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# 1 Occurrence of nodular gill disease in farmed brown trout (*Salmo trutta* 2 L.)

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10 *Keywords:* nodular gill disease, brown trout, *Salmo trutta*, amoeba.

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## 14 ABSTRACT

15 In March 2017, during an investigation on NGD in salmonid farms of the Northern Italy, the first cases of branchitis  
16 associated with amoebic infection in farmed brown trout (*Salmo trutta* L.) was observed. The episodes were detected in  
17 two commercial trout farms, where outbreaks of NGD in rainbow trout (*Oncorhynchus mykiss*) occurred periodically.  
18 Clinical examination of the affected brown trout evidenced respiratory distress with dyspnea and abnormal swimming  
19 behavior. In both tanks the cumulative mortality was less than 5%. The respiratory symptoms and mortality appeared  
20 less serious in brown trout compared to previous episodes of NGD in rainbow trout. From March to May 2017 twenty-  
21 five live and moribund brown trout from each tank were collected at monthly intervals and submitted for necropsy,  
22 parasitological analysis and histology. The gill tissue appeared pale and swollen at necropsy with whitish nodules in the  
23 distal parts of filaments. The histology showed multi-focal epithelial hyperplasia of the gills causing lamellar fusion  
24 with presence of limited number of amoebae along the surface of the affected filaments. Further investigations appear  
25 necessary to clarify pathogenesis, biological and environmental determinants of NGD in brown trout and to define the  
26 involvement of this species as potential reservoir of the disease.

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29 Amoebae, highly variable and diverse eukaryotic organisms, are ubiquitous in freshwater and salt  
30 water, in soil and can live as commensals or parasites in fish. Some free-living amoebae may  
31 change their mode of life and become harmful. Pathogenic potential of these so called amphizoic  
32 amoebae is rather high and several outbreaks of diseases associated with amoebic infections was  
33 reported (Scholz 1999). Among amoebae known to be able to colonize fish gills, representatives of  
34 only a few genera were described as agents of gill disease conditions. The most important  
35 outbreaks of amoebic gill disease resulting in mortality were recorded in both marine and  
36 freshwater cultures of salmonids (Dyková & Lom 2004).

37 Nodular gill disease (NGD) is an emerging ectoparasitic condition by amoeboid protozoa causing  
38 significant mortality in some species of salmonids farmed in freshwater environment, especially  
39 rainbow trout (*Oncorhynchus mykiss*). In 1999 Speare described a mixed infection of bacterial gill  
40 disease (BGD), caused by *Flavobacterium branchiophilum*, and NGD in arctic char (*Salvelinus*  
41 *alpinus*) and rainbow trout farmed in Eastern Canada. This was the first and only report of NGD in  
42 a salmonid species other than rainbow trout, until 2010, when Tubbs *et al.* reported an episode of  
43 NGD in Chinook salmon farmed in freshwater raceways in New Zealand.

44 NGD appears to be caused by different species of amoebae, both testate (*Roghostoma minus*) and  
45 naked amoebae belonging to five genera, *Acanthamoeba*, *Vermamoeba* (formerly *Hartmannella*),  
46 *Naegleria*, *Protacanthamoeba*, and *Vannella*, as reported by Dykova *et al.* (2010; 2016). NGD was  
47 described in rainbow trout farms of North America and Europe (Denmark, Germany, Poland, Czech  
48 Republic and Italy) (Daoust & Ferguson 1985; Daoust & Ferguson 1986; Speare & Ferguson 1989;  
49 Hoffman *et al.* 1992; Buchmann *et al.* 2004; Antychowicz 2007; Quaglio *et al.* 2016).

50 NGD in highly intensive farming systems plays a major role in production losses and its outbreaks  
51 can result in cumulative mortality exceeding 60%, especially during the winter months and early

52 spring. Additional economic losses due to NGD, which are difficult to calculate but no less  
53 significant, result from impacts on feed conversion rate and the downgrading or rejection of the  
54 product. NGD affected fish show lethargy, anorexia, surface swimming particularly at the end of the  
55 tanks and signs of respiratory distress associated with mortality. Macroscopically the most  
56 prominent features of the disease are excessive mucus on the gills, that appears pale and swollen  
57 with whitish patches. Microscopically, it is characterized by a proliferation of gill tissue that causes  
58 fusion of lamellae and, in the most serious cases, of filaments with amoebic organisms along the gill  
59 surface. Histological examination shows an intense hyperplastic reaction of epithelial cells with  
60 presence of amoebae on the gill surface and within proliferative tissue.

61 In the last few years NGD is wide-spread in rainbow trout farms of Northern and Central Italy. This  
62 disease was detected for the first time in Trento Province, where, during winter months of 2014,  
63 serious episodes of gill disease characterized by respiratory failure and high and prolonged  
64 mortality was reported. Histological examinations of the affected gills allowed to diagnose the first  
65 cases of NGD in Italy (Quaglio *et al.* 2016). Successively NGD was observed in other Italian  
66 regions (Lombardia, Veneto, Friuli-Venezia Giulia, Piemonte and Marche) causing serious mortality  
67 only in rainbow trout farms located in mountain areas or characterized by cold waters (< 12°C).  
68 Currently studies are in progress to clarify the epidemiology and pathogenesis of NGD in Italy.

69 In March 2017, during epidemiological investigations on NGD in salmonid farms of the Northern  
70 Italy, the first cases of branchitis referable to amoebic infection in juvenile brown trout (*Salmo*  
71 *trutta* L.) were observed. The episodes occurred in two commercial trout farms hydrographically  
72 unrelated that breed both brown trout and rainbow trout. High mortalities in rainbow trout due to  
73 NGD occurred annually in these farms causing the loss of 30% of fish in the affected tanks. The  
74 investigated brown trout sectors of both farms were located upstream respect to the tanks of  
75 rainbow trout. The brown trout were stocked in two earth tanks (tank 1 and tank 2, respectively  
76 from farm1 and farm 2) supplied with groundwater. The average weight of fish was 23g in tank 1  
77 and 46g in tank 2 at the time of the outbreaks. The water temperatures during the episodes ranged  
78 from 5.5 to 11.2°C. In both tanks the cumulative mortality was less than 5%. The main  
79 environmental parameters and the monthly mortality of each tank are reported in Table 1. Clinical  
80 examination of affected fish evidenced respiratory distress with dyspnoea and abnormal swimming  
81 behaviour (by gathering and by gasping at the water's surface). Opercula of these fish were  
82 bilaterally flared. The respiratory symptoms and mortality appeared less serious in brown trout  
83 compared to rainbow trout.

84 Twenty-five live and moribund brown trout from each tank were collected at monthly intervals from  
85 March to May 2017 for a total of 150 fish.

86 The samples were submitted for necropsy, parasitological analysis, histology and bacteriological  
87 and virological examinations for diagnostic purposes.

88 Pools of spleen, heart and head kidney were screened for Viral Haemorrhagic Septicaemia (VHS),  
89 Infectious Haematopoietic Necrosis (IHN) and Infectious Pancreatic Necrosis (IPN) infection by  
90 virus isolation in EPC and BF-2 cell cultures and Real time PCR (rRT-PCR).

91 Bacteriological examination has been performed from head kidney, spleen and liver inoculating  
92 Tryptone Soya Broth (TSB), Tryptone Soya Agar (TSA) and Blood Agar (BA) incubated at 15 and  
93 22°C for one week.

94 Portions of gills and visceral organs of all the collected specimens were fixed in 10% neutral  
95 buffered formalin solution for histological studies. The samples were routinely processed,  
96 dehydrated through a graded ethanol series, embedded in paraffin wax, sectioned (4 µm), stained  
97 with hematoxylin-eosin and Giemsa solution and subsequently mounted on slides.

98 Since the scoring system, proposed by Clark & Nowak (1999) for Amoebic Gill Disease, doesn't fit  
99 with lesion observed in NGD outbreaks, a new score system is proposed. The first left gill arch of  
100 each fish was subjected to histological analysis with Giemsa stain and rated, with a score to 0 from  
101 5, according to the severity and spread of hyperplastic lesions (Table 2) and the number of amoebae  
102 observed on the epithelial surface (Table 3).

103 Significant lesions were limited to the gills. The macroscopic observations revealed swelling,  
104 excessive production of mucus and multiple whitish nodules (around 1 mm diameter) localized  
105 mainly in the distal part of filament (Fig. 1) on a third of collected fish. Gross gill lesions seemed to  
106 be less severe compared to those observed in the rainbow trout sampled in other tanks of the same  
107 farms. An external fungal infection caused by *Saprolegnia* sp. was observed on the skin and gills in  
108 over half of the brown trout in tank 1. Other external and internal lesions were not evidenced during  
109 the necropsy.

110 Portion of the gills examined under light microscopy, exhibited swollen and clubbed profile,  
111 especially in the distal part for extensive cellular proliferation. In the most severe cases several  
112 filaments (from 2 to 4) coalesced into a single mass (Fig. 2). Affected filaments were covered with a  
113 thick layer of mucus. The gill surface revealed a low number of amoeboid organisms, particularly  
114 associated to the hyperplastic tissue. The size of the oval to round-shaped amoebae varied from 15  
115 to 20  $\mu\text{m}$  (Fig. 3). It was not possible identify the amoebae on the basis of morphological features.  
116 Several monogenean trematodes belonging to *Gyrodactylus* spp. were detected at the microscopic  
117 examination of skin scrapings in more than half of brown trout collected in tank 1.

118 Histological examination of diseased and moribund samples of brown trout showed characteristic  
119 pathological signs of NGD infection. The most significant change was the development of gill  
120 lesions due to multifocal hyperplasia of mucous cells and gill epithelium. This resulted in extensive  
121 lamellar fusion, especially in the distal part of filaments, with obliteration of interlamellar spaces  
122 (Fig. 4, Fig. 5). The epithelial cells showed, in more severe cases, hypertrophy, nuclear  
123 pleomorphism (anisokaryosis), necrosis and superficial desquamation. Spongiosis was also noted  
124 particularly in the distant portion of the filaments (Fig. 6). Additionally, there were many findings of  
125 hypertrophic mucous cells. Inflammatory infiltrate with several lymphocytes, macrophages and  
126 eosinophilic granule cells/mast cells (EGC) were seen within the filamental interstitium between the  
127 fused lamellae.

128 A limited number of amoebic organisms was detected along the surface of the gills (Fig. 7). In most  
129 cases, the parasites were included within hyperplastic epithelium (Fig. 8). The amoebae appeared  
130 uniform and pleomorphic with a proper nucleus and an eosinophilic cytoplasm. The amoeba cells in  
131 Giemsa stained sections presented a polyhedral structure with basophilic nucleus of 3-4  $\mu\text{m}$  of  
132 diameter surrounded by an achromatic halo. Cytoplasm was vacuolated showing numerous  
133 inclusions, a large numbers of basophilic spherical bodies and in fewer quantities eosinophilic.  
134 There was no evidence of amebae in internal organs.

135 The histological evaluation of the gills in affected brown trout reveals slight hyperplastic lesion  
136 with a score between 1 and 2. The histologic gill lesions in affected rainbow trout, reared in the  
137 same farms with similar environmental conditions, reached a score lesion between 4 and 5.  
138 Similarly, isolated groups of amoebae were detected on the tips of the filaments in brown trout  
139 (score between 1 and 2) whereas in the rainbow trout were observed numerous amoebae along the  
140 entire surface of the hyperplastic filaments (score 4).

141 Virological and bacteriological exams were negative in the investigated samples.

142 The signs observed by the light microscopy on fresh mounts and by histopathology of the infected  
143 brown trout gills confirm to be less serious than in affected rainbow trout of the same farm.  
144 Therefore, this investigation, which reports for the first time NGD in brown trout, has shown that  
145 this species would seem to be less susceptible to this pathology compared to recurrent episodes in  
146 rainbow trout.

147 It was not possible to establish whether it could be due to an effective resistance of the brown trout  
148 to the NGD or to a better environmental condition upstream, related to water parameters, amount of  
149 suspended solid and/or organic matter, environmental bacterial load, etc. The lack of descriptions on  
150 NGD in this species does not allow to evaluate the sensibility to the parasite. The disease was  
151 observed in only two farms and it is not possible with the currently available data to determine the  
152 trend and pathogenesis of NGD in brown trout.

153 The common denominator among the outbreaks was that the disease occurred at temperature <12°C  
154 and optimal oxygen concentrations (>9 mg/L) during the three months of sampling.

155 Despite the water temperatures have been registered higher in tank 2 than tank 1 and the pH  
156 resulted higher in the tank 1 than in the tank 2 during the survey, no apparent differences in  
157 mortality rates were evident (Tab. 1).

158 There are many gaps in the knowledge of this pathology, both at European and global level,  
159 including aspects of epidemiology of the disease, risk factors conditioning the emergence of clinical  
160 disease, reservoirs of pathogen, proper species identification, biology and life cycle of the parasite  
161 and aspects of the immunological response and pathological aspects in salmonids to the parasite as  
162 well as the pathogenesis and recovery from the disease (Rodgers 2014). Additional studies are  
163 necessary to better define the spread of NGD in freshwater salmonid farms, to evaluate the  
164 transmissibility of the etiological agent between the different species.

165 The episodes of NGD described by Speare (1999) in arctic char and Tubbs *et al.* (2010) in the  
166 Chinook salmon, showed that species of freshwater salmonids other than rainbow trout, may be  
167 heavily affected by this disease. Tubbs *et al.* (2010) reported a daily mortality rate of 1.5% in  
168 Chinook salmon fry concomitant with non-optimal water conditions due to the presence of  
169 suspended solids. Speare (1999) described a case of NGD and BGD co-infection in the Arctic char  
170 with high mortality rate.

171 In Northern Italy the trout farms produced different species of freshwater salmonids, such as brook  
172 trout (*Salvelinus fontinalis*), marble trout (*Salmo trutta marmoratus*), grayling (*Thymallus*  
173 *thymallus*) and Carpione del Garda (*Salmo carpio*) for commercial and restocking purposes in  
174 addition to rainbow and brown trout. In the absence of epidemiological studies, the spread of NGD  
175 in these species of salmonids needs to be further investigated, in order to assess a possible impact of  
176 NGD on wild fish populations when infected fish are restocked, since farming conditions amplified  
177 amoebae gill load.

178 Molecular investigations will be fundamental to isolate the eventual different species of amoebae  
179 observed in brown trout gills. Previous studies carried out on rainbow trout during NGD outbreaks,  
180 identify the presence of several species of naked (Dykova *et al.*, 2006) and testate (Dykova & Tylml,  
181 2010) amoebae, some considered potentially pathogenic and other only as accidental finding.  
182 Besides the “simple” species identification of amoebae, one of the next research challenge on this  
183 emerging disease will be the association of amoebic species to presence of lesions and/or  
184 pathological changes at the histology and the role of the environmental factors as diseases  
185 determinants.

186 The present study emphasizes the importance to carry out further investigations to understand the  
187 susceptibility of the different species of salmonids to NGD and the role of brown trout as potential  
188 reservoir of the disease.

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192

	March 2017		April 2017		May 2017	
	Tank1	Tank2	Tank1	Tank2	Tank1	Tank2
T (C°)	5.5 C°	8.9 C°	6.7 C°	9.7 C°	7.2 C°	11.2 C°
O <sub>2</sub> (mg/L)	9.2 mg/L	9.5 mg/L	9.6 mg/L	9.2 mg/L	9.3 mg/L	8.9 mg/L
pH	7.97	7.14	8.03	7.03	7.91	7.19
Monthly mortality (%)	0.7%	0.8%	0.9%	0.6%	0.5%	0.6%

193

194 **Table 1** Environmental parameters (temperature, dissolved oxygen and pH) and monthly mortality  
 195 monitored in tank 1 and tank 2 in the months of March, April and May 2018.  
 196

Gill Score	Histological description
0	Absence of proliferative lesions, filaments and lamellae present physiological appearance.
1	Slight epithelial hyperplasia, with lamellar fusions, limited to the tips of the filaments.
2	Less than half of the filaments are affected by epithelial hyperplasia for a third of their length.
3	Diffuse hyperplasia in half of the gill tissue, presence of sporadic fusions of filaments.
4	Severe hyperplasia diffused in most of the gill tissue, fusion of groups of 3 or 4 filaments.
5	Gill arch completely affected by proliferative reactions, fusion of 5 or more filaments.

197  
 198 **Table 2** Gill score system to estimate the severity and spread of hyperplastic lesions of NGD.

Amoeba Score	Histological description
0	Total absence of amoebae, both free and included in gill tissue.
1	Occasional, single amoebae included on the tips of the filaments
2	Rare groups of 3 or 4 amoebae located on the surface of the affected filaments.
3	Presence of numerous groups of 5 or more amoebae on the hyperplastic epithelium.
4	Rows of amoebae lined up side by side around affected filaments.
5	Clusters of amoebae completely surrounding proliferative tissue.

199  
 200 **Table 3** Amoeba score based on the number of amoebas observed along the surface of the filaments.

201  
 202  
 203  
 204

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