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# Fungi isolated from cultured eggs, alevins and broodfish of brown trout in a hatchery affected by saprolegniosis

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The aquatic fungi cultured from eggs, alevins and broodfish of brown trout *Salmo trutta* belonged to the genus *Saprolegnia* and were identified as *S. diclina*, *S. australis*, *S. ferax*, *S. furcata*, *S. hypogyna*, *S. unispora* and *S. parasitica*. The species obtained from infected eggs and alevins were different to those from infected fish. Several *Saprolegnia* species were isolated from eggs and alevins, whereas all the isolates obtained from broodfish were the pathogenic *S. parasitica*.



Key words: aquaculture; diseases; salmonids; *Saprolegnia*; *S. diclina*; *S. parasitica*.



## INTRODUCTION

Fungal infection of fish eggs is a major economic problem with many freshwater species, especially salmonids in hatcheries (Neish & Hughes, 1980; Srivastava, 1987; Willoughby, 1994). Members of the family Saprolegniaceae (Oomycetes), mainly the genus *Saprolegnia*, are responsible and they can also affect adult fishes (Scott & O'Bier, 1962; Czeczuga & Woronowicz, 1993). Many species of fungi have been isolated from salmonid eggs (Czeczuga & Muszynska, 1996; Kitancharoen *et al.*, 1997; Hussein *et al.*, 2001) a probable reflection of the diversity of fungi found in the aquatic environment. In contrast, studies on adult salmonids with cutaneous lesions show that one type of *Saprolegnia* predominates, having characteristics such as long bundles of hairs in the secondary cysts and indirect germination (septate secondary cyst germ tubes). These features differentiate it from other *Saprolegnia* species (Pickering & Willoughby, 1982; Willoughby, 1985; Aller-Gancedo & Fernández-Díez, 1987; Beakes *et al.*, 1994). Depending on the taxonomic criteria used, these salmonid-pathogenic

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*Saprolegnia* isolates have been identified as *S. parasitica*, *S. diclina*, *S. diclina* type 1, *S. diclina*-*S. parasitica* complex, *Saprolegnia* sp. and 'salmonid *Saprolegnia*'. Some of these names, however, have been used for saprophytic isolates of *Saprolegnia*, which has lead to considerable taxonomic confusion. Since the epizootic of saprolegniosis that occurred in the 1980s, causing a high mortality rate among wild brown trout *Salmo trutta* L. in various rivers in the Province of Leon (north-west Spain), these salmonid *Saprolegnia* isolates have been the only pathogen isolated from lesions of fish (Aller-Gancedo & Fernández-Díez, 1987; Fregeneda-Grandes *et al.*, 2000). Beakes *et al.* (1994) and Diéguez-Uribeondo *et al.* (2007) recommend the name *S. parasitica* to designate these salmonid *Saprolegnia* isolates.

There have been no studies of the aquatic fungi developing on brown trout eggs in Spain. Hence, the aim of this study was to identify the aquatic fungi, limited to the Oomycetes, that colonize brown trout eggs and alevins in a hatchery and to compare the results with those obtained from broodfish suffering saprolegniosis in the same hatchery.

## MATERIALS AND METHODS

The study was based on a brown trout hatchery at Vegas del Condado in the Province of Leon, north-west Spain, owned by the Castile and Leon Regional Government (Junta de Castilla y León). Eggs were incubated in trays inside troughs with *c.* 7000 eggs per tray distributed as a monolayer. Continuously flowing well water (at 7–9° C) was used throughout egg incubation at a rate of *c.* 15 l min$^{-1}$. During incubation, the eggs received routine prophylactic treatments with Proxitane® 0510 (Solvay Interox S.A., Barcelona, Spain) or Pyceze® (Novartis Animal Vaccines Ltd, Braintree, U.K.).

Ninety-two samples were obtained: 74 from eggs, nine from alevins (yolk-sac fry) and nine from broodfish, all of them bearing macroscopic fungal colonization. To isolate the fungi, eggs coated with fungal mycelia (Fig. 1) were removed from trays and taken to the laboratory in 28 ml glass bottles filled with sterile distilled water (one egg per bottle). Here the samples were washed several times with sterile distilled water, then egg membranes with fungal mycelia were separated, washed again and placed in plastic Petri dishes with 20 ml of paper-filtered and autoclave-sterilized river water (SRW).

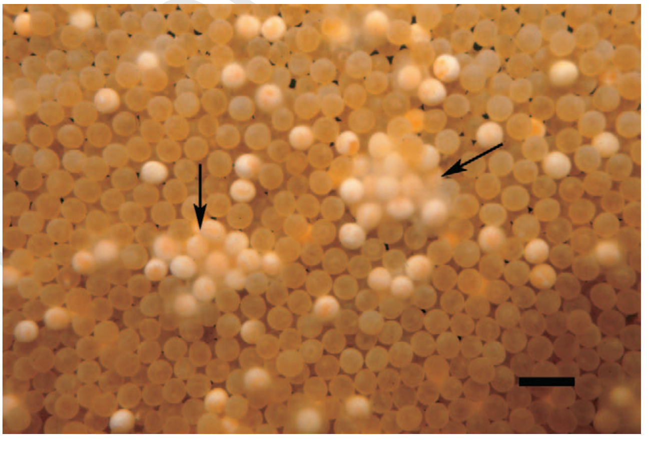

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FIG. 1. Brown trout eggs colonized with *Saprolegnia* (⟶) incubated in trays in a hatchery. Scale bar = 10 mm.

The dishes were incubated for 24 h at 20° C and examined stereomicroscopically for the morphology of the colonies, to determine if one or more types of fungi were growing concurrently. A sample of the mycelium was seeded onto glucose-peptone agar (Willoughby, 1994) with chloramphenicol (200 μg $ml^{-1}$) and bacteria-free cultures were obtained by successive passages on this medium. Isolation from broodfish with cutaneous lesions (Fig. 2) and infected alevins (Fig. 3) was carried out on the same medium using a small portion of mycelium from the lesions, previously washed in sterile distilled water.

For identification, the isolates were cultivated on glucose-peptone agar with hemp seeds *Cannabis sativa* L. Subsequently, the fungi were cultivated in Petri dishes with SRW (one colonized seed per dish) and incubated in darkness at 20 or 7° C with at least two dishes for each temperature. Cultures were examined periodically for 2 months to monitor the development of sexual structures. The fungi were identified from classical morphological features following Coker (1923), Seymour (1970) and Johnson *et al.* (2002). *Saprolegnia parasitica* was identified from bundles of long hairs on secondary cysts and their indirect germination (Willoughby, 1985; Beakes *et al.*, 1994; Diéguez-Uribeondo *et al.*, 2007).

## RESULTS

All the isolates belonged to genus *Saprolegnia*. The 74 isolates obtained from colonized eggs were identified as: *S. diclina* (38 isolates), *S. australis* (11 isolates), *S. ferax* (four isolates), *S. furcata* (four isolates), *S. hypogyna* (three isolates), *S. parasitica* (two isolates) and *S. unispora* (one isolate). The remaining 11 isolates from eggs could not be identified to species because they did not develop sexually, so they were designated *Saprolegnia* spp. The nine isolates obtained from alevins were identified as: *S. diclina* (four isolates), *S. ferax* (two isolates), *S. parasitica* (two isolates) and *S. australis* (one isolate). The nine isolates obtained from broodfish with saprolegniosis were identified as *S. parasitica* (Table I).

The six sexual *Saprolegnia* species identified had the morphological characteristics of the oogonia, oospores and antheridial branches described for their respective species (Table II and Fig. 4). The *S. parasitica* isolates, in contrast

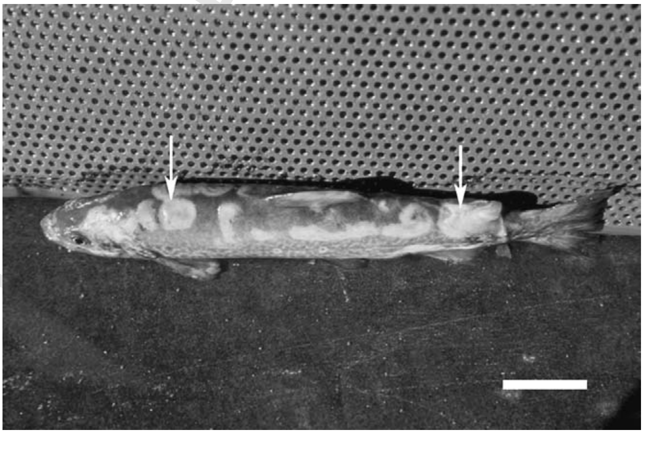


FIG. 2. Four year-old farmed male brown trout broodfish with skin lesions (—▻) caused by *Saprolegnia parasitica*. Scale bar = 50 mm.

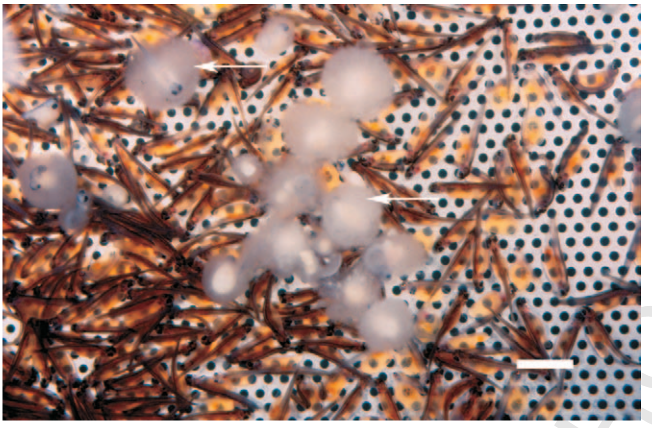


FIG. 3. Seven days-old brown trout alevins colonized with *Saprolegnia* (—▻) incubated in trays in a hatchery. Scale bar = 10 mm.





with these six species and the asexual *Saprolegnia* sp. isolates, had secondary cysts with bundles of long hairs and indirect germination that were visible with phase-contrast microscopy. Of the 13 isolates identified as *S. parasitica* (two from eggs, two from alevins and nine from broodfish), 12 were asexual isolates lacking oogonia (*Saprolegnia* sp. according to classical taxonomic criteria), even after incubation for 80 days at 7° C. One isolate had oogonia (obtained from a single, infected alevin) which developed at 7° C after 30 days of incubation but they were very scarce and generally grouped together in the same zone of the colony. The morphological characteristics of the sexual structures (oogonia,

TABLE I. *Saprolegnia* spp. isolated from eggs, alevins and broodfish of brown trout in a hatchery suffering from saprolegniosis

| Source | Number of isolates | Identification (Number of isolates/%) |
|---|---|---|
| Eggs | 74 | *S. diclina* (38/51·4) |
| | | *S. australis* (11/14·9) |
| | | *S. ferax* (4/5·4) |
| | | *S. furcata* (4/5·4) |
| | | *S. hypogyna* (3/4·1) |
| | | *S. parasitica* (2/2·7)* |
| | | *S. unispora* (1/1·4) |
| | | *Saprolegnia* sp. (11/14·9)* |
| Alevins | 9 | *S. diclina* (4/44·4) |
| | | *S. ferax* (2/22·2) |
| | | *S. parasitica* (2/22·2)† |
| | | *S. australis* (1/11·1) |
| Broodfish | 9 | *S. parasitica* (9/100)* |
| Total | 92 | |

*Asexual.

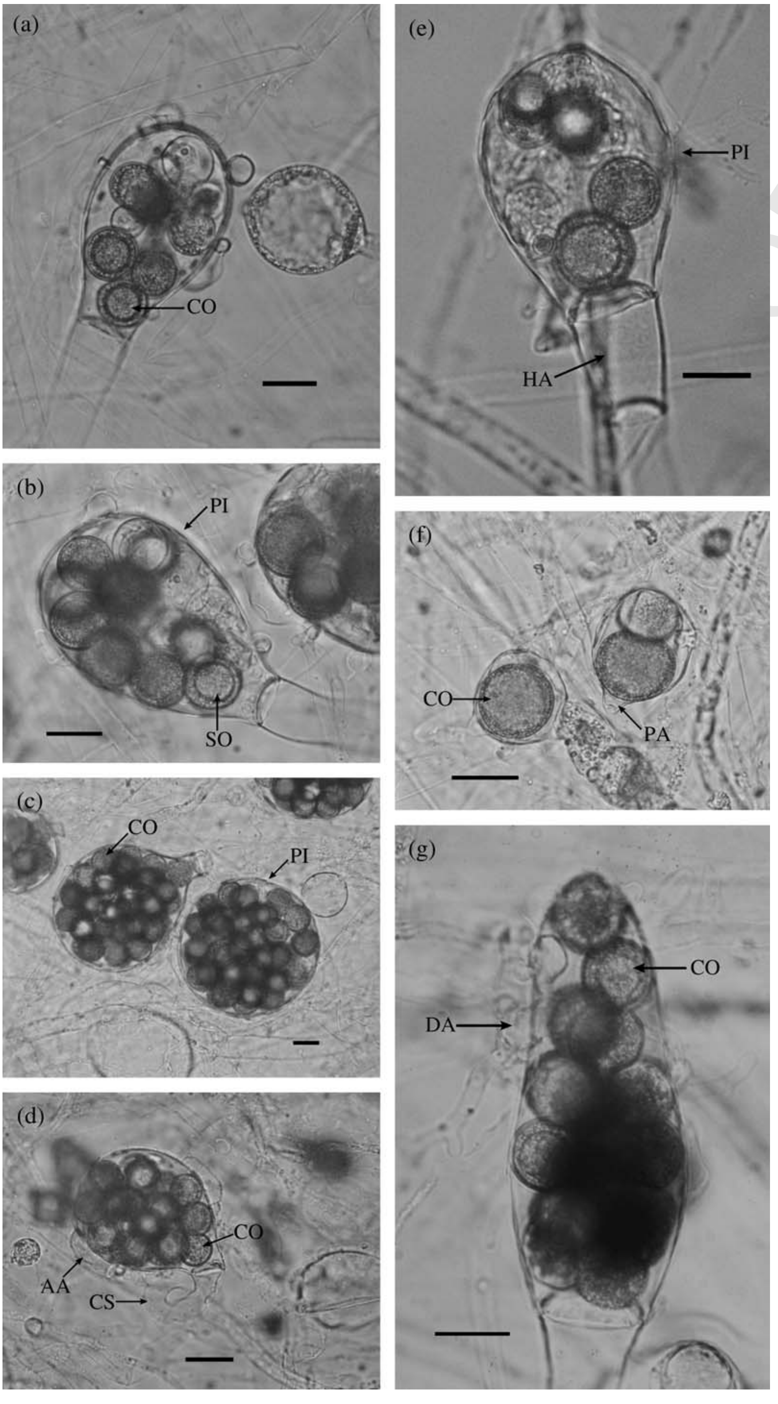
†One asexual and one with oogonia.

TABLE II. Main morphological characteristics of the sexual structures of *Saprolegnia* spp. isolated from eggs, alevins and broodfish of brown trout in a hatchery suffering from saprolegniosis

| Identification | Oogonial formation* | Oogonial shape | Oogonial wall | Oospores | Antheridial branches | Bundles of long hairs |
|---|---|---|---|---|---|---|
| *S. diclina* | ++ | Spherical or pyriform | Unpitted or few pits but inconspicuous | Centric or subcentric | Diclinous | No |
| *S. australis* | ++ | Spherical or pyriform | Unpitted or few pits but conspicuous | Subcentric | Diclinous, occasionally androgynous | No |
| *S. ferax* | +++ | Spherical | Conspicuously pitted | Centric or subcentric | Androgynous, monoclinous or absent | No |
| *S. furcata* | ++ | Spherical or subspherical | Conspicuously pitted | Centric | Androgynous | No |
| *S. hypogyna* | +++ | Spherical or subspherical | Conspicuously pitted | Centric | Hypogynous | No |
| *S. unispora* | +† | Spherical | Unpitted | Subcentric | Absent | No |
| *Saprolegnia* sp. | – | | | | | No |
| *S. parasitica* | –‡ | | | | | Yes |

*–, no formation; +, scarce; ++, abundant; +++, very abundant.
†Only at 7° C and after prolonged incubation (20 to 30 days).
‡Asexual, with the exception of one isolate that developed oogonia at 7° C after 30 days of incubation similar to oogonia of the former *S. diclina* type 1 (Willoughby, 1978) but with non-antheridial investment.


(a)
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(f)
CO
PA
(g)
CO
DA

oospores and antheridia) of this isolate were very similar to the former *S. diclina* type 1 described by Willoughby (1978) but with non-antheridial invested oogonia (Fig. 4).

## DISCUSSION

The aquatic fungi able to colonize fish eggs, particularly those of salmonids, are mostly species of *Achlya*, *Aphanomyces*, *Saprolegnia*, or less often, *Dictyuchus*, *Leptolegnia* and *Thraustotheca*, all belonging to the Saprolegniales. Species of the genera *Leptomitus* and *Pythium* are the commonest within the orders Leptomitales and Pythiales, respectively (Czeczuga & Woronowicz, 1993; Czeczuga *et al.*, 1996, 2005; Kitancharoen *et al.*, 1997; Hussein *et al.*, 2001). These data suggest that species of *Saprolegnia* are the principal microorganisms associated with fungal colonization of salmonid eggs. In the present study, all the isolates obtained from colonized brown trout eggs were *Saprolegnia*. On two occasions, however, fungi growing directly on egg membranes, were identified as *Leptolegnia* spp., but were mixed with *Saprolegnia* spp. Attempts to grow them as pure cultures, produced only *Saprolegnia* spp. Among the species of *Saprolegnia*, *S. diclina* was the most frequent, followed by *S. australis*, although 11 non-sexual isolates remained unidentified to species. Similar results were obtained from alevins. All isolates belonged to the genus *Saprolegnia* and the most frequent species was *S. diclina*. A statistical comparison between the isolates obtained from eggs and those from alevins was not possible because of the small number of isolates of some species. Routine prophylaxis with antifungals are used during the incubation of eggs in hatcheries. Such treatment was in use at the fish farm where the study was undertaken. Hence, the results reflect the situation in a fish farm under normal production conditions. Kitancharoen *et al.* (1997) investigated the aquatic fungi developing on the eggs of five salmonid species at six fishery stations in Japan and similarly found that *S. diclina* was commonest. In contrast, Czeczuga *et al.* (2005) studying the eggs of sea trout (anadromous brown trout) in Poland found the commonest species were *Achlya polyandra*, *S. ferax*, *S. parasitica* and *Leptomitus lacteus*. These differences might be explained because fungal colonization normally originates on dead eggs (Smith *et al.*, 1985; Kitancharoen & Hatai, 1996) and all these fungi are saprobes in aquatic systems. Thus, the different species found on fish eggs at different locations could be a reflection of the diversity of aquatic fungi present, depending on the physical and chemical conditions of the water.

FIG. 4. Main morphological characteristics of the sexual structures of *Saprolegnia* spp. isolated from eggs, alevins and broodfish of brown trout in a hatchery with saprolegniosis. (a) *Saprolegnia diclina*: pyriform unpitted oogonium with centric oospores. (b) *Saprolegnia australis*: pyriform oogonium with pits and subcentric oopores. (c) *Saprolegnia ferax*: spherical pitted oogonia with centric oospores and no antheridial branches. (d) *Saprolegnia furcata*: subspherical oogonium with coiled stalk, centric oospores and androgynous antheridial branches. (e) *Saprolegnia hypogyna*: subspherical pitted oogonium with hypogynous antheridial cell. (f) *Saprolegnia unispora*: spherical papillate oogonia with centric oospores and no antheridial branches. (g) *Saprolegnia parasitica*: elongated oogonium with centric oospores and diclinous antheridial branches. CO, centric oospore; SO, subcentric oospore; PI, pitting; CS, coiled stalk; PA, papillae; AA, androgynous antheridium; HA, hypogynous antheridium; DA, diclinous antheridium. Scale bars = 25 μm.



 

In contrast to the results obtained from eggs, when studies were carried out on live adult salmonids with cutaneous lesions from extensive geographical areas, normally only one type of *Saprolegnia* isolate was found (Neish, 1977; Willoughby, 1978; Aller-Gancedo & Fernández-Díez, 1987; Hatai *et al.*, 1990). In fact, a number of authors have named this type *S. parasitica*, distinguished from the description of the species given by Kanouse (1932). These isolates have certain characteristics, such as long bundles of hairs in the secondary cysts, indirect germination in low nutrient media and internal transcribed spacer (ITS) sequences that allow them to be differentiated from other *Saprolegnia* species (Diéguez-Uribeondo *et al.*, 2007). They frequently do not develop sexual structures under laboratory conditions, however, which prevents species identification from classical morphological criteria. These pathogenic isolates of salmonids are rarely found on infected eggs (Kitancharoen *et al.*, 1997). The results of present study agree with these observations, since only two such isolates were obtained from infected eggs (out of 74 isolates), whereas all the isolates from broodfish were identified as *S. parasitica*. On the other hand, these results contradict those found by Czeczuga *et al.* (2005), who were able to isolate *S. parasitica* from the eggs of all 42 sea trout females investigated on three fish farms in Poland. It may be that these authors are using the name *S. parasitica* in the original sense assigned by Coker (1923), meaning any asexual *Saprolegnia* isolate obtained from fishes or fish eggs, or by Kanouse (1932), and not in the sense given by Beakes *et al.* (1994) and Diéguez-Uribeondo *et al.*, 2007. In the present study, the 11 asexual isolates and the two isolates of *S. parasitica* obtained from colonized eggs could be identified as *S. parasitica* in accordance with Coker's concept, therefore this would be the second commonest species found. One conclusion of the present study was that eggs and alevins of brown trout were infected or colonized after death by various *Saprolegnia* saprophytic species, whereas trout (salmonids) were infected by pathogenic *S. parasitica* isolates.

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