment at concentrations of between 30 and 100 mg l^{-1}. Pyceze appears to qualify as a safe and effective replacement for malachite green and formalin in the prevention of fungal infections in the aquaculture environment.

KEY WORDS: *Saprolegnia* · Fungal infection · Salmonid · Bronopol · Pyceze · Cortisol

INTRODUCTION

Mycotic infections of farmed fish, primarily by water moulds or pseudofungi of the genus *Saprolegnia*, represent a significant economic and welfare problem. Sexually mature broodstock and fertilised ova of salmonid fish (*Oncorhynchus* sp., *Salmo* sp.) are particularly susceptible to *Saprolegnia*, as are other cultured species such as channel catfish *Ictalurus punctatus* (Bly et al. 1992). This state of affairs has been exacerbated by existing and anticipated restrictions on the use of the most effective fungicide available, malachite green. The n-methylated diaminotriphenylmethane dye malachite green has long been employed as a fungicide and ectoparasiticide (see Alderman 1985, van Heerden et al. 1995 for references). However, concerns regarding the mutagenicity, carcinogenicity, and teratogenicity of malachite green (Meyer & Jorgenson 1983, Culp & Beland 1996) have led to restrictions on its use (Alderman 1994, Marking et al. 1994).

There has therefore been significant effort expended to identify a therapeutant which is as effective as malachite green in combating mycotic infections of fish and fish eggs, but is safer for the operator, the fish, and the environment. Among the alternative compounds tested for antifungal activity are sodium chloride (Edgell et al. 1993, Marking et al. 1994, Waterstrat & Marking 1995, Bly et al. 1996, Schreier et al. 1996), potassium permanganate (Bly et al. 1996), copper sulphate (Bly et al. 1996), formalin (Marking et al. 1994, Waterstrat & Marking 1995, Bly et al. 1996, Schreir et al. 1996), the herbicides diquat, simazine, hydrothol 191, aquathol K (Bly et al. 1996), iodine (Fitzpatrick et al. 1995), glutaraldehyde (Marking et al. 1994, Fitzpatrick et al. 1995), and hydrogen peroxide (Marking et al. 1994, Fitzpatrick et al. 1995, Waterstrat & Marking 1995, Schreir et al. 1996).

Formalin is reported to be effective in treating fungal infections on eggs (Waterstrat & Marking 1995, Schreir et al. 1996) but raises safety concerns in relation to the user and to the environment which render it a far from ideal alternative to malachite green (Marking et al. 1994, Burka et al. 1997). Hydrogen peroxide has also been identified as possessing effective antifungal activity (Marking et al. 1994, Fitzpatrick et al. 1995, Waterstrat & Marking 1995, Schreir et al. 1996) but

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requires further toxicological and efficacy studies to validate its use at the concentrations found to be most effective. The margin of safety applicable to hydrogen peroxide is reportedly narrow (Burka et al. 1997). To date, no wholly effective alternative treatment to malachite green has been identified.

Bronopol (2-bromo-2-nitropropane-1,3-diol) is a biocide which is widely used as a preservative in medical and pharmaceutical products, cosmetics and shampoos (Bryce et al. 1978, Toler 1985, Kumanova et al. 1989). Following *in vitro* evaluation (D. J. Alderman unpubl.) and a series of studies carried out at various trout farms within the UK by Vericore Ltd. (J.C. Braidwood, C. Müller, J.L. Hart, R.P. Hunter, unpubl.) preliminary evidence was obtained which suggested that bronopol may be a suitable therapeutic/prophylactic treatment for mycotic infections of salmonid fish and fertilised ova. Bronopol is believed to have a dual toxic action in bacteria, with growth suppression being ascribed to the catalytic oxidation of accessible thiols while cell death is thought to be caused by the generation of free radicals (Shepherd et al. 1988). Because this compound is a broad-spectrum biocide, and presents no serious toxicological hazard to humans (Bryce et al. 1978, Croshaw & Holland 1984) or fish (Vericore Ltd. unpubl. data), the possible application of bronopol within the aquaculture environment requires thorough investigation. The experiments described in the present paper were designed to expand upon the previously conducted, unpublished studies, and to provide a preliminary assessment of the fungicidal efficacy of bronopol under controlled conditions.

Although salmonid fish are naturally susceptible to *Saprolegnia* infection at certain stages of their development and most UK waters present a continuous background fungal spore challenge, it was decided that for the purposes of these studies a reproducible disease model would provide the most appropriate approach to quantitatively estimating the efficacy of bronopol in combating *Saprolegnia* infection. The disease model adopted was composed of 2 elements: (1) a reproducible and controlled *Saprolegnia* spore challenge; (2) a susceptible fish host. The development and use of this model in assessing the efficacy of bronopol as a fungicide are described. We also describe a preliminary assessment of the use of bronopol in treating mycotic infection of salmonid ova.

METHODS

***Saprolegnia* strain employed and stock-culture maintenance regime.** *Saprolegnia parasitica* RAT 1 (RAinbow Trout 1) was isolated by Dr L. G. Willoughby (Freshwater Biological Association) from a diseased rainbow trout (*Oncorhynchus mykiss* Walbaum) obtained from the Institute of Freshwater Ecology (IFE) hatchery in January 1997. On purification the isolate was examined for diagnostic characters including the development of long fine hairs on the secondary spore cyst (Pickering et al. 1979). The occurrence of this diagnostic feature was taken as confirmation of its identification. Master stock cultures were maintained by routine serial subculture (monthly transfers to fresh medium) on glucose peptone (GP) agar slopes (Willoughby & Pickering 1977).

Secondary spore production and development and administration of the infective challenge. Secondary spore suspensions were produced by adapting the method of Willoughby et al. (1983) and Cross & Willoughby (1989). Dried hemp seeds were sterilised by autoclaving in a 250 ml flask to which an equal volume of distilled water was added. Sterilised seeds were infected with *Saprolegnia parasitica* by transferring seeds (50 per Petri-dish) into Petri-dishes containing GP agar. The dishes were then inoculated with *S. parasitica*, and incubated for 4 d at 20°C. After 4 d 'bearded' hemp seeds were aseptically transferred to coarse weave muslin bags (50 seeds per bag). The fungal spore challenge was administered to each tank in Expts 1 to 3 by anchoring the muslin bags within 500 ml glass flasks attached 'in-line', via Drechsel bottle heads (Quickfit), to the water supply to each aquarium. In Expt 4 the spore challenge was provided by 10 batches of hemp seeds infected with *S. parasitica* retained within muslin bags anchored within a 10 l plastic bucket which was suspended beneath the inflow of each tank. In Expt 5 incoming water passed through a glass beaker within which were anchored 3 muslin bags each containing fifty *S. parasitica*-infected hemp seeds. The source of the fungal challenge was thus protected from contact with the contents of the tank or trough. A preliminary study (data not shown) confirmed that this approach resulted in an elevation of spores judged adequate to initiate an infection in susceptible fish and in ova.

Quantifying spores produced by the infective challenge: sampling, and processing of samples to germinate secondary spores. The numbers of spores released into the experimental tanks were assayed using a method based on that of Willoughby et al. (1984). Water samples (500 ml) were collected in presterilized bottles directly from each tank. GP medium (5 ml) and antibiotic solution (1 ml; 7.5 mg ml^{-1} streptomycin sulphate and 7.5 mg ml^{-1} Penicillin G in deionized water; filter sterilized) were aseptically added to each sample. The sample bottles were then resealed and inverted once to ensure that the negatively geotactic spores were uniformly distributed within the sample. Aliquots (100 ml) of this material were asepti-

cally transferred to sterile glass 250 ml flasks, with the closure protected by a foam bung and covered by an aluminium foil cap. The flasks were then incubated overnight at 7°C.

Enumeration of secondary spores. Assay flasks were manually shaken to ensure uniform distribution of the germinated spores; replicate 1 ml samples from duplicate assay flasks were then transferred to a Sedgewick-Rafter cell (Fisher Scientific Ltd.) and examined/enumerated, under phase contrast microscopy (×50 magnification). A mean germinating spore density was obtained from a minimum of 4 separate counts on samples derived from each of the tanks in the study.

Fish. Rainbow trout (hatched February 1996, IFE Stirling strain) were maintained in four 1500 l outdoor circular glassfibre stock tanks (1.8 m diameter), each supplied with a constant flow of Windermere lake water (25 l min^{-1}) (Windermere Lake, Cumbria, UK). Each tank contained approximately 250 fish. Fish were fed 3 d per week with commercial feed (Trouw Aquaculture Standard Expanded) at the manufacturers recommended (daily) rate according to fish size and water temperature.

Administration of implants. Fish were transferred from the experimental aquaria to a bucket containing anaesthetic (2-phenoxyethanol; 1:2000) and given an intraperitoneal injection of molten coconut oil (0.5 ml) containing a suspension of steroid (cortisol and/or testosterone; Sigma; see below for details of dose).

Statistical analysis. Significant treatment-related differences in mean fungal infection scores in Expts 3, 4 and 5 were identified by analysis of variance (Genstat) with mean scores for fish within each tank providing replication within treatment groups. Significant differences between treatments were determined using the estimated standard error of the difference between means.

Expt 1. Comparison of cortisol and testosterone as immunosuppressants. This study was carried out to establish whether the susceptibility of rainbow trout to infection by *Saprolegnia parasitica* from an enhanced spore challenge could be increased by treatment with the steroid hormones cortisol and testosterone. It is well established that cortisol is an immunosuppressant in fish. Both under conditions of chronic stress when blood cortisol levels are elevated naturally, and following the administration of exogenous cortisol to otherwise unstressed fish, an increased susceptibility to disease, including mycotic infection, is observed (Pickering & Pottinger 1989). In addition, the frequency of mycotic infection in salmonids, particularly males, increases dramatically during the spawning period (Richards & Pickering 1978, Pickering & Christie 1980), and androgen-dependent changes in the integument have been suggested to contribute to this phenomenon (Cross & Willoughby 1989). The administration of a predisposing agent to the fish was considered to be less invasive and more reproducible than alternative approaches such as scarification or induction of a 'natural' stress response by exposure to a chronic stressor.

One week pre-study: 8 glass aquaria (50 l), each supplied with a constant flow of lake water (0.2 l min^{-1}, 11°C) were populated with juvenile rainbow trout, 8 fish per tank (weight 50 to 100 g). On Day 0 of the study 4 treatment groups, 2 tanks $group^{-1}$, each received either 20 mg of cortisol, 20 mg testosterone, 10 mg of each steroid together, or coconut oil only. A spore challenge was administered as described above and the number of spores within each tank was enumerated on Days 0 to 4 and 7 to 11. The number of fish displaying evidence of mycoses or other disease symptons was recorded daily for 11 d, at which point the study was terminated.

Expt 2. Determination of cortisol implant dose. This study was carried out to optimise the dose of cortisol administered to the fish. Experimental conditions were as for Expt 1 except that the implants administered contained either 5, 10 or 20 mg cortisol. Study duration was 9 d with a spore challenge present from Day 0. Spores were counted in samples collected from each tank on Days 0, 4, 7 and 9. Fish were inspected daily for evidence of mycotic infection or other disease symptoms.

Expt 3. Range-finding trial—determination of the effective concentration range of bronopol. This study was carried out to evaluate the effects of dose and duration of exposure to bronopol on *Saprolegnia parasitica* using challenged, predisposed, fish. Seven day pre-study: 20 glass aquaria (50 l) supplied with a constant flow of Windermere lake water (flow rate 3 l min^{-1}) were each populated with 5 rainbow trout (weight 134 ± 8.3 g; mean ± SEM, n = 30). On Day 0 fish were given cortisol-containing implants (20 mg $fish^{-1}$) and were marked individually using alcian blue dye administered via a Panjet needleless injector (Wright Dental Group). Fish were distinguished by placing marks adjacent to the left and right pectoral and pelvic fins, and the ventral fin. An additional group of 25 rainbow trout, maintained in a further 5 aquaria, were treated identically. These fish were anaesthetised in groups of 5 on Days 0, 1, 3, 6, and 10. A blood sample was removed from the caudal vessels of each fish with a heparinized syringe for the subsequent determination of blood cortisol levels. Blood was kept on ice until centrifuged; plasma was removed and frozen at –20°C until required for assay. Cortisol was determined according to Pickering & Pottinger (1987). The spore challenge was administered to each tank as described

for Expt 1 and spore numbers within each tank were enumerated on Days 3 and 10, immediately prior to the administration of the bronopol.

Bronopol (2-bromo-2-nitropropane-1,3-diol) was provided as a 50% solution in glycol (Pyceze; Vericore Ltd.). On Days 0 to 10 Pyceze was administered to the test tanks to provide the following nominal concentrations of bronopol, in quadruplicate; 0, 2, 5, 15, 30 mg l^{-1}. The water supply to each tank was switched off immediately prior to the administration of the bronopol. In 2 tanks of each treatment group, the water was reconnected 15 min after administration of the bronopol, in the remaining 2 tanks water was reconnected 60 min after dosing. Additional aeration was provided to each tank for the duration of the dosing.

pH was determined on Days 3 and 7 (Jenway model 3050 pH meter). Bronopol levels were determined on Days 8 and 9. Samples were collected from the 60 min exposure tanks at 5 and 55 min after the addition of bronopol and from the 15 min exposure tanks 5 min after the introduction of bronopol. A 500 ml water sample was removed from each test tank. A 0.25 ml aliquot of 85% orthophosphoric acid solution was added and the sample was thoroughly mixed. Approximately 100 ml of the stabilised sample were stored chilled before being assayed for bronopol content. Dissolved bronopol concentrations were determined by HPLC-UV according to method reference MBR/97/7 with a limit of detection of 7.47 mg l^{-1}. Analyses were conducted at the analytical laboratories of Vericore Ltd.

The degree of mycotic infection apparent on each fish was recorded daily (0: absent; 1: mild; 2: moderate; 3: severe; 4: dead). The scoring was carried out by an operator with no prior knowledge of the treatment administered to each tank. Severely infected fish were removed and humanely killed.

Expt 4. Confirmation of effective concentration of bronopol. This study was carried to evaluate under semi-field conditions the efficacy of the doses identified as effective in the previous study. One week prestudy: 30 trout (weight 246 ± 19 g; mean ± SEM, n = 10) were transferred to each of 8 experimental tanks (flow rate 10 l min^{-1}, volume 500 l). On Day 0 of the study each fish received a 30 mg cortisol implant. A larger implant was employed to maintain proportionality to fish size which was approximately 50% larger than the fish employed to establish the dose regime. Spore numbers were enumerated on Days 3 and 10, immediately prior to the administration of the bronopol, as described above.

On Days 0 to 14, pairs of tanks received 0, 10, 15, or 20 mg l^{-1} of bronopol. The water supply to each tank was turned off approximately 2 min prior to administration of the test substance and was reconnected 30 min after administration of bronopol.

Dissolved oxygen levels were measured daily (Oxy-Guard Handy Mk III portable DO meter) and pH was determined on Days 2 and 7. On Day 7, 2 water samples were removed from each tank for the determination of bronopol levels.

Because it was not possible to carry out assessment of levels of mycotic infection on individual fish within the tanks during the study, an assessment of the degree of infection of each fish was made on Day 15. During Days 0 to 14, any dead fish were removed from the tanks. On Day 15, the surviving fish were anaesthetized before being killed by a blow to the head. Each fish was inspected and scored according to the level of mycotic infection present as described above.

Expt 5. The effects of Pyceze on the growth of *Saprolegnia parasitica* on fertilised rainbow trout ova. This experiment was carried out to provide a preliminary assessment of the efficacy of bronopol in combating *S. parasitica* infection of fertilised rainbow trout eggs. Eggs were stripped from anaesthetized ovulated female rainbow trout and fertilised with milt from males of the same age and strain. The fertilised eggs were transferred to each of 2 mesh-bottomed egg trays (0.4 × 0.4 × 0.14 m) within each of 12 troughs (3.5 × 0.4 × 0.16 m) such that each trough contained a total of ~9000 eggs. Care was taken to ensure that each fertilised pool of eggs was equally represented within each trough. Each trough was supplied with a constant flow of lake water (5 l min^{-1}). Pyceze was administered to the troughs daily, for 21 d. The water supply to each trough was switched off and Pyceze was mixed with the water within the trough to provide final nominal concentrations of 5, 20, 30, 50 and 100 mg l^{-1} of bronopol. Each dose was administered to duplicate troughs which, together with 2 control troughs receiving no bronopol, provided a total of 6 treatments. After 30 min, the water supply was reconnected. On a daily basis, the eggs in each tray were inspected for evidence of mycotic infection by an operator with no prior knowledge of the treatment administered to each tank and scored as 0: no obvious infection; 1: 1 to 5 eggs infected; 2: >10 eggs <10% coverage; 2.5: >10% <50% coverage; 3: >50% <75% coverage; 3.5: >75% <100% coverage; 4: 100% coverage. During the course of the study no dead or infertile eggs were removed and the trays were not physically disturbed. The dissolved oxygen concentration and water temperature were recorded daily in each trough and pH was recorded from each trough on Days 7 and 18. On Days 3 and 15, the numbers of spores within each trough were enumerated as described above. On Day 12, water samples were removed from each trough and treated as described above for the determination of bronopol concentrations.

Expt 6. The effects of Pyceze on the growth of *Saprolegnia parasitica in vitro*. As a consequence of the observations made during the studies described above, i.e. that bronopol killed spores at concentrations below the maximum nominal (during washout of the dose), this experiment was carried out to establish the concentrations of bronopol which inhibit spore production, spore germination, and vegetative growth of *S. parasitica*. The strain of *S. parasitica* employed was the same as that used for the fish challenge studies. Forty-two single hemp seeds, colonised with *S. parasitica* as detailed above, were transferred inividually to flasks containing 100 ml of sterile lake water. After 48 h incubation at 7°C Pyceze was administered to the flasks, in replicate groups of 6 flasks, to give the concentration range 2, 5, 10, 15, 20, 30 mg l^{-1} of bronopol together with 6 control flasks which received no bronopol. After 30 min incubation at 7°C a 1.0 ml aliquot of medium was removed from 3 flasks at each concentration and transferred to the enumeration system to determine the number of spores present. The hemp seeds from these flasks were washed in sterile lake water and transferred to 100 ml of sterile lake water in clean flasks to assess the capacity of the colonies for continued spore production following bronopol treatment. Hemp seeds from the remaining 3 flasks from each bronopol treatment group were washed in sterile lake water and transferred to sterile Petri-dishes containing GP agar to assess the capacity of the colonies for hyphal/mycelial growth following bronopol treatment.

RESULTS

Expt 1. Comparison of cortisol and testosterone as immunosuppressants

The mean spore levels (l^{-1}) for each of the treatment groups, and the cumulative incidence of infection on the experimental fish within each experimental group are shown in Fig. 1. Spore concentrations were low in all tanks until between Days 2 and 3 when marked increases were observed rising from background levels of 100 to 250 spores l^{-1} on Day 0 to 4000–6000 spores l^{-1} on Days 3 and 4. In the tanks in which no infected fish were observed (control, testosterone-treated) spore numbers declined consistently until the termination of the study (Fig. 1a,c). However, in the tanks in which infected fish were observed (Fig. 1b,d), high spore numbers were sustained for longer. No evidence of disease was apparent in any group until Day 7 of the study, at which point fungal lesions were observed on fish within the cortisol-treated (Fig. 1b) and cortisol+testosterone-treated groups (Fig. 1d). After 11 d exposure to the challenge 44% of cortisol-treated and 38% of cortisol+testosterone-treated fish had either died or were showing significant levels of mycotic infection. Inspection of the fish revealed no evidence of bacterial lesions either externally or internally. Microscopic examination of material isolated from the infected fish confirmed that *Saprolegnia parasitica* was the cause of infection. The fish were not screened for ectoparasites but no gross symptoms were evident either on the body surface or gills. On the basis of these data it was decided that cortisol implantation was an appropriate method with which to predispose the experimental fish to infection with *S. parasitica*.

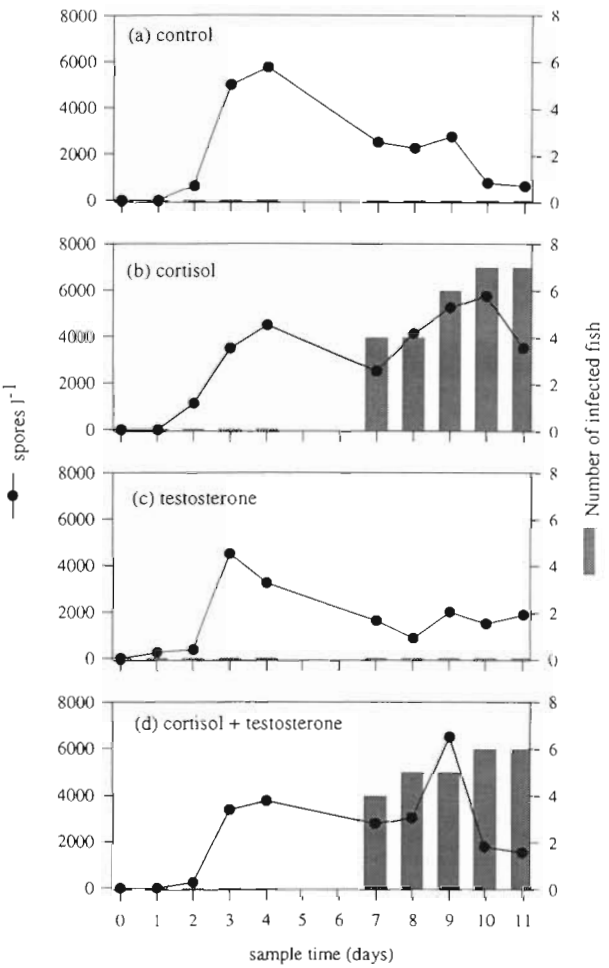

Fig. 1. Expt 1. *Saprolegnia parasitica* infecting rainbow trout. Mean concentration of *S. parasitica* spores and total number of fish displaying fungal infection in pairs of duplicate tanks containing fish implanted with (a) 0.5 ml of coconut oil alone, or 0.5 ml coconut oil containing 20 mg (b) cortisol, (c) testosterone, (d) cortisol+testosterone and exposed to a continuous spore challenge. Each spore estimate represents the mean of 2 determinations in each replicate tank. Numbers of fish within each treatment group which displayed any evidence of fungal infection are denoted by the solid bars

Expt 2. Determination of cortisol implant dose

The mean spore densities (l^{-1}) for each of the treatment groups, and the cumulative incidence of infection on the experimental fish within each experimental group are shown in Fig. 2. The mean spore levels achieved in each tank after introduction of the in-line inoculum and the rate of increase were similar to those observed during Expt 1 (~2000 to 4000 spores l^{-1} within 4 d). However, numbers of spores dramatically increased to a maximum of >20 000 l^{-1} in the tanks containing fish which had received 20 mg cortisol implants and in which the highest levels of mycotic infection were observed (Fig. 2d). The first evidence of mycotic infection was visible by Days 5 to 6 (Fig. 2), similar to the lag between the onset of the challenge and infection which was observed in Expt 1. There was a clear dose-dependent relationship between the amount of cortisol administered to the fish and the level of infection which was subsequently observed following spore challenge. In the groups receiving 5, 10, and 20 mg cortisol 31, 63 and 88% of the fish were infected, respectively. No infection was observed in either of the control, vehicle-implanted groups. As was the case for Expt 1, no evidence of bacterial infection or ectoparasitic problems were observed in any of the experimental groups.

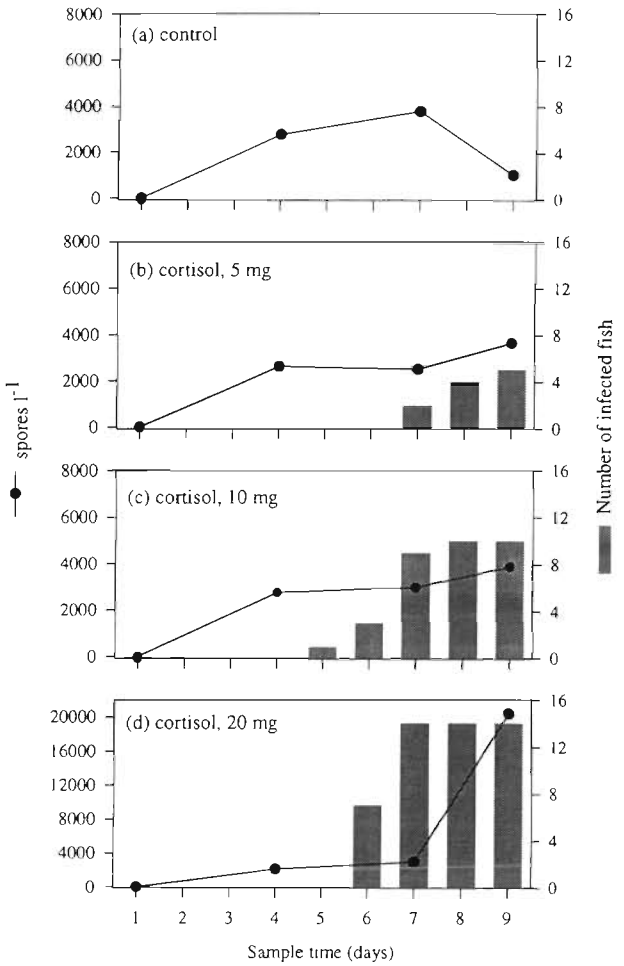

Fig. 2. Expt 2. *Saprolegnia parasitica* infecting rainbow trout. Mean concentration of *S. parasitica* spores and total number of fish displaying fungal infection in pairs of duplicate tanks containing fish implanted with (a) 0.5 ml of coconut oil alone, or 0.5 ml coconut oil containing (b) 5 mg, (c) 10 mg, (d) 20 mg cortisol and exposed to a continuous spore challenge. Each spore estimate represents the mean of 2 determinations in each replicate tank. Numbers of fish within each treatment group which displayed any evidence of fungal infection are denoted by the solid bars

Expt 3. Range-finding trial—determination of the effective concentration range of bronopol

Blood cortisol levels for the group of cortisol-implanted fish which were sampled at intervals during this experiment are presented in Fig. 3. Maximum cortisol levels were observed within 24 h of implantation, rising from a pre-implantation value of 0.4 ± 0.1 ng ml^{-1} (n = 5) to 569 ± 35 ng ml^{-1} within 24 h and thereafter declining steadily to a level still markedly elevated above that in unimplanted fish after 10 d (137 ± 8 ng ml^{-1}; Fig. 3). The concentration of spores detected within 3 d of the introduction of the challenge was ~3000 l^{-1}, similar to that observed after this period in Expts 1 and 2, and numbers were consistent between treatments, and between exposure times (Fig. 4a,b). However, after 10 d, greater variability in the concentration of spores present was observed between treatment groups; high spore counts were present in the

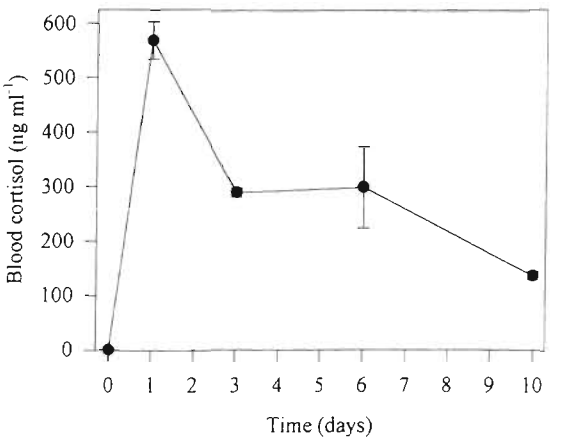

Fig. 3. Expt 3. *Oncorhynchus mykiss*. Blood cortisol levels immediately prior to implantation and at intervals following implantation in rainbow trout which received 0.5 ml coconut oil implants containing 20 mg cortisol. Each point is the mean ± SEM, n = 5

control and low bronopol dose tanks (0, 2, 5 mg l^{-1}) while in the high bronopol dose tanks (15, 30 mg l^{-1}) numbers of spores had declined to <1000 l^{-1}.

The concentrations of bronopol detected by direct assay of samples collected from each tank are presented in Table 1. Overall, there is good agreement between nominal and actual concentrations although the assay sensitivity precluded accurate measurement of bronopol concentrations in the 2 lowest treatments. Water temperature during the course of the study increased gradually from 14.5 to 16.3°C by Day 10 and pH was 6.4.

No evidence of mycoses was observed in any treatment until Day 4, at which point low levels of infection (scored 'present') were observed in the tanks receiving 0, 2, and 5 mg l^{-1} of bronopol for both 15 and 60 min (Figs. 5 & 6). Levels of infection increased steadily in these tanks for the remainder of the study. No infection was observed throughout the course of the study in tanks receiving bronopol at 15 and 30 mg l^{-1} for 60 min

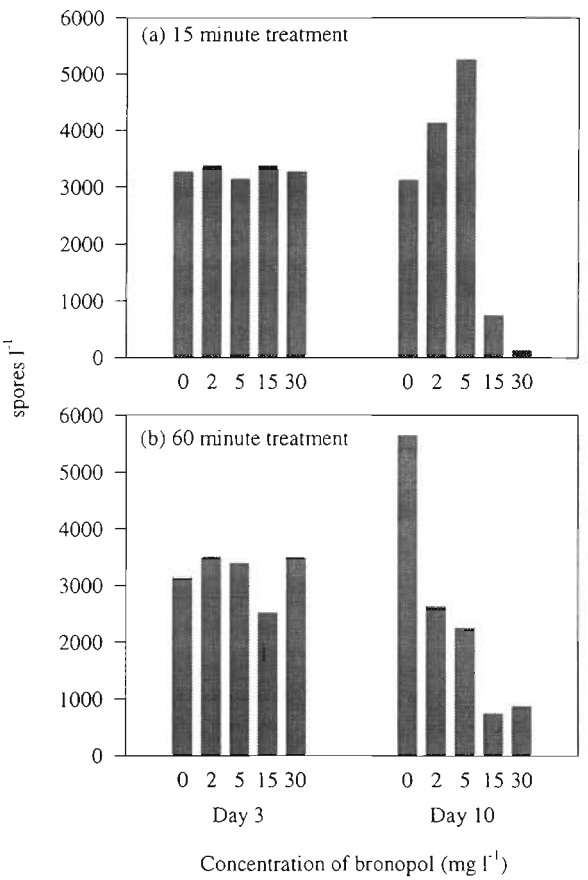

Fig. 4. Expt 3. *Saprolegnia parasitica*. Mean concentration of spores on Day 3 and Day 10 in duplicate tanks receiving various doses of bronopol for either (a) 15 min or (b) 60 min combined with a continuous spore challenge. Each spore estimate represents the mean of 2 determinations in each replicate tank

Table 1. Expt 3. Concentrations of bronopol in the test aquaria (50 l) at intervals after administration. conc. = concentration, ND = not detectable (limit of detection = 7.5 mg l^{-1})

Duration of treatment (min)	Time of sample removal after start of treatment (min)	Nominal conc. of bronopol (mg l^{-1})	Actual conc. of bronopol (mg l^{-1})
60	+5	0	ND
60	+5	2	ND
60	+5	5	ND
60	+5	15	15.7, 15.2
60	+5	30	28.9, 30.4
60	+55	0	ND
60	+55	2	ND
60	+55	5	ND
60	+55	15	15.6, 14.6
60	+55	30	28.9, 29.9
15	+5	0	ND
15	+5	2	ND
15	+5	5	ND
15	+5	15	13.5, 14.6
15	+5	30	28.7, 29.0

(Fig. 6) and only low levels of infection were observed in tanks receiving bronopol at these doses for 15 min (Fig. 5). *Saprolegnia parasitica* was confirmed to be present on infected fish by culturing and examining

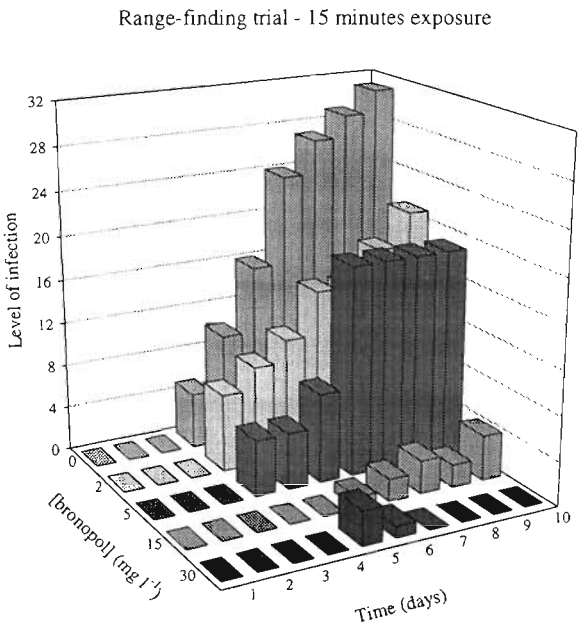

Fig. 5. Expt 3. *Saprolegnia parasitica* infecting rainbow trout. Level of fungal infection (summed for all fish; scored as 0: absent; 1: mild; 2: moderate; 3: severe; 4: fish dead) in groups of rainbow trout (5 fish per tank, 10 fish per treatment) exposed to a continuous *S. parasitica* spore challenge and treated daily with bronopol at concentrations of 0, 2, 5, 15 or 30 mg l^{-1} for 15 min

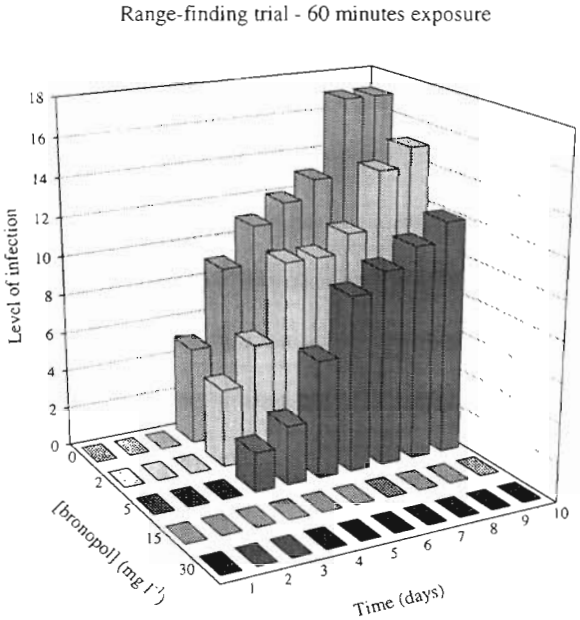

Fig. 6. Expt 3. *Saprolegnia parasitica* infecting rainbow trout. Level of fungal infection (summed for all fish; scored as 0: absent; 1: mild; 2: moderate; 3: severe; 4: fish dead) in groups of rainbow trout (5 fish per tank, 10 fish per treatment) exposed to a continuous *S. parasitica* spore challenge and treated daily with bronopol at concentrations of 0, 2, 5, 15 or 30 mg l^{-1} for 60 min

isolates removed from infected fish during the study. ANOVA revealed that the levels of infection in fish receiving 2 mg l^{-1} bronopol for 15 min were significantly lower than those of the untreated fish ($p < 0.001$) but not different to those of fish receiving 5 mg l^{-1} bronopol. The infection scores for fish receiving 15 and 30 mg l^{-1} bronopol for 15 min were both significantly lower than for groups receiving the lower doses ($p < 0.001$). At 60 min exposure, there was no significant difference between the level of infection in the untreated groups and the lowest dose of bronopol. However, the infection levels in the groups receiving 5, 15 and 30 mg l^{-1} bronopol were all significantly lower than those in the controls ($p < 0.001$).

Expt 4. Confirmation of effective concentration of bronopol

Water temperature during this experiment varied between 7.1 and 9.1°C. pH was consistently within the range 6.3 to 6.7. Dissolved oxygen levels were within the range 6.5 to 10.4 mg l^{-1}. The concentrations of bronopol detected by direct assay of water samples collected during this study are presented in Table 2. Overall, the actual concentrations achieved were below the intended nominal figures of 10, 15 and 20 mg l^{-1}. At 5 min after dosing the mean levels in each treatment were 8.2, 9.4 and 14.4 mg l^{-1} and after 25 min the figures were 8.3, 10.8 and 16.4 mg l^{-1}; 70 to 80% of the nominal concentrations.

There was a clear dose-dependent effect of bronopol on the incidence of mycotic infection on the fish within the treatment tanks. In the control tanks which received no bronopol, almost all fish were heavily infected with *Saprolegnia parasitica* (total scores of 99.5 and 89.5 compared a theoretical maximum of 120). The presence of *S. parasitica* was confirmed by examining cultures of material isolated from infected fish. Mortality in these groups was high with almost 62% of the fish dying as a consequence of mycotic infection (Fig. 7a). No evidence of internal or external bacterial lesions was observed in any fish during the study. Nor was there any evidence of excessive ectoparasite infestation. The cause of death was therefore assumed to be the *Saprolegnia* infection. In contrast, no mortality was observed among the fish receiving bronopol treatment at 15 and 20 mg l^{-1} (Fig. 7a) and only a limited amount of mycotic infection was observed on 5 fish from a total of 60 in the tanks receiving bronopol at 15 mg l^{-1} at the end of the study (Fig. 7c). Only 1 fish from the 60 fish receiving bronopol at 20 mg l^{-1} displayed evidence of *Saprolegnia* infection at the completion of the study. The fungal scoring system employed in the study, which weights the extent of mycelial coverage and occurrence of mortality, provides a clear picture of the efficiency of bronopol treatment at 15 and 20 mg l^{-1} with scores of 1 and 7, respectively, compared to scores of 99.5 and 89.5 for the control tanks (Fig. 7c). Overall, the mean fungal scores for fish in the control tanks were significantly greater than for all 3 bronopol treated tanks ($p < 0.001$) and the scores for fish in the group receiving 10 mg l^{-1} bronopol were significantly greater than those in the 2 highest dose groups ($p < 0.05$). There was no significant difference between fungal scores in the 2 groups receiving the highest bronopol doses.

Table 2. Expt 4. Concentrations of bronopol in the test tanks (500 l) at intervals after dosing on Day 7 of the study. Two measurements were made from each of 2 replicate tanks at 5 and 25 min after the addition of bronopol. conc. = concentration, ND = not detectable (limit of detection = 7.5 mg l^{-1})

Nominal conc. of bronopol (mg l^{-1})	Actual conc. of bronopol (mg l^{-1}) at +5 min	Actual conc. of bronopol (mg l^{-1}) at +25 min
0	ND	ND
10	8.3, 8.8; 7.8, 7.8	8.5, 8.6; 8.1, 8.0
15	7.8, 9.9; 10.0, 10.0	10.5, 10.6; 10.2, 11.9
20	14.6, 15.9; 15.8, 11.1	13.8, 16.7; 17.3, 17.6

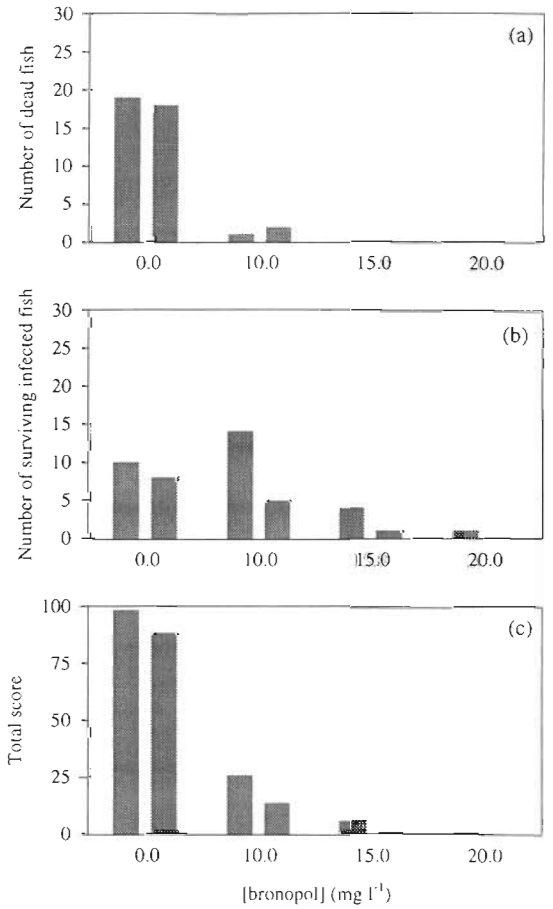

Fig. 7. Expt 4. *Saprolegnia parasitica* infecting rainbow trout. (a) Total mortality at the end of the experimental period; (b) total number of infected fish surviving at the end of the experimental period and (c) the total fungal infection score summed for all fish in each tank at the end of the experimental period (scored as 0: absent; 1: mild; 2: moderate; 3: severe; 4: fish dead) among rainbow trout exposed to a constant *S. parasitica* spore challenge and treated daily for 14 d with bronopol at concentrations of 0, 10, 15 or 20 mg l^{-1}. Each bar represents the result for 1 of 2 duplicate tanks per treatment (30 fish per tank)

Expt 5. The effects of Pyceze on the growth of *Saprolegnia parasitica* on fertilised rainbow trout ova

The spore challenge system was successful in sustaining a substantial concentration of spores in the troughs during the course of the experiment; mean spore numbers on Days 3 and 15 were 1393 ± 119 spores l^{-1} and 500 ± 98 spores l^{-1}, respectively (mean ± SEM, n = 12). Water temperature was 5.3°C at the start of the experiment and rose gradually to 6.9°C on Day 21. Dissolved oxygen levels were within the range 11 to 12 mg l^{-1} and pH was within the range 6.7 to 7.7. Bronopol concentrations measured in the troughs following dosing on Day 12 are presented in Table 3.

Evidence of mycotic infection was first noted on Day 7 of the study on eggs receiving 20 mg l^{-1} of bronopol daily and within 2 days was apparent in the 0 and 2 mg l^{-1} groups (Fig. 8). The initial infections were associated with dead eggs, those which appeared opaque and white in colour rather than the translucent yellow/orange of live eggs. We did not observe primary infection of live eggs although the mycelial mat which was initially associated with dead eggs spread with time to engulf live eggs also. By the end of the study period the control trays receiving no bronopol displayed up to 75% coverage with mycelium. In contrast, in trays receiving 100 mg l^{-1} bronopol the infection was restricted to fewer than 10 individual eggs or 2 or 3 small clumps of eggs. Low levels of infection were also observed in trays receiving 30 mg l^{-1} bronopol although in trays receiving 50 mg l^{-1} infection coverage was actually slightly greater. A variety of fungi/pseudofungi including *Leptomitus* sp. and *Saprolegnia* sp. were identified to genus level in samples taken from troughs containing infected eggs; however, *S. parasitica* was the dominant organism recovered. Analysis of the data set by ANOVA revealed that the level of infection was significantly reduced, relative to the controls, by all treatments ($p < 0.001$) except 5 mg l^{-1} which was statistically indistinguishable from the 0 mg l^{-1} group.

Expt 6. The effects of Pyceze on the growth of *Saprolegnia parasitica in vitro*

Assay of spore germination, following treatment with Pyceze, indicated that the response of *Saprolegnia parasitica* to bronopol was dose dependant (Table 4). At relatively low levels of bronopol (2 to 10 mg l^{-1}) only minor reductions in spore levels were observed, whereas higher doses (15 and 20 mg l^{-1}) led to >50% reduction in spore level and the highest dose tested (30 mg l^{-1}) resulted in ~80% reduction in spore level

Table 3. Expt 5. Concentrations of bronopol in egg-rearing troughs after dosing on Day 12 of the study. The means of single measurements made from each of 2 replicate troughs at 5 and 25 min after the addition of bronopol are presented. ND = not detectable (limit of detection = 7.5 mg l^{-1})

Nominal concentration of bronopol (mg l^{-1})	Actual concentration of bronopol (mg l^{-1})
0	ND
5	18.2
20	39.7
30	46.2
50	59.1
100	116.7

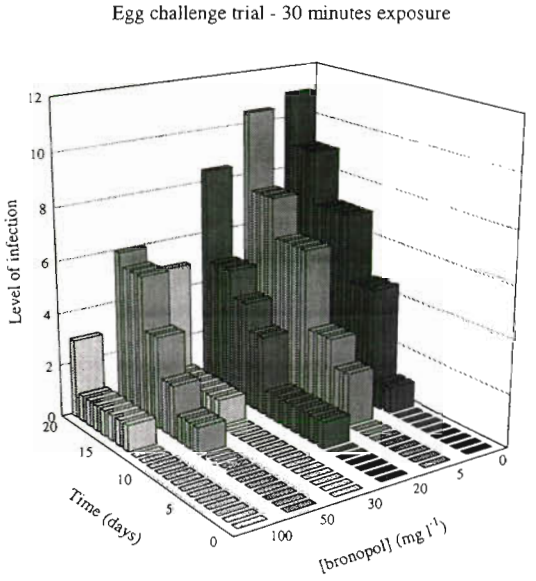

Fig. 8. Expt 5. *Saprolegnia parasitica* infecting rainbow trout. Fungal infection score in duplicate egg-rearing troughs, each containing 2 trays with a total of ~9000 eggs per trough, treated daily for 21 d with bronopol at concentrations of 0, 5, 20, 30, 50, 100 mg l^{-1}. Each bar represents the combined score for the duplicate trays within 2 replicate troughs

(Table 4). Furthermore, 1 flask treated with 30 mg l^{-1} bronopol, failed to yield any spores capable of germinating. At all dose levels employed, the transfer of ~1% of the bronopol present, leading to an initial concentration in the assay flasks of 0 to 0.3 mg l^{-1}, had no influence on the ability of the spores present to germinate (Table 4).

After washing bronopol-exposed hemp seeds in sterile water, and transferring to sterile solid medium, *Saprolegnia parasitica* was recovered from all of the treated seeds (Table 5). No obvious difference between the growth of control material and that treated

Table 4. Expt 6. *Saprolegnia parasitica* spore germination following bronopol treatment. Each value is the mean ± SEM of 2 determinations on each of 3 replicates (n = 6)

Bronopol conc. (mg l^{-1})	No. of spores ($\times 10^{-3}$ l^{-1}) Post-bronopol treatment	Residual bronopol assay[a]
0	10.3 ± 1.0	13.6 ± 1.0
2	8.0 ± 1.3	13.2 ± 0.2
5	6.8 ± 3.5	15.8 ± 1.3
10	8.2 ± 0.6	13.6 ± 0.6
15	4.3 ± 1.0	13.6 ± 0.6
20	4.5 ± 1.7	13.0 ± 0.9
30	2.0 ± 2.0	13.5 ± 1.0

[a]Transfer of 1 ml of bronopol-treated water to the spore induction system to assess whether residual bronopol transferred from the treatment flasks would affect the assay

Table 5. Expt 6. *Saprolegnia parasitica* hyphal growth and spore production following bronopol treatment. Each value is the mean ± SEM of 2 determinations on each of 3 replicates (n = 6)

Concentration of bronopol (mg l^{-1})	Hyphal growth (mm) 24 h	72 h	Spore production (no. $\times 10^{-3}$ l^{-1})
0	3	13.5	10.2 ± 0.3
2	3	13.5	8.8 ± 0.3
5	3	13.0	9.5 ± 1.5
10	3	13.5	4.8 ± 2.3
15	3	12.5	5.5 ± 0.5
20	1.5	9.0	3.7 ± 1.6
30	1.5	10.0	1.8 ± 0.3

with bronopol up to 15 mg l^{-1} was noted (Table 5). However, at higher dose levels the growth rate of *S. parasitica* was reduced, suggesting that the fungal hyphae at the periphery of the hemp seed were killed by the initial treatment (Table 5). Furthermore, treatments employing bronopol at 10 mg l^{-1} and above had a significant influence on the production of spores (Table 5). The reduction in the capacity of the colony to produce spores was apparently dose dependent, with recoverable spore numbers comparable to those observed in the initial assay (Table 4).

DISCUSSION

The disease model

The challenge system employed in these studies was effective in elevating the spore concentration markedly above background levels (~4000 spores l^{-1} cf. ~150 spores l^{-1}) and in providing a sustained, though declining, challenge for a period of 10 to 14 d. However, we were unable to infect rainbow trout by exposure to the spore challenge without first predisposing the fish to infection by elevating blood cortisol levels using slow-release cortisol implants; sham-implanted control fish did not exhibit evidence of colonisation by *Saprolegnia parasitica*. Carballo et al. (1995), although employing more invasive techniques than in the present study, also reported that elevated blood cortisol levels were a major factor in successfully inoculating rainbow trout with *S. parasitica*, and it is well-established that cortisol-implanted salmonids display an increased susceptibility to infection by the 'natural' *Saprolegnia* spore challenge (Pickering & Duston 1983, Pickering & Pottinger 1989). Rainbow trout clearly have a very effective protective mechanism preventing the successful attachment and germination of *S. parasitica* spores which is compromised by cortisol administration, allowing *S. parasitica* to act as a primary

pathogen. This strongly supports earlier assertions that outbreaks of mycotic infection in various species under aquacultural conditions or in the natural environment must normally arise as a consequence of a predisposing/immunosuppressive factor(s) (Bly et al. 1994, Pickering 1994). In the current studies we were surprised to observe no secondary disease problems other than the deliberately induced *S. parasitica* infections, despite the likely presence of opportunist fish pathogens in the lake water within which the studies were executed. Bly et al. (1992) also reported an absence of bacterial pathogens in catfish suffering from 'winter kill' Saprolegniasis. These authors considered that low water temperatures and the possible production of antimicrobial substances by *Saprolegnia* may have accounted for the absence of other disease symptoms. It is unlikely that temperature was a factor in preventing bacterial infections in the present studies, particularly during Expt 3 in which water temperatures reached 16°C.

The elevation of blood androgen levels by the administration of implants containing the steroid testosterone was ineffective in enhancing the susceptibility of trout to *Saprolegnia parasitica* infection. This is somewhat surprising when the following factors are considered. Sexually mature male and female salmonids show an enhanced vulnerability to mycotic infection (Richards & Pickering 1978, Pickering & Christie 1980); testosterone levels are elevated in rainbow trout in both sexes during the spawning period (Pottinger et al. 1996); the androgen 11-ketotestosterone was reported to enhance susceptibility of rainbow trout to *S. parasitica* challenge (Cross & Willoughby 1989); and testosterone displays apparent suppressive effects on the immune system of salmonid fish (Slater et al. 1995). However, it is possible that the enhanced susceptibility to *Saprolegnia* infection which is associated with cortisol administration occurs via a mechanism other than suppression of the immune system, or via an immunosuppressive route which is not sensitive to androgen regulation. It is also possible that the teleost androgen 11-ketotestosterone, rather than testosterone, would have been more potent in enhancing susceptibility.

The permissive link between stress and mycotic infections in mammals is well established but as yet the mechanisms underlying the relationship are not fully understood (Mishra et al. 1994). In fish also, the defence mechanisms which afford protection from mycotic infection are not well understood and one can only speculate by what mechanism cortisol renders fish susceptible to *Saprolegnia parasitica* infection. The 2 defensive strategies suggested to be employed by fish to overcome the challenge presented by pathogenic fungi are (1) the removal of the spore from the surface of the fish before it establishes infection and (2) inhibition of the growth of the germinating spore within the mucus layer (Pickering & Willoughby 1982a). It has been shown that the rate of loss of viable spores from the surface of brown trout *Salmo trutta* L. is rapid, with 95% of viable spores being removed within 2 h of exposure (Pickering & Willoughby 1982b), suggesting that physical removal is an important protective element. Mucus secreted by epidermal cells of brown trout has been reported to provide a nutritive growth medium *in vitro* for *Saprolegnia* spores (Willoughby et al. 1983). However, subsequent work suggested that if spores come into contact with mucus while on the surface of the fish, there is an inhibitory effect on growth of the fungus (Wood et al. 1986, 1988). A number of compounds with potentially fungicidal activity have been reported to occur in fish mucus including trypsin, lysozyme, immunoglobulins, and agglutinins (Ourth 1980, Dalmo et al. 1997), some of which might account for inhibitory effects on fungal growth. It is not known whether cortisol has any effects on the dynamics of mucus production by epidermal goblet cells, but given the broad range of effects of cortisol on elements of the specific and non-specific immune system (Barton & Iwama 1991, Wendelaar Bonga 1997), there is clearly scope for corticosteroid interference with the integumental defence system.

Evaluation of Pyceze

Pyceze was found to be effective in reducing or preventing mycotic infection in challenged fish. Administered daily, at concentrations of 15 and 30 mg bronopol l^{-1}, a 60 min bath was completely effective in protecting immunosuppressed rainbow trout from a continuous *Saprolegnia parasitica* spore challenge (Expt 3). In contrast, untreated fish showed heavy and persistent *Saprolegnia* infections. When an exposure duration of 15 min was employed, the incidence of infection remained low in the groups receiving bronopol at 15 and 30 mg l^{-1}. When the experiment was repeated in outdoor full-size tanks (Expt 4), bronopol remained effective, at concentrations of 15 and 20 mg l^{-1}, in protecting the immunosuppressed, challenged, fish from *S. parasitica* infection.

It must be noted that the exposure times reported are an underestimate of the actual residence time of bronopol within the experimental tanks because they do not take into account the period required for washout of the residual bronopol from the tanks. In Expt 3 the additional exposure time was significant; within 4 h of the resumption of water flow the concentration of bronopol would have declined to 11 mg l^{-1} from a starting point of 30 mg l^{-1} with a flow rate of 0.2 l min^{-1} and total volume of 50 l. However, in Expt 4, with

a flow rate of 10 l min^{-1} and a total volume of 500 l, the concentration of bronopol would have declined from a start point of 20 mg l^{-1} to <1 mg l^{-1} within 2.5 h and within 1 h would have declined to 5 mg l^{-1}, a concentration with little evident effect on the growth of *Saprolegnia parasitica*. Nonetheless, viable spores were detected in all treatments within 2 to 4 h of reestablishing the water supply. The agreement between the estimated most-effective doses in the low-flow small aquarium study (Figs. 5 & 6) and the higher flow large tank studies (Fig. 7) suggests that the effectiveness of bronopol is not primarily a function of prolonged exposure times but that absolute concentration is critical.

The effects of bronopol *in vitro* on spore germination, hyphal growth, and spore production were in broad agreement with the results of the fish exposure studies. Germination of spores was reduced by up to 80% by concentrations of bronopol shown to protect fish from infection (15 to 30 mg l^{-1}) but hyphal growth was less affected by exposure to bronopol. Continued growth was observed even following treatment with bronopol at a concentration of 30 mg l^{-1}. Spore production was greatly curtailed at higher concentrations of bronopol also. These results suggest that the role of bronopol in protecting fish from infection may be to prevent the germination of attached spores for sufficiently long to allow their removal by turnover of the mucus layer. The lack of effect on hyphal growth suggests that bronopol will be less effective against an established infection although the marked inhibitory effects of bronopol on spore release suggest that fish with an existing fungal burden would pose less of an infective risk to other fish in the immediate vicinity.

Pyceze was effective in protecting rainbow trout eggs from infection when administered as a single daily 30 min bath/flush treatment at a concentration of 100 mg bronopol l^{-1}. There was strong evidence from the preliminary experiment that concentrations of 30 and 50 mg l^{-1} may also offer protection. As is the case for the fish experiments the actual time in contact with bronopol exceeded the duration of the bath period and in the case of the 100 mg l^{-1} treatment, 2.5 h would have been required to reduce the residual concentration of bronopol to <1 mg l^{-1} after reconnecting the water supply. During the course of this experiment we noted that live eggs were not colonised by *Saprolegnia parasitica*; initial infections were always associated with dead eggs. After nodes of infection had become established on dead eggs, the expanding mycelial growth engulfed live eggs, ultimately leading to egg mortalities. This is consistent with the observations of Kitancharoen & Hatai (1996), who reported that live rainbow trout eggs were not susceptible to infection when challenged with any of 4 different isolates of *Saprolegnia* spp., and Smith et al. (1985), who observed the colonization of live eggs by hyphal contact only, not by spore infection. It has been suggested that nutrient loss from dead eggs is critical in attracting and/or sustaining initial spore contact (Smith et al. 1985) and positive chemotaxis by *Saprolegnia* zoospores has been demonstrated towards concentration gradients of extracts of live salmonid eggs (Rand & Munden 1993). The fertilization envelope of viable rainbow trout eggs has been demonstrated to possess antifungal activity (Kudo & Teshima 1991, Kudo 1992), which is presumably a primary factor in preventing the infection of live eggs by *Saprolegnia* spores. The presence of lysozyme in the yolk of coho salmon *Oncorhynchus kisutch* Walbaum eggs may also be of significance in this context (Yousif et al. 1994).

In conclusion, the data reported here provide evidence that bronopol (Pyceze) is an effective therapeutic agent for the preventive treatment of mycotic infections in the aquaculture environment. This compound is a safer alternative to malachite green and formalin, currently considered to be the most effective aquacultural fungicides.

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