

Prevalence of serum antibodies against *Saprolegnia parasitica* in wild and farmed brown trout *Salmo trutta*

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ABSTRACT: The prevalence of serum antibodies against *Saprolegnia parasitica* in wild and farmed brown trout *Salmo trutta* from the province of León (NW Spain) was studied by enzyme-linked immunosorbent assay (ELISA). Blood samples from healthy and *Saprolegnia*-infected brown trout were collected over 2 yr with a seasonal periodicity (January, April, July and October) from a hatchery and river with frequent presence of saprolegniosis (River Porma) and from a river in which the disease was rarely observed (River Omaña). The individual prevalence was 30.1%, but statistically significant differences were observed between the prevalence in trout from the hatchery (43.0%), from River Porma (31.8%) and from River Omaña (6.4%) and also between the prevalence observed in October (42.9%) and the values obtained in January (24.8%), April (22.7%) and July (27.5%). There was no difference between the seroprevalence in females (34.8%) and males (38.2%), but a positive correlation between raised serum antibody levels and larger (older) fish was found. The low prevalence of antibodies observed in *Saprolegnia*-infected trout (18.0%) suggests possible immune suppression and the lack of an effective specific immune response in fish with saprolegniosis.

KEY WORDS: *Saprolegnia parasitica* · Brown trout · Serum antibodies · Prevalence · ELISA

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INTRODUCTION

Saprolegniosis, caused by *Saprolegnia* spp., is an important freshwater fish disease, which often affects wild and farmed salmonids. In salmonid fish farms saprolegniosis affects mainly broodfish and incubating eggs, causing major financial losses (Pickering & Willoughby 1982, Noga 1993, Bruno & Wood 1999). In rivers and lakes saprolegniosis can reduce the natural salmonid populations, as has occurred amongst wild brown trout *Salmo trutta* in several rivers in the province of León in Spain (Aller-Gancedo & Fernández-Díez 1986).

It has been suggested that the mucus layer, the integrity of the epidermis and a cellular response based on non-specific phagocytic activity of inflammatory cells play an important role in the defence mechanisms of fish against saprolegniosis, whereas the par-

ticipation of a specific immune response is uncertain (Hodkinson & Hunter 1970, Sohnle & Chusid 1983, Wood et al. 1986, Pickering 1994). Results from studies on the production of antibodies against *Saprolegnia* infection in fish are contradictory, depending on the species studied, the water temperature, and the antigens and serological techniques used (Hodkinson & Hunter 1970, El-Feki 1987, Sohnle & Chusid 1983, Bly et al. 1994). For example, El-Feki (1987) did not find any serological response in carp intraperitoneally injected with soluble or hyphal extracts of *S. diclina* or in carp experimentally infected with *S. diclina*, but he did detect antibodies in rainbow trout with saprolegniosis from a fish farm. Moreover, Sohnle & Chusid (1983) and Bly et al. (1994) did not find precipitating antibodies in rainbow trout or channel catfish, respectively, following experimental inoculation with *Saprolegnia* sp. isolates. In contrast, Hodkinson & Hunter

(1970) found them in 93% of the wild salmon studied but only 66% of these were colonized by *Saprolegnia* spp. Fregeneda-Grandes et al. (2007) demonstrated that brown trout injected with antigenic extracts from a pathogenic isolate of *Saprolegnia parasitica* are able to develop specific antibodies against them that can be detected by standard immunological techniques such as enzyme-linked immunosorbent assay (ELISA), immunofluorescence (IF) and western blotting (WB). The results obtained by using these various techniques were similar, showing there is cross-reaction between the antigenic extracts used, but ELISA detected the greatest number of positive samples. The aim of the present study was to research the prevalence of serum antibodies against *S. parasitica* in wild and farmed brown trout from both healthy and naturally *Saprolegnia*-infected fish to gain a better understanding of the specific immune response against *Saprolegnia* infections.

MATERIALS AND METHODS

Fish and samples. The study was conducted in the province of León in northwest Spain. Healthy and *Saprolegnia*-infected brown trout *Salmo trutta* were collected over a period of 2 yr (October 2002 to July 2004) with a seasonal periodicity (January, April, July and October). Samples were collected from 3 locations: 2 with frequent presence of saprolegniosis during the spawning season in recent years, a hatchery situated on the River Porma and owned by the Castile and Leon Regional Government (Junta de Castilla y León) and the River Porma itself; and the third a river in which the disease has never or rarely been observed, the River Omaña. At the River Omaña, samples were taken 5 times between October 2002 and October 2003. As saprolegniosis affects mainly sexually mature fish, blood samples were taken from broodfish at the hatchery and from wild brown trout >17 cm in length. At the hatchery, broodfish were maintained in raceways (one for females and another for males) situated in the open with a continuous supply of well water. They were sampled when 3 yr old (in the first year of the study) and 4 yr old (in the second year). Wild brown trout were captured by electrofishing at different sampling points between Candanedo de Boñar and Santibañez del Porma on the River Porma, and between Villanueva de Omaña and Guisatecha on the River Omaña. The water temperature at the sampling points varied between seasons from 7 to 13°C at the hatchery, from 6 to 14°C in the River Porma and from 4 to 14°C in the River Omaña. Required sample sizes were calculated using Win Episcopo 2.0 (CLIVE), based on the population data for broodfish at the hatchery ($n =$

1600), an expected prevalence of 50%, an accepted error of 10% and a 95% confidence level. The sample size calculated was 91, but to avoid loss of information in the analyses, the sample was increased by 5%. In the end, 96 samples were randomly selected in each season and taken from the 3 populations studied, except the River Omaña, where it was only possible to take 84 samples in January 2003. At the hatchery, half of the samples were taken from females and half from males, except at the first sampling (October 2002, all broodfish kept together until first spawning). In total, 2004 blood samples were investigated (1236 from wild brown trout and 768 from broodfish), of which only 23 (1.1%) were from *Saprolegnia*-infected fish (see Table 1).

In view of the small number of samples from fish with saprolegniosis, a further 2 specific groups of *Saprolegnia*-infected male broodfish from the hatchery were sampled over 6 to 12 wk in order to study the presence of serum antibodies related to the disease. The first group (Group 1) was made up of 57 males with saprolegniosis, which were kept separate in a raceway and did not receive any treatment. Group 1 had 100% mortality within a period of 51 d (January to March 2003). These fish were sampled on the first day, and then samples were taken from fish alive at 7, 14, 21, 35 and 43 d to obtain a total of 148 blood samples (57, 43, 24, 16, 5 and 3 samples, respectively). The second group (Group 2) were trout from a raceway that contained 253 males kept under normal hatchery conditions; they received preventive treatments against saprolegniosis (Proxitane 0510, Solvay Interlox). Fish with saprolegniosis were observed very frequently in this raceway and they presented a cumulative mortality of 36.8% during the study (January to April 2004). Blood samples were taken from 48 trout from Group 2 every 4 wk (Days 0, 28, 55 and 83) with a total of 192 samples, 68 from *Saprolegnia*-infected fish (48 in January, 17 in February and 3 in March) and 124 from trout without saprolegniosis (31 in February, 45 in March and 48 in April). All the trout were anaesthetized with 50 mg l⁻¹ of MS 222 (tricaine methane sulphonate), weighed and measured, then individual blood samples were obtained by puncturing the caudal vein. All the trout were returned alive to the water with the exception of trout with saprolegniosis from the rivers. After clotting overnight at 4°C, the blood samples were centrifuged at 1000 × *g* for 45 min to obtain the serum. Sera were stored at -20°C until they were used.

Antibody detection. The presence of antibodies in the serum was analyzed by ELISA using a soluble antigenic extract from sonicated formalinized mycelia of a pathogenic isolate of *Saprolegnia parasitica*, as described in Fregeneda-Grandes et al. (2007). Serial 2-fold dilutions

(1:20 to 1:10 240) of trout serum were used and the ELISA titre was defined as a reciprocal value of the highest serum dilution giving an optical density (OD) value >0.5 (mean OD values for the negative control sera plus 3 SD). Only titres of 40 or higher were considered positive.

In addition, 271 representative trout sera from the various fish samples, including some from the 2 groups of males with saprolegniosis (218 from trout without saprolegniosis and 53 from trout with saprolegniosis), were selected to perform a WB analysis, in order to identify individual proteins in the soluble antigenic extract recognized by the trout sera. WB was performed as described in Fregeneda-Grandes et al. (2007).

Statistics. Chi-squared tests were used to compare seroprevalence rates with relation to origin, season, sex, length-group and the presence of saprolegniosis. Confidence limits for the proportions were established by an exact binomial test with a 95% CI. For statistical purposes the titres were transformed to a logarithmic scale and differences in the mean \log_{10} titres for positive fish were analysed statistically by means of the parametric ANOVA or the non-parametric Kruskal-Wallis test, depending on whether the conditions for a classical ANOVA were fulfilled or not. All statistical tests were done with Epi Info software for Windows, Version 3.3 (Centers for Disease Control); $p < 0.05$ was taken as statistically significant.

RESULTS

Antibodies were found in 30.1% (95% CI: 28.1–32.2) of the serum samples analyzed (604 of 2004) in the seasonal study. However, statistically significant differences ($p < 0.001$) were observed between the prevalence in the trout from the hatchery (43.0%, 39.4–46.6), the River Porma (31.8%, 28.5–35.2) and the River Omaña (6.4%, 4.4–9.1) (Table 1). With regard to the season, the prevalence observed in October (42.9%) was significantly different ($p < 0.001$) from that obtained in January (24.8%), April (22.7%) and July (27.5%). The seroprevalences among males (38.2%, 33.4–43.1) and females (34.8%, 30.0–39.9) were not significantly different ($p = 0.37$) but a positive

Table 1. Seroprevalence of antibodies against *Saprolegnia parasitica* in wild and farmed brown trout *Salmo trutta* (with or without saprolegniosis) by origin, season, sex and length-group (includes fish from the seasonal study only). Enzyme-linked immunosorbent assay (ELISA) considered positive when titre ≥ 40 . CI: confidence interval

	Fish tested (n)	Relative distribution (%)	ELISA positive	Prevalence (%)	95% CI
Origin					
Hatchery ^a	768	38.3	330	43.0	39.4–46.6
River Porma ^a	768	38.3	244	31.8	28.5–35.2
River Omaña ^b	468	23.4	30	6.4	4.4–9.1
Total	2004	100	604	30.1	28.1–32.2
Season					
January	468	23.3	116	24.8	21.0–29.0
April	480	24.0	109	22.7	19.1–26.8
July	480	24.0	132	27.5	23.6–31.8
October	576	28.7	247	42.9	38.8–47.0
Total	2004	100	604	30.1	28.1–32.2
Sex					
Female	368	18.4	128	34.8	30.0–39.9
Male	401	20.0	153	38.2	33.4–43.1
Not recorded	1235	61.6	323	26.2	23.7–28.7
Total	2004	100	604	30.1	28.1–32.2
Length-group (cm)					
>16–21	404	20.2	86	21.3	17.5–25.7
>21–26	462	23.0	80	17.3	14.0–21.1
>26–31	344	17.2	90	26.2	21.7–31.2
>31–36	342	17.1	133	38.9	33.7–44.3
>36–41	247	12.3	117	47.4	41.0–53.8
>41–46	172	8.6	81	47.1	39.5–54.8
>46–50	28	1.4	14	50.0	30.6–69.4
>50	5	0.2	3	60.0	14.7–94.7
Total	2004	100	604	30.1	28.1–32.2
^a Frequent presence of saprolegniosis					
^b Saprolegniosis has never or rarely been observed					

relation was found between size (length-group) and seroprevalence ($p < 0.001$): prevalence tended to increase with the length of the trout. The lowest seroprevalence values were found in trout less than 26 cm

Table 2. Distribution of serum ELISA titres of antibodies against *Saprolegnia parasitica* in wild and farmed brown trout *Salmo trutta* (includes fish from the seasonal study and Groups 1 and 2)

Titre	Without saprolegniosis no. sera (% total)	With saprolegniosis no. sera (% total)
40	207 (33.3)	13 (30.2)
80	189 (30.5)	15 (34.9)
160	113 (18.2)	9 (20.9)
320	71 (11.4)	3 (7.0)
640	25 (4.1)	0 (0)
1280	12 (1.9)	2 (4.7)
2560	4 (0.6)	0 (0)
5120	0 (0)	0 (0)
10240	0 (0)	1 (2.3)
Total	621 (100)	43 (100)

in length and the highest values were found in trout greater than 36 cm in length (Table 1). The titres of positive fish (including seasonal study and Groups 1 and 2) varied greatly among individuals, from 40 to 2560 (median = 80). In general, most of the serum samples showed titres between 40 and 160, and 18 samples showed titres between 1280 and 2650 (Table 2). Although the highest mean \log_{10} titres of positive fish were found in fish from the River Omaña, in so far as origin was concerned; in those sampled in July with regard to the season; and in males with respect to sex, these differences were not significant in any case (results not shown).

From the total of trout randomly sampled in the seasonal study ($n = 2004$), only 23 (1.1%) were from individuals with *Saprolegnia* infection. Of these 23 fish, 6 were from the River Porma and the remaining 17 from the hatchery, 20 were males and 21 were captured in January. The prevalence of serum antibodies in these fish with saprolegniosis was 8.7% (Table 3). In the 2 specific groups of males with saprolegniosis studied at the hatchery the seroprevalence was significantly different (Table 3). In Group 1 (infected fish), 39 of 148 samples (26.4%) were positive. However, a major reduction in the prevalence was observed as sampling progressed. Seroprevalence in the first sampling was 47.4% (27 of 57) but it decreased to 20.9% (9 of 43) in the second, 8.3% (2 of 24) in the third, 6.3% (1 of 16) in the fourth and 0% in the fifth (0 of 5) and sixth (0 of 3) samplings. In Group 2, only 2 samples out of 68 (2.9%) of trout with saprolegniosis were positive. In this last group the seroprevalence of trout without saprolegniosis was 15.3% (19 of 124). The titres of positive trout

Table 3. *Salmo trutta* infected by *Saprolegnia parasitica*. Seroprevalence of antibodies against *S. parasitica* in wild and farmed brown trout with cutaneous lesions of saprolegniosis. Enzyme-linked immunosorbent assay (ELISA) considered positive when titre ≥ 40 . CI: confidence interval

	Samples tested (n)	ELISA positive	Prevalence (%)	95% CI
Random sampling ^a	23	2	8.7	1.1–28.0
Group 1 ^b	148	39	26.4	19.5–34.2
Group 2 ^c	68	2	2.9	0.4–10.2
Total	239	43	18.0	13.3–23.5

^aTrout from the River Porma and a hatchery on this river
^b57 males with saprolegniosis kept separate in a hatchery raceway that did not receive any treatment. This group was sampled at the beginning of the experiment; all survivors were sampled on 5 occasions over 43 d
^c253 hatchery males, many affected by saprolegniosis. Samples taken 4 times (4 wk apart) from among all the *Saprolegnia*-infected fish

with saprolegniosis were similar to those obtained from fish without saprolegniosis (Table 2) and the difference between the mean \log_{10} titres of positive fish with (2.02) or without saprolegniosis (1.99) was not significant ($p = 0.68$).

The number of positive samples obtained by WB was lower than by ELISA. By WB, only 15 of the 271 samples (5.5%) analysed were positive, whereas 115 samples (42.4%) were positive when ELISA was used. Only 1 of the 15 positive samples was from a trout with saprolegniosis. Positive samples only recognized 1 or 2 bands of the 28 bands of the soluble antigenic extract from *Saprolegnia parasitica*. These 2 bands corresponded to 2 proteins of 25 and 29 kDa (Fig. 1).

DISCUSSION

The present study has shown that brown trout are able to produce anti-*Saprolegnia* serum antibodies in natural conditions and that seroprevalence varies according to the origin of fish, in relation to the pres-

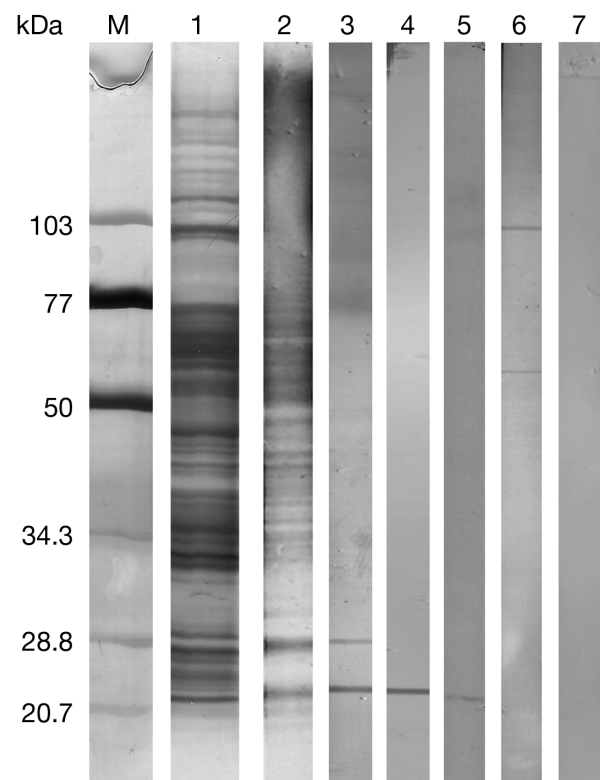


Fig. 1. *Saprolegnia parasitica*. Western blot on nitrocellulose membrane after SDS-PAGE (gel 4–20%) of *S. parasitica* soluble extract. Lane M: low range molecular weight protein marker (Bio-Rad, catalog no. 161-0304); Lane 1: silver-stained SDS-PAGE of soluble extract; Lane 2: positive control rabbit polyclonal serum; Lanes 3 to 6: positive trout sera; Lane 7: negative trout serum

ence or absence of the disease, the season and the size of the fish, which is directly related to their age. It is well known that temperature, photoperiod and other environmental conditions, which vary seasonally, may have an impact on the immune system of fish (Bowden et al. 2007). Generally, higher temperatures (in the physiologically normal range) have been reported to enhance immune responses in fish, whereas lower temperatures adversely affect immune function (Bly & Clem 1992). However, in the present study, the prevalence of anti-*Saprolegnia* antibodies observed in the samples taken in January (with a water temperature between 4 and 7°C) was no different from that observed in April (7 to 11°C) or July (10 to 14°C). The differences in seasonal prevalence observed might be related to the seasonal periodicity of *Saprolegniaceae* in northwestern Spain (Fregeneda-Grandes 1998); higher concentrations of spores were found to be present in water at the end of the summer (August to September) and early autumn (October). This might explain the fact that the highest prevalence of antibodies was found in October (Table 1). In the present study, the difference between the prevalence in males and in females was not significant, despite the fact that Wilson (1976) noted a reduced immune response capacity against *Saprolegnia* antigens in mature males.

Surprisingly, the prevalence of serum antibodies in brown trout with saprolegniosis was lower than in clinically healthy fish and, curiously, in those trout that showed the most severe lesions. These results are in agreement with Hodkinson & Hunter (1970), who used double gel diffusion to test sera from wild salmon *Salmo salar* L. affected by saprolegniosis and observed less reactivity in sera from more heavily colonized fish, because they showed a smaller average number of precipitation lines than salmon not affected or having only slight lesions. This phenomenon could be related to the haemodilution observed in *Saprolegnia*-infected fish (Pickering & Willoughby 1982), more severe in the present study because some trout from the 2 specific groups studied at the hatchery were bled several times in a short period. It is also possible that during the acute phase of the disease, serum antibodies were removed from the blood by adsorption with the antigens and could not be detected by standard serological techniques (Hodkinson & Hunter 1970, Wilson 1976). However, the same distribution and mean log titres were observed in positive samples from fish with or without saprolegniosis. Other authors have tried to characterize the immune response, both cellular and humoral, against *Saprolegnia* infection and have shown evidence of a lack of an effective response, which might favour the course of the disease (Sohnle & Chusid 1983, El-Feki 1987, Álvarez et al. 1988, 1995, Bly et al. 1994). Results from

the present study are along the same lines. It is not clear whether fish with saprolegniosis are immunocompromised prior to infection (as a consequence of low water temperature or high circulating corticosteroids), or whether *Saprolegnia* can produce cytotoxic or other virulence factors to evade or suppress the immune system. Host defence suppression by *Saprolegnia* remains poorly understood, but recently Kales et al. (2007) have suggested that inhibition of phagocytosis due to the large spore size and the reduction of macrophage gene expression of the class II major histocompatibility (MH II) receptor and associated molecules, which play a crucial role in the recognition of exogenous antigens, may suppress the host immune response. Moreover, Torto-Alalibo et al. (2005) generated a large number of expressed sequence tags from a mycelial cDNA library of *Saprolegnia parasitica* and discovered several proteins that may be implicated in protein translocation process into host cells; Van West (2006) speculated that some of these proteins might be responsible for suppressing the immune response in the host.

In the present study, a smaller number of positive samples were found by WB than by ELISA; similar results were obtained previously by using various techniques to detect serum antibodies in brown trout intraperitoneally injected with antigenic extracts from a pathogenic isolate of *Saprolegnia parasitica* (Fregeneda-Grandes et al. 2007). Nevertheless, a greater number of positive samples was detected by ELISA, followed by immunofluorescence and finally by WB. Moreover, trout experimentally injected with *S. parasitica* antigens recognized a larger number of bands (1 to 8) than trout in the present study (1 to 2 bands), which may reflect differences in the immune response against *Saprolegnia* antigens when fish are naturally exposed. Lorenzen & LaPatra (1999) noted the poor reactivity of rainbow trout sera to viral antigens in WB. They found that sera from trout that survived natural infection with viral haemorrhagic septicaemia virus did not react in WB; these fish showed a detectable antibody response only to certain viral proteins and only after immunization with whole virus particles. The 2 bands most frequently recognized in brown trout naturally and experimentally exposed to *Saprolegnia* antigens were 2 proteins of 25 and 29 kDa (Fregeneda-Grandes et al. 2007, present study). This suggests that these 2 proteins may be the most immunogenic and studies to characterize them are in progress.

In conclusion, the present study contributes to a better understanding of the specific immune response against *Saprolegnia* infections. Further studies will focus on the factors that might influence the humoral immune response and the possible role of the antibodies in protection against saprolegniosis.

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