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Temporal changes in infections with some pathogens associated with gill disease in farmed Atlantic salmon (*Salmo salar* L)

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ABSTRACT

Gill disease (GD) in farmed Atlantic salmon may be due to the impact of several agents that may interact. We followed the infections of four gill disease-associated agents during the first year of production in six farms; examining the correlation between their densities in the gills. These farms were located in an area with high risk of developing gill disease, and three of the farms were diagnosed with GD in the autumn.

In the present study we show temporal changes in pathogens in salmon from all 6 farms, revealing recurring infection patterns by *Desmozoon lepeophtherii* and *Candidatus* Branchiomonas cysticola (C. B. cysticola), and large variation without an apparent pattern in densities of *Ichthyobodo salmonis*. Infections with the microsporidian *D. lepeophtherii* show a clear seasonal pattern, and high densities are associated with GD. Ca. B. cysticola appears not to be directly connected to the disease, albeit densities of this bacterium may coincide with high *D. lepeophtherii* densities. Amoebae (*Paramoeba* spp.) were only sporadically detected with low densities. Salmon with low condition had elevated densities of *D. lepeophtherii* and *Ichthyobodo* spp., while no such pattern was seen for the other pathogens.

Challenge studies are needed to substantiate the role of *D. lepeophtherii* in GD, but realistic challenge model (i.e. bath) has so far not been developed. Based on the present molecular screening, we suggest that *D. lepeophtherii* infections both may be responsible for GD, and cause runting in farmed salmon populations.

Statement of relevance: This is an epizootiological study of Atlantic salmon and gill diseases. There is much known about pathogen in connection with GD outbreak, less is known about the occurrence of potential pathogen in healthy farmed salmon or how such infections develop over time.

High densities of the microsporidian parasite *Paranucleospora theridion* in the gills coincide with gill disease, making *P. theridion* a possible primary source to this condition.

Densities of *Paranucleospora theridion*, *Candidatus* Branchiomonas cysticola and *Ichthyobodo salmonis* may be correlated in farmed salmon populations with gill disease.

P.theridion and *I. salmonis* densities are elevated in Atlantic salmon with low condition ('runts').

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1. Introduction

Gill disease (GD) is an increasing problem in Atlantic salmon (*Salmo salar*) seawater aquaculture in Norway (Kvellestad et al., 2005; Steinum et al., 2009; Steinum et al., 2010; Mitchell et al., 2013). Gill pathologies occur in the autumn five to seven months after spring smolt is transferred to sea (Steinum et al., 2010). Fish suffering from GD usually show pale gills, have poor appetite, low stress tolerance and show increased mortality (Nylund et al., 2008a; Steinum et al., 2010). There are numerous agents associated with GD and a range of clinical and pathological presentations (Kvellestad et al., 2005; Nylund et al., 2008a; Steinum et al., 2008; Steinum et al., 2010; Mitchell and Rodger, 2011; Rodger et al., 2011). GD with hyperplasia and infiltration of inflammatory cells is often referred to as 'proliferative gill inflammation' (PGI) (Kvellestad et al., 2005), while when gill inflammation is not apparent as 'proliferative gill disease' (PGD) (Nylund et al., 2008a). The common occurrence of large numbers of hypertrophic epithelial cells containing inclusions with intracellular bacteria (epitheliocysts) often

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cause the condition to be referred to as epitheliocystis. The most commonly identified agents associated with GD are the epitheliocystis forming bacteria *Candidatus* Branchiomonas cysticola (Mitchell et al., 2013), *Candidatus* Piscichlamydia salmonis (Draghi et al., 2004), salmonid gill poxvirus (SGPV), (Nylund et al., 2008b), the parasites *Ichthyobodo salmonis* (Isaksen et al., 2011) and *Desmozoon lepeophtherii* (syn. *Paranucleospora theridion*) (Nylund et al., 2011, Steinum et al., 2010). Infections with the amoeba *Desmozoon lepeophtherii* may also occur (Steinum et al., 2008), but amoebic gill disease (AGD) tend to show a distinct clinical and histopathological picture (Taylor et al., 2009; Adams et al., 2004; Munday et al., 1990). Atlantic salmon paramyxovirus (ASPV) infections, characterized from salmon with PGI (Kvellestad et al., 2005), have proved to be uncommon among fish with the disease (Nylund et al., 2011; Steinum et al., 2010). There is also strong evidence that Cand. P. salmonis infections, which may originate in freshwater (i.e. hatchery origin) {Draghi, 2010 #7730}, is not a major cause of GD (Mitchell and Rodger, 2011; Mitchell et al., 2013; Steinum et al., 2015; Toenshoff et al., 2012). Hence existing data pinpoints *D. lepeophtherii* and Ca. B. cysticola as the most likely causative agents of GD in addition to *P. perurans*. However, claims that the flagellate parasite *Ichthyobodo* sp. (likely *I. salmonis*, reported as *I. necator*) may cause GD was presented in the past, prior to the discovery of the other microbial agents (Ellis and Wootten, 1978; Poppe and Håstein, 1982; Urawa et al., 1998).

Elevated densities with the microsporidian *D. lepeophtherii* show an association with PGI (Steinum et al., 2010; Hamadi, 2011; Nylund et al., 2011). In addition to Atlantic salmon and other salmonids, this microsporidian infects also salmon lice (*Lepophtheirus salmonis*) (Freeman and Sommerville, 2009; Nylund et al., 2009; Nylund et al., 2010).

D. lepeophtherii has two development cycles in salmon, a systemic infection involving leucocytes and blood vessels endothelial cells where spores are produced in the cell-cytoplasm, and a second cycle confined to the nucleus of gill and skin epidermal cells resulting in intranuclear spores. Sporogony in phagocytes leads to their lysis, and the released autoinfective spores may subsequently infect new phagocytes or gill cells (Nylund et al., 2010). The epitheliocyst forming betaproteobacterium *Candidatus* Branchiomonas cysticola (Ca. B. cysticola) appears to be the main cyst-forming agent in epitheliocystis of Atlantic salmon in both Norway and Ireland (Mitchell and Rodger, 2011; Mitchell et al., 2013; Toenshoff et al., 2012). Steinum et al. (2010) found a relationship between Ca. B. cysticola load and increased numbers of hypertrophic epithelial cells containing inclusions with intracellular bacteria (epitheliocysts) (Steinum et al., 2015; Mitchell et al., 2013). However, infections with the bacterium are common also in apparently healthy salmon, and a causal relationship has not been shown.

Heavy infections of *Ichthyobodo* spp. are common in fish with GD (Ellis and Wootten, 1978; Poppe and Håstein, 1982; Isaksen et al., 2011). Infections of salmonids in the marine environment was in the past associated with *I. necator*, but recent studies has shown that *I. necator* is a freshwater flagellate, while another species, *I. salmonis*, infects salmonids in seawater (Isaksen et al., 2007; Isaksen et al., 2011). Hence, cases of ichthyobodosis in seawater farms were likely associated with *I. salmonis* and not *I. necator* (Isaksen et al., 2011). It now appears possible that some of these GD cases were caused by other agents. Studies on GD tend to consider these flagellate infections as secondary (Kvellestad et al., 2005; Rodger et al., 2011; Mitchell et al., 2013).

A major reason that any causative agent(s) of GD (except AGD), has not been identified is that controlled challenge experiments with the candidate pathogens have been difficult to perform. No realistic and controllable challenge models with *D. lepeophtherii* or Ca. B. cysticola has been published.

Studies on GD in seawater reared salmon emphasizes that the condition is multifactorial (Mitchell and Rodger, 2011; Nylund et al., 2011; Steinum et al., 2010; Kvellestad et al., 2005; Steinum et al., 2015), with several agents involved in, or responsible for pathology and disease. Synergistic effects may be important, such as immune-modulation or pathology due to one pathogen may favor infections with others (Wongtavatchai et al., 1995a; Wongtavatchai et al., 1995b; Miller, 2014; Miller and Kiss, 2014; Hebert-Dufresne and Althouse, 2015). While past studies have revealed an suite of agents associated with GD, an consideration of their importance suffer from a lack of information on the occurrence and dynamics of these potential gill pathogens in a healthy farmed salmon (Steinum et al., 2010; Aunsmo, 2009; Snow, 2011). Therefore, we embarked on a PCR based screening study where we followed some selected GD associated agents in several farms during the first year of a production, the period when GD tend to occur. In the study area in western Norway, it was very likely that some populations would develop GD. Hence, our aim was to examine the infection dynamics and co-variation in pathogen densities in the gills of Atlantic salmon from farms with and without GD, providing evidence for causality.

2. Material and methods

2.1. Samples

Atlantic salmon (*Salmo salar*) transferred to sea in the spring of 2011 were sampled from six farming sites in western Norway (Hordaland, Sogn & Fjordane counties) (Fig. 1). The farming sites were all located in areas with a history of repeated outbreaks of GD. We aimed at sampling all populations at four different sampling dates, 30 fish at each sampling, during the first year at sea, summer (July–August), autumn (September–October), winter (February) and spring (May) (Table 1).

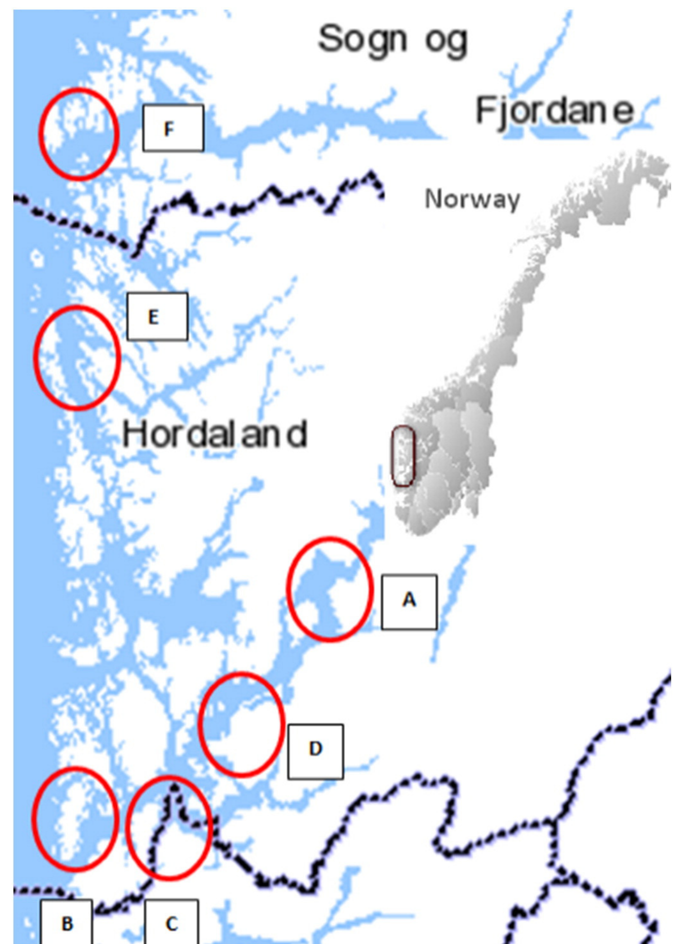

Fig. 1. Areas surrounded by a circle showing where the farms were located.

Table 1
Salmon was sampled from six fish farms (A–F). Number (N) and sampling dates for each fish farm are presented in table.

Farm	Sea-transfer	Sample 1 'Summer'		Sample 2 'Autumn'		Sample 3 'Winter'		Sample 4 'Spring'	
Site	Month(s)	N	Date	N	Date	N	Date	N=	Date
A	April/May	27	29.8.2011	30	20.10.2011	20	13.2.2012	30	8.5.2012
B	April/May	31	26.8.2011	11	13.10.2011	30	17.2.2012	29	15.5.2012
C	April/May	30	25.8.2011	30	3.10.2011	30	15.2.2012	30	21.5.2012
D	April/May	30	25.8.2011	31	26.9.2011	30	21.2.2012	30	21.5.2012
E	May	30	11.8.2011	30	13.10.2011	30	28.2.2012	30	31.5.2012
F	April/May	10	2.7.2011	10	16.9.2011	30	22.2.2012	30	29.5.2012

Large samples of fish were caught by purse seine tailings and 30 fish were randomly selected. All 30 fish were killed by blow to their head and each individual was put into a single plastic bag that was individually labeled and thereafter frozen pending transportation to the laboratory. Near surface sea temperature (3 to 5 m depth) and monthly mortality were registered by personal at the farms. GD diagnoses were provided by the responsible veterinarians and fish health personnel at the farms. Gill samples were taken immediately after the salmon was thawed. The fish were then measured (length, weight) and examined for possible signs of disease. Condition factor (CF) was calculated by the formula $CF = weight\ (g) * 100 / (length\ (cm))^3$.

2.2. Analyses

All samples were tested for *Desmozoon lepeophtherii*, *Candidatus* Branchiomonas cysticola, *Ichthyobodo* spp., *Paramoeba* spp. and Piscine orthoreovirus (PRV) on the basis of RNA using reverse-transcription real-time polymerase chain reaction (RT-PCR) (see below).

2.3. RNA and DNA extraction

RNA from gills was extracted in the following way: 50 to 100 mg of tissue was homogenized by adding 1 ml of Isol-RNA Lysis Reagent (5 Prime) to each sample and shaking the tubes in a Qiagen TissueLyser LT at 50 Hz for 5 min. The samples were placed on the benchtop for 5 min to promote dissociation of the nucleoprotein complexes. 0.2 ml chloroform was added to the homogenate; the tubes were vigorously vortexed for 15 s and placed on the benchtop again for 5 min. The samples were centrifuged at 13,800 ×*g* for 15 min at 4 °C for phase separation and 200–450 μl of the upper, colorless, aqueous phase, which contains RNA, was transferred to a new tube. The RNA was precipitated by adding 500 μl of isopropanol (2-propanol), vortexing for 15 s and incubating the samples for 10 min at room temperature. Samples were centrifuged at 13,800 ×*g* for 15 min at 4 °C, which resulted in a pellet of RNA at the bottom/side of the tube. The supernatant was removed, 1 ml of 75% ethanol was added and the tubes were spun for 5 min at 13,800 ×*g* (4 °C). The ethanol was removed and another washing step was performed by adding 1 ml of 99% ethanol and spinning for 5 min at 8700 ×*g* (4 °C). The supernatant was removed and the RNA pellet air-dried for about 10 min, before being dissolved in an appropriate amount of RNase-free water, which had been heated to 70 °C. All RNA samples were stored at −20 °C until further use.

DNA was extracted from tissues using the DNA kit from Omega Biotec as outlined in the E.Z.N.A tissue DNA kit protocol.

2.4. Real time RT PCR

The AgPath-ID™ one step RT-PCR kit (ThermoFisher Scientific) was used to test the extracted RNA from the gill tissues for the presence of pathogens using the following real time RT PCR assays: the 'Nuc' assay for *D. lepeophtherii* 16S (Nylund et al., 2010), the 'Costia' assay for *Ichthyobodo* spp. 18S (Isaksen et al., 2012), the NeoNy assay for *Paramoeba* spp. (F: TTG TCA GAG GTG AAA TTC TTG GA TT, probe: ATG AAA GAC GAA CTT CTG, R: TGA AAA CAT CTT TGG CAA ATG C), the Epit assay for *Candidatus* Branchiomonas cysticola (F: GAG TAA TAC ATC GGA ACG TGT CTA GTG, probe: ACT TAG CGA AAG TTA AGC, R: CTT TCC TCT CCC AAG CTT ATG C), and the PRV-M2 assay for Piscine orthoreovirus (PRV) (F: CAA TCG CAA GGT CTG ATG CA, probe: CTG GCT CAA CTC TC, R: GGG TTC TGT GCT GGA GAT GAG). The housekeeping gene elongation factor 1 alpha ($EF1A_A$ assay) was used as an internal control (Olsvik et al., 2005).

Standard curves were generated using 10-fold serial dilutions of RNA in three parallels. Regression analysis, standard curve slopes s (cycle threshold, Ct, versus log quantity), amplification efficiency E ($E = [10^{1\ /\ (-slope)}] - 1$), and the coefficient of determination, R^2, were calculated for all assays (Muller et al., 2002; Simon, 2003).

Each run of the real time RT PCR consisted of 45 cycles and the samples were considered positive when the fluorescence signal increased above a set threshold of 0.1. Negative controls, RNA extraction controls (lacking target RNA) and no template control, were included in all runs at a rate of 1 control per 10 samples.

2.5. Relative quantification

The C_T's of target and endogenous control (EF1A, reference gene) and PCR amplification efficiencies (E) were used to calculate the normalized expression (NE) of target RNA from the different pathogens using

$$(NE = (E_{reference})^{CT}{}_{reference} / (E_{target})^{CT}{}_{target})$$

NE, a measure of density of the different pathogens in the gill tissue samples, was used as a proxy for abundance (Bush et al., 1997) in the analyses.

The primers and probes used were obtained from Life Technologies (https://www.lifetechnologies.com), and the amounts of probes and primers were optimized for each assay. The real-time master mixture consisted of 6.25 μl 2 × RT-PCR buffer together with 0.25 μl of 25 × RT-PCR enzyme. Primers and probes at their respective concentrations were added to the master mixture and adjusted with double-distilled H_2O to a total volume of 10.5 μl prior to adding 2 μl of RNA template. The real-time RT-PCR reaction were run in a 7500 and a 7500 Fast Real-Time PCR System cycler (Applied Biosystems) using the following conditions: reverse transcription at 40 °C for 10 min followed by activation of the DNA polymerase at 95 °C for 15 min prior to amplification with 45 cycles of 95 °C for 15 s and 60 °C for 45 s (denaturation and annealing/extension). The threshold was fixed for all runs, and samples above the threshold were considered positive. Negative controls (RNA extraction controls lacking RNA, and no template controls) were included in all runs to avoid false positives.

2.6. Statistical testing

Prevalence was compared using Chi square or Fisher's Exact tests (in cases of low expected values). Bonferroni corrections were employed in multiple comparisons (temporal changes). Temporal changes in density (NE) were examined using analysis of variance (ANOVA). The NE data (0 when negative) were usually non-normal, and heteroscedastic

(Levene's tests). Therefore, the data were log10 (NE + 1) transformed. Post-hoc testing was performed with Newman-Student-Keul's tests (NSK). Correlations were examined using Spearman's rank-order correlation coefficients (r_s). The Kendall coefficient of Concordance (W) (Siegel and Castellan, 1988) was used to examine concordance in temporal pathogen density levels.

2.7. Diagnosis of GD

Gill tissue for histology was not collected in the fish farms. It was therefore not possible to give precise diagnosis on type of GD. Clinical description from the fish farms health personnel was increased mortality, decreased appetite and findings of individuals with lethargy, swimming close to the water surface and with increased respiratory rate. Gross pathological findings were areas of thickened or paler gills. The expression gill disease (GD) was chosen to describe the gill condition the salmon was suffering from.

3. Results

3.1. Temperature

The variation in temperature followed the same pattern at all farming sites during the production period (Fig. 2). The warmest period was in July–August and the lowest temperatures were registered in January.

3.2. Mortality

Mortality varied between farms throughout this study (Fig. 3).

GD was associated with elevated mortality in fishfarm C, D, E (September–November) whereas no GD was seen in farm A, B and F (Table 2). In this period the average cumulative mortality in the diseased salmon populations was more than four times higher than in those that appeared to be healthy (5% versus 1.2%, respectively) (Fig. 3 & Table 2).

Farm B had elevated mortality in April–June caused by Infectious pancreas necrosis (IPN). A peak in mortality occurred in farms A and E in April–July caused by pancreas disease (PD). High mortality arose in Farm D in Jan.–Feb., with unknown cause. Lowest mortality, mean across all farms, was in December, January and February (0.5%) but the highest mortality, mean across all farms, was in August, September and October (1.02%). The average accumulated mortality was 11.9%. Highest cumulative mortality was in farm E (22%), and lowest mortality was in farm F (5.4%).

3.3. *Desmozoon lepeohtherii*

Prevalence of *D. lepeophtherii* was 100% in all samples from the six farms in this study.

However, *D. lepeophtherii* NE varied significantly, with a pattern concordant across farms (W = 0.43, $p < 0.01$). NE was higher in summer and particularly in autumn than in the following winter and spring samples ($p < 0.05$) (Fig. 4). At peak *D. lepeophtherii* NE in autumn, salmon from farms C, D and E showed highest *D. lepeophtherii* NE ($p < 0.05$) (Fig. 4). These farms were diagnosed with GD. The salmon in these farms showed an on average 3.7 fold higher density of *D. lepeophtherii* than those from farms with no such diagnosis.

3.4. *Candidatus Branchiomonas cysticola (Ca. B. cysticola)*

Ca. B. cysticola infections were detected in all samples (Table 3). Five of the farms showed a high Ca. B. cysticola prevalence (96–100%) throughout this study. The sixth, Farm D, showed a significant decrease in prevalence from 100% in winter to 87% in the spring ($p < 0.05$).

The Ca. B. cysticola mean NE was not temporally concordant in the salmon samples from the 6 farms (W = 0.11, NS). Overall, NE was higher in summer and autumn than in winter and spring ($p < 0.05$; Fig. 4). A decrease (1.4–13 fold) in NE was seen from autumn to winter in three farms (A, E, F), while the salmon from two other farms had a peak in density in summer followed by a decrease (9–352 fold) in autumn (farms B, C; $p < 0.05$ and $p < 0.001$, respectively). There was no significant temporal variation in Ca. B. cysticola NE in farm D.

3.5. *Ichthyobodo* spp.

A considerable variation in prevalence of *Ichthyobodo* spp. was observed but all the farmed populations were at some point infected with the flagellate. There was no clear pattern detected in prevalence of this parasite in the study period (Table 3).

Ichthyobodo spp. mean NE were not concordant among farms (W = 0.28, NS), and did not change significantly during the study in most farms (Fig. 4). The exception was fish sampled from farm E (farm with GD diagnosis) that showed a marked increase (213 fold) from summer to autumn ($p < 0.001$), followed by a decrease (127 fold) to winter ($p < 0.001$).

3.6. *Paramoeba* spp.

Paramoeba spp. infections were only occasionally detected in salmon sampled from three farms (A, B, D), in August and May (Table 3), with low NE.

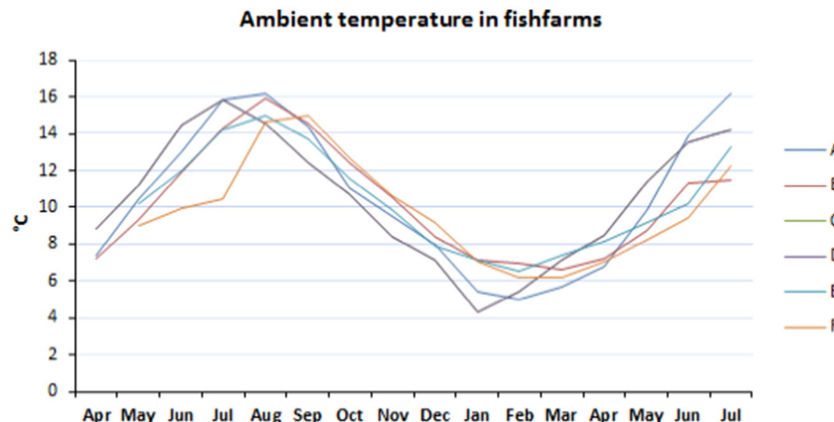

Fig. 2. Ambient temperatures in the period, April 2011 to July 2012 for all the six farms included in this study.

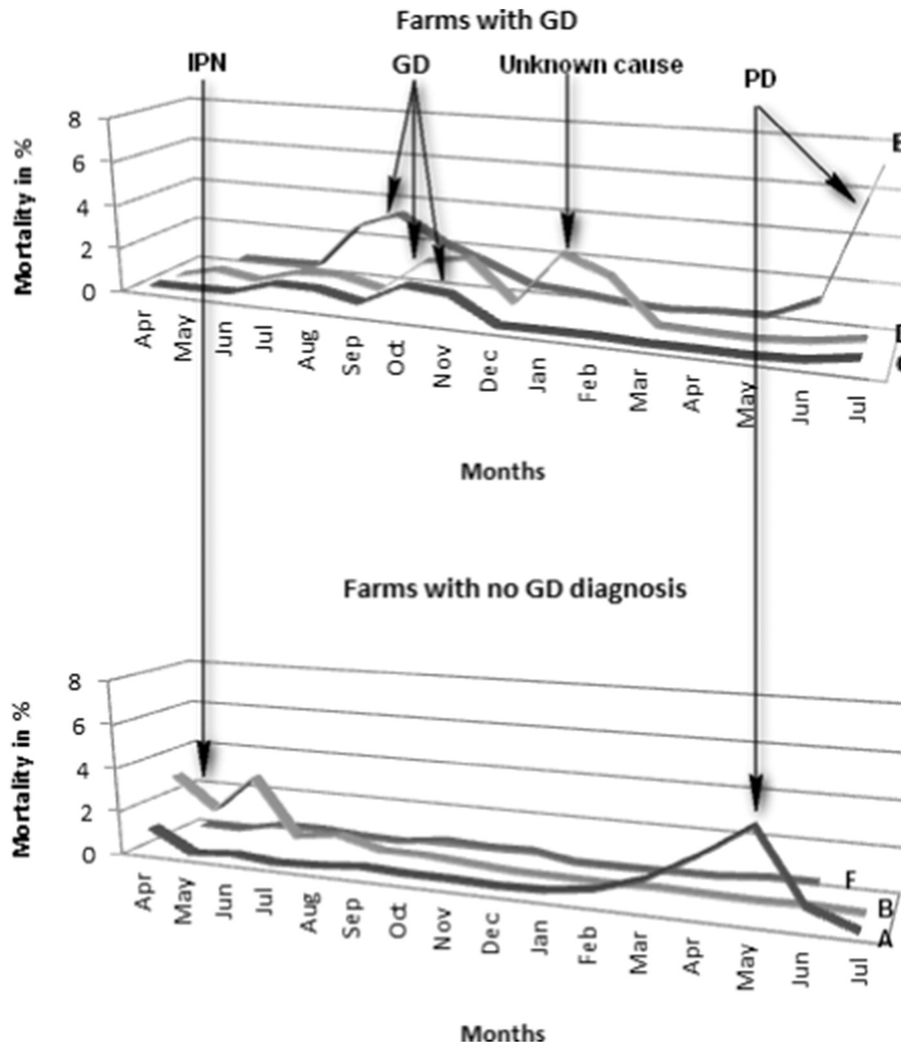

Fig. 3. Mortality given as percentage in the period April 2011 to July 2012. Mortality peaks associated with diseases are marked with an arrow. Abbreviations are IPN (Infectious pancreas necrosis), GD (gill disease) and pancreas disease (PD).

3.7. Piscine orthoreovirus (PRV)

PRV prevalence based on the analyzed gill samples varied between 69% and 100% (Table 3). Three farms (A, D, F) had no temporal variation whereas two showed significant drop from winter-spring (B, E) ($p < 0.05$). Farm C had a significant increase in prevalence from summer-winter ($p < 0.05$).

Table 2
The period of gill disease (GD) outbreak (September–November). The numbers are mortality in % per month and the column to the right is accumulative mortality in % in the disease period. Diagnoses are from veterinarians and fish health personnel serving the fish farms. Information on mortality is from the farming sites. Farms marked with bold digits and shaded with gray color are farms with GD diagnosis.

Farm	September	October	November	Accumulated mortality in %
A	0.34	0.25	0.24	0.83
B	0.48	0.46	0.3	1.24
F	0.34	0.6	0.49	1.43
Average	0.39	0.44	0.34	1.17
C	**0.44**	**1.42**	**1.24**	**3.10**
D	**0.44**	**1.91**	**2.26**	**4.61**
E	**3.43**	**2.25**	**1.52**	**7.20**
Average	**1.44**	**1.86**	**1.67**	**4.97**

PRV mean NE was concordant ($W = 0.47$, $p < 0.05$), the pattern being elevated densities summer-autumn followed by decrease to low levels in spring. PRV NE differed between salmon in autumn where farms with GD had three fold higher PRV mean NE than those without this diagnosis ($p < 0.05$) (Fig. 4).

3.8. Relationship between pathogens

D. lepeophtherii, Ca. B. cysticola and PRV showed high prevalence (70 to 100%) throughout this study (Table 3). *Ichthyobodo* spp. prevalence was variable, while *Paramoeba* spp. infections were infrequent. Correlations between the most prevalent agents were examined when present in the highest densities, in autumn (Fig. 4). In this period, *D. lepeophtherii* densities were generally high, while Ca. B. cysticola densities were particularly high in fish from three farms (A,E,F). Their overall correlation in these 3 samples was significant ($r_s = 0.29$, $p < 0.02$). The relationship was strongest in farm A ($r_s = 0.77$, $p < 0.001$), but $r_s > 0.1$ in all 6 farms this period. The relationship between *D. lepeophtherii* and PRV NE was examined in the four farms with highest PRV densities. Densities were significantly correlated in one farm (B, $r_s = 0.39$), but not supported by $r_s > 0$ in the other farms (r_s range-0.31–0.08). High Ca. B. cysticola NE coincided with high PRV NE in salmon from farm E in the autumn, and densities were then significantly correlated ($r_s =$

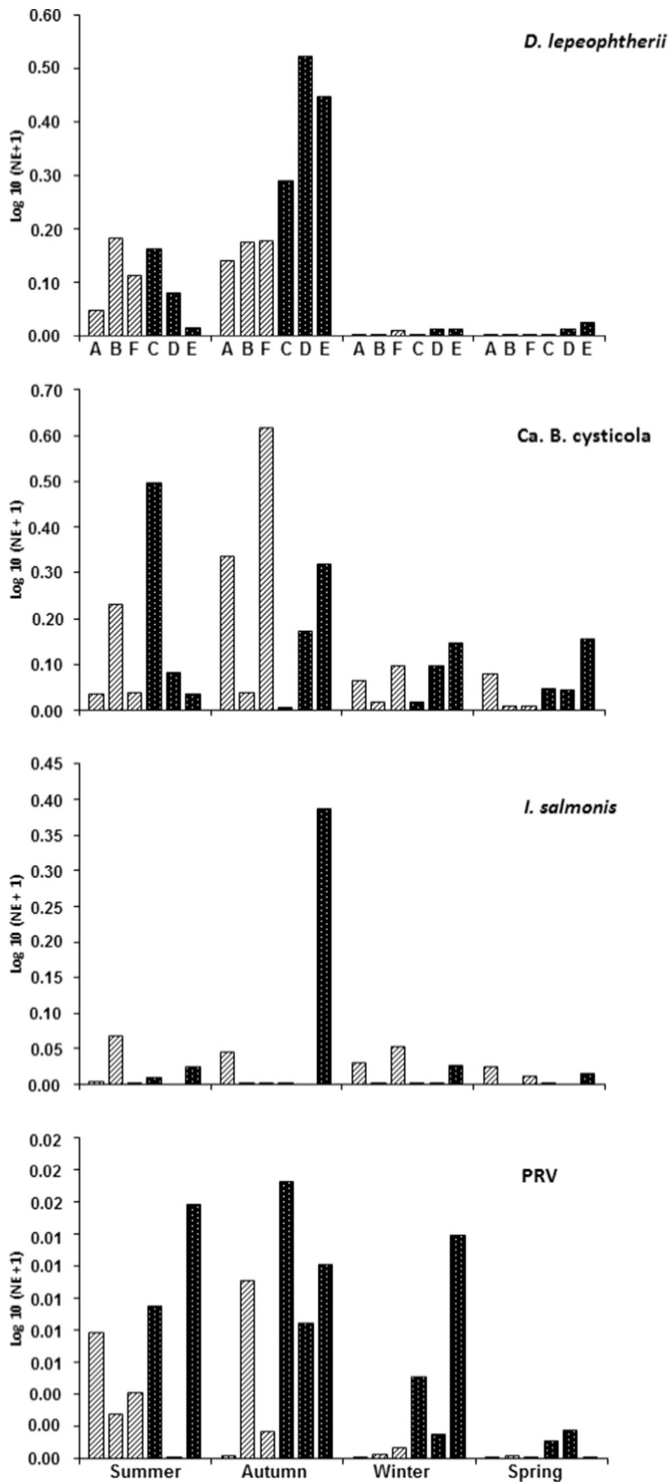

Fig. 4. Temporal changes in pathogen density in the six studied fish farms. Columns representing farms that were diagnosed with GD during the study are shown with black background and bright dots. At the top normalized expression (NE) for*D. lepeophtherii* and then the following order Ca. B. cysticola, *Ichthyobodo* spp. and PRV. Letters in top graph indicate different farms and at bottom the seasonal samples.

0.56, $p < 0.01$). *Ichthyobodo* spp. showed particularly high densities in farm E in the autumn. At that time NE was significantly correlated with *D. lepeophtherii* ($r_s = 0.46$, $p < 0.01$), and Ca. B. cysticola ($r_s = 0.33$, $P = 0.05$), but not with PRV NE ($r_s = 0.08$).

Table 3
Fish farms and prevalence of pathogens from August 2011 to July 2012. The prevalence of *D. lepeophtherii* (not shown) was 100% in all samples throughout the study period. Abbreviations for the pathogens are *Candidatus* Branchiomonas cysticola ('CBC'), *Ichthyobodo* spp. ('Ich.'), Piscine orthoreovirus (PRV) and *Paramoeba* spp. ('Para').

Farm	Sampling	CBC %	Ich %	Para %	PRV %
A	Summer	100	96	0	100
	Autumn	100	83	0	96
	Winter	100	100	0	100
	Spring	100	80	17	93
B	Summer	100	55	10	90
	Autumn	96	70	0	100
	Winter	97	37	0	100
	Spring	10	0	14	69
C	Summer	100	83	0	80
	Autumn	100	47	0	100
	Winter	100	73	0	97
	Spring	97	30	0	93
D	Summer	100	0	0	87
	Autumn	100	0	0	100
	Winter	100	60	0	97
	Spring	83	0	8	96
E	Summer	100	100	0	100
	Autumn	100	97	0	97
	Winter	100	77	0	100
	Spring	100	77	0	87
F	Summer	100	30	0	100
	Autumn	100	20	0	100
	Winter	100	100	0	100
	Spring	97	83	0	100

3.9. Relationship between pathogens and condition factor (CF)

A relationship was observed between low CF, *D. lepeophtherii* and *Ichthyobodo* spp. density summer-autumn (ANCOVA, $F_{1,280} = 25.0$, $p < 0.001$; $F_{1,280} = 8.4$, $p < 0.01$). The relationship is visualized in CF categories in Fig. 5. Average *D. lepeophtherii* NE were 2–7 fold higher in salmon with low CF (Fig. 5) (ANOVA, $F = 29$, $p << 0.0001$). *Ichthyobodo* spp. density in salmon with low CF, was between 14 and 37 folds higher in the summer and between 26 and 267 folds higher in the autumn samples ($F = 14$, $p < 0.001$). Ca. B. cysticola, *P. perurans* and PRV did not show any significant relationships with CF.

3.10. Association with GD diagnoses

Three farms were diagnosed with GD during this study, in the autumn period. We therefore examined the NE of the different agents in farms with GD versus farms without that diagnosis in the autumn. Densities of *D. lepeophtherii* (MW, $p << 0.001$), *Ichthyobodo* spp. ($p < 0.05$)

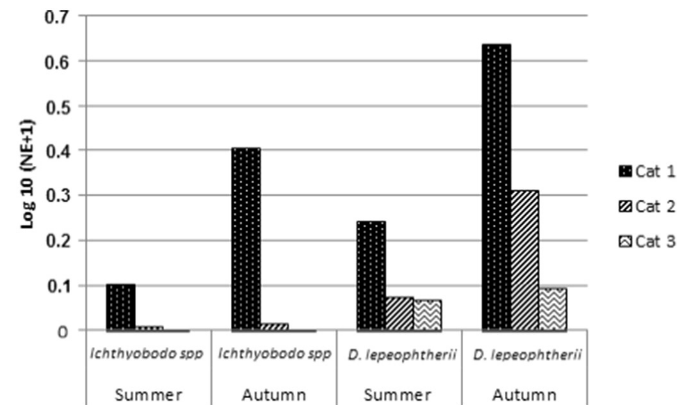

Fig. 5. *D. lepeophtherii* and *Ichthyobodo* spp. density (NE) in salmon with different condition factor. CF categories: Low (Cat 1), CF < mean-1SD, Normal (Cat 2), mean-1SD < CF < mean + 1SD, high (Cat 3), CF > mean + 1SD. SD calculated sample vice.

and PRV ($p < 0.001$) were significantly higher in fish with GD, the average difference being 2.6, 7.2 and 2.3 fold, respectively.

4. Discussion

While much is known about pathogens found in connection with diseases outbreaks, much less is known about the occurrence of potential pathogens in a healthy farmed salmon, or how such infections develop over time (Steinum et al., 2010; Aunsmo, 2009; Crockford et al., 1999).

We followed the infections of four gill disease-associated agents, using RT PCR, during the first year of production in six farms. These farms were located in an area with high risk of developing gill disease, and three of the farms were diagnosed with GD in autumn, in one farm the diseases was apparent already in August. This allowed us to examine the densities of the studied agents in farms with this diagnosis, versus those without. It has been suggested that GD is due to the impact of several agents that may interact (Steinum et al., 2010, Steinum et al., 2009, Rodger et al., 2011). This hypothesis was tested, by examining the correlations between the densities of the different agents during the period with GD. During preliminary testing for infections in the present fish groups, an apparent association of high PRV levels with gill disease was noted, leading us to also analyze the fish for this virus, albeit not known to be a gill pathogen.

4.1. *Desmozoon lepeophtherii*

The most prevalent agent detected was the microsporidian *Paranucleospora theridion*, always being present in our samples. Since all fish in all farms were infected in our first samples ('Summer', July–August), some 12–16 weeks after sea transfer, the infection pressure with this parasite must be tremendous in western Norway. The parasite showed a clear pattern in infection densities, high in summer, peaking in autumn and then falling to relatively low levels in the subsequent winter-spring samples. The results from our study supports the results from a previous study by Sveen et al. (2012), who found that *D. lepeophtherii* density was highest in autumn (high temperatures) and lowest in winter and spring (low temperatures). This pattern is likely temperature dependent, propagating infections acquired during summer-autumn, being arrested at low temperatures (Nylund et al., 2011, Sveen et al., 2012).

The high levels in autumn coincide with the period of GD problems in Western-Norway. We observed that the densities of this parasite were particularly high in the fish populations diagnosed with GD, some 4 fold higher. This observation reinforce previous observations to the same effect (Nylund et al., 2011), and an observation by Steinum et al. (2010) that 14 fish individuals with PGI had some 30 + times higher load of the microsporidian than fish without the disease (Steinum et al., 2010).

Matthews et al. (2013) found an association between the presence of minute cytoplasmic spores of the microsporidian and interlamellar hyperplasia in the gills. Cytoplasmic propagation, common also in phagocytes, appears to culminate in the lysis of the infected cell (Nylund et al., 2010). How the parasite may induce hyperplasia is not known. However, the lysis of important immune cells (phagocytes) suggests that *D. lepeophtherii* infections may affect immune functions in the host (Nylund et al., 2009, Nylund et al., 2010, Steinum et al., 2015). There is evidence that other microsporidia of fish may be immunosuppressive (Laudan et al., 1986; Wongtavatchai et al., 1995a,b). Immunosuppression is one way that *D. lepeophtherii* infections may affect infections with other agents in cases of GD, both by increasing susceptibility and/or facilitate proliferation of pathogens already present in the fish (Magnadottir, 2006, Sitja-Bobadilla, 2008). A typical finding is concurrent epitheliocystis, and less frequently *Ichthyobodo salmonis* infections (see below).

4.2. *Candidatus Branchiomonas cysticola*

Candidatus Branchiomonas cysticola (Ca. B. cysticola) is the most common epitheliocystis forming bacterium in the gills of both healthy and gill diseased salmon in Norway and Ireland (Steinum et al., 2015, Mitchell et al., 2013, Toenshoff et al., 2012). The prevalence of Ca. B. cysticola in this study was very high throughout the study period. Densities were highly variable, with a tendency for higher densities summer-autumn. However, The densities of Ca. B. cysticola in summer and autumn were not significantly different between fish with GD and healthy fish ($p > 0.05$), and some farms with high Ca. B. cysticola NE showed relatively low mortality. Hence our results do not show a clear coincidence between gill disease diagnoses and high Ca. B. cysticola densities. This parasitic bacterium appears to show seasonality in occurrence similar to *D. lepeophtherii*, and tendencies for a positive correlation of Ca. B. cysticola with *D. lepeophtherii* do suggest that this agent may benefit from concurrent infections with the other (facilitation). It has been suggested that Ca. B. cysticola could be the aethiological agent for PGI (Mitchell et al., 2013, Toenshoff et al., 2012). No such association was found with the observed GD in this study.

4.3. *Ichthyobodo spp.*

Ichthyobodo spp. are ectoparasite flagellates that infect wide range of marine fish species worldwide, and has repeatedly been implicated in diseases and mortality among ornamental and farmed fish (Urawa et al., 1998). We observed *Ichthyobodo* spp. infections in all farms and seasons, but did not detect any pattern in flagellate densities. A single farm ('E') showed a significant peak in *Ichthyobodo* spp. density, in October during a GD outbreak with high mortality, but another farm with gill disease and high mortality in this period had no *Ichthyobodo* spp. infection. At high prevalence and peak density in farm E in October, there was no correlation between *Ichthyobodo* spp. densities and the gill pathogens *D. lepeophtherii* and Ca. B. cysticola. This observation does not exclude the possibility that infections with this flagellate are opportunistic, but seems to preclude a direct link to these agents. Clearly, *Ichthyobodo* spp. infections were not responsible for the gill disease cases involved in the present study. This also shed doubt on past studies, prior to the discovery of most of the GD-associated microorganisms, that *Ichthyobodo necator* (i.e. *I. salmonis*) caused a proliferative gill disease with high mortality (Ellis and Wootten, 1978; Poppe and Håstein, 1982).

4.4. *Paramoeba perurans*

Amoebic gill disease, caused by *Paramoeba perurans*, was unknown to Norwegian aquaculture until 2006, when 4 cases were registered. In late autumn 2012, AGD was again diagnosed at five fish farm sites at the South-West coast of Norway. The pattern in 2012–2014 has been that the amoeba emerge on the salmon in autumn, and disappear (fish real-time PCR negative) during winter (Karlsbakk et al., 2015). Hence our few *Paramoeba* sp. positive salmon gills in 2011–12 occurred in a period with no registered AGD outbreaks and in the case of the positive fish in May, at a time of year when *P. perurans* infections tend to be absent (Karlsbakk et al., 2015). The rare and slight detections could therefore represent amoebae from the water, and possibly other *Paramoeba* spp. than *P. perurans*.

4.5. *PRV*

Piscine orthoreovirus (PRV) is most likely the causative agent of heart and skeletal muscle inflammation (HSMI) an important disease in farmed Atlantic salmon in Norway (Kongtorp et al., 2004, Palacios et al., 2010). The virus appears to predominantly infect erythrocytes the first weeks after infection, with a subsequent increase in density in

heart and skeletal musculature after 6–8 weeks (Finstad et al., 2014). The mechanisms by which PRV causes disease are largely unknown (Finstad et al., 2014), but gills are not known to be involved. Lovoll et al. (2012) found in their study that 36% of the pre-smolt were infected by PRV and that there is a general increase in virus load shortly after transport to sea (Lovoll et al., 2012). Hence, a PRV infection soon after sea-transfer in our study is not surprising. At our first sampling in August PRV infections were already established with prevalence between 80 and 100% based on our gill analyses. The likely explanation is PRV infected blood cells in the gills (Finstad et al., 2014). Therefore, the significant temporal pattern in PRV densities in the gills was likely due to varying viremia, peaking in autumn and declining winter-spring. Following this, a peak density of PRV in the gills in our autumn sample could simply reflect the common PRV dynamics in the blood. However, higher densities of PRV were observed in the fish populations with GD and elevated *D. lepeophtherii* densities, but the densities of these agents were not clearly correlated. PRV is not known to replicate in any gill cells, while D. lepeoptherii develop both in the cytoplasm and in the nuclei of gill epithelial cells. PRV does however replicate in red blood cells, during the months following infection. Hence, our interpretation is that the elevated D. lepeoptherii levels are due to true gill infections, while the PRV levels reflect a peaking viremia in the same period. A higher level of PRV in the three cases with gill disease is interesting, and could reflect a weakened immune system in the diseased fish, allowing increase viral replication. However, if that was the case, a correlation of PRV levels with *P. theridion* density could be expected. That was not observed. Also, the studied populations were not subsequently diagnosed with HSMI, so there is no evidence that the PRV infections led to disease.

4.6. Pathogens and host condition factor (CF)

Salmon with low CF had significantly higher densities with the microsporidian parasite *D. lepeophtherii* and the flagellate *Ichthyobodo* spp. whereas there was no such connection observed for Ca. B. cysticola, *P. perurans* and PRV.

Salmon with low CF tends to stay in the upper part of the water column in the pens, and one possibility is exposure to a higher infection pressure there. Runting is a well-known phenomenon in salmon farms, often appearing following disease episodes such as IPN (Infectious pancreas necrosis) and pancreas disease (PD) (Lillehammer et al., 2013, Mc Vicar, 1987), or due to *Parvicapsula pseudobranchicola* infections (Hansen et al., 2015). The present observations suggest that also *D. lepeophtherii* infections could be responsible for this problem.

4.7. Shortcoming of this study

The main purpose of the project was to describe, using RT PCR, development of four known pathogens associated with gill disease for one year. Tissue for histopathology was not sampled, this might be considered to be a shortcoming of the study. However, the main point was to follow development of four known gill pathogens over time.

Correlation testing was used to reveal relationships between RNA levels of the different pathogens. Possible drawbacks could be the detection of RNA also from the environment, or from dead and disintegrating parasites such as *D. lepeoptherii* during spring-summer. It is also important to note that correlation does not prove causality.

GD has been known for decades in Norway but causality is unknown. In resent years, with new technology, a suite of agents have been found to be associated with the condition, causing (e.g. epitheliocysts) or being associated with various histopathological changes in the gills. Depending on the agents present and the observed pathology, GD can be classified as PGI, PGD, epitheliocystis or AGD. AGD may be a distinct diagnosis, but concurrent epitheliocystis and PGI is common. Since *Paramoeba* spp. infections were rare and of low density, AGD was not present in this study. While our aim was to reveal evidence for causality in the common and recurring autumn GD based on real-time PCR screening for some commonly detected agents, it cannot be excluded that other agents occasionally cause such diseases.

In the present study we show temporal changes in pathogens in salmon from 6 farms, revealing recurring infection patterns by some, and large variation without apparent patterns in others. Infections with the microsporidian *D. lepeophtherii* shows a clear seasonal pattern, and particularly high densities in the gills coincide with GD. Ca. B. cysticola, previously put forward as an likely causative agent, appears not to be directly connected to the GD seen in this study, albeit proliferation of this bacterium may be facilitated by high *D. lepeophtherii* densities. Challenge studies are needed to substantiate the role of *D. lepeophtherii* in GD, but an realistic challenge model (i.e. bath) has so far not been developed. With the advent of challenge models, experiments may also be used to examine if synergy occur e.g. in concurrent *D. lepeophtherii* and Ca. B. cysticola infections.

Author contributions

Conceived and designed the experiments: GSG, EK, AN, SH. Performed the experiments: GSG, SH, SB and HP. Analyzed the data: GSG, EK, AN, AKI. Contributed reagents/materials/analysis tools: GSG, AN, HS. Wrote the paper: GSG, EK, AN, SH.

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