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ABSTRACT

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

Portion of the gills examined under light microscopy, exhibited swollen and clubbed profile, especially in the distal part for extensive cellular proliferation. In the most severe cases several filaments (from 2 to 4) coalesced into a single mass (Fig. 2). Affected filaments were covered with a thick layer of mucus. The gill surface revealed a low number of amoeboid organisms, particularly associated to the hyperplastic tissue. The size of the oval to round-shaped amoebae varied from 15 to 20 µm (Fig. 3). It was not possible identify the amoebae on the basis of morphological features.

Several monogenean trematodes belonging to *Gyrodactylus* spp. were detected at the microscopic examination of skin scrapings in more than half of brown trout collected in tank 1.

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

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

The histological evaluation of the gills in affected brown trout reveals slight hyperplastic lesion with a score between 1 and 2. The histologic gill lesions in affected rainbow trout, reared in the same farms with similar environmental conditions, reached a score lesion between 4 and 5.

Similarly, isolated groups of amoebae were detected on the tips of the filaments in brown trout (score between 1 and 2) whereas in the rainbow trout were observed numerous amoebae along the entire surface of the hyperplastic filaments (score 4).

Virological and bacteriological exams were negative in the investigated samples.

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

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

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

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

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

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

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

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

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

	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_2 (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%

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

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.

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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.

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

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