Differences in gill responses in ballan wrasse

(*Labrus bergylta*)

Thesis for the degree

Master of science in Aquamedicine

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Abbreviations

Abbreviations	Full words
AB-PAS	Alcian blue – Periodic acid-Schiff
AGD	Amoebic Gill Disease
ANOVA	Analysis of Variance
cd	Challenge Dose
Cs/L	Cells/L
CVS	Central Venous Sinus
EGC	Eosinophilic Granular Cell
F	F-statistics
H & E	Haematoxylin and Eosin
HES	Haematoxylin and Eosin saffron
ILU	Interlamellar Unit
IMR	Institute of Marine Research
IS	Increase in Severity
PCNA	Proliferating Cell Nuclear Antigen
PCR	Polymerase Chain Reaction
RNG	Random Number Generator
SP	Sampling Point
W	Watt

Abstract

The ectoparasite salmon lice *(Lepeoptheirus salmonis)* continues to trouble the Norwegian aquaculture. Over the course of the last decade there has been a significant drop in effective treatments with the ectoparasites resistance to medicinal and chemical treatments. This has paved way for the non-medicinal treatments; however, recent studies have shown that the use of such methods lead to increased mortality due to stress and handling. As a result, cleaner fish like ballan wrasse (*Labrus bergylta*) and lumpfish (*Cyclopterus lumpus*) have been introduced to fish pens as a less invasive alternative to supress the salmon lice infestation. Recent studies have shown that cleaner fish are susceptible to amoebic gill disease (AGD), raising the issue that ballan wrasse may function as a vector for disease in salmonids. As ballan wrasse still is a relatively new species on the job in Norwegian aquaculture little is known about the progression of disease. This study was part of a 11 weeks challenge experiment, investigating variances in susceptibility and severity in AGD between Atlantic salmon and ballan wrasse. Samples from week 1, 3 and 5 where used to compare progression of disease and further investigating the inflammatory response and variations in pathology. There was a significant absence of lesions and gross pathology in the ballan wrasse, where only 1,1% of the fish developed clinical signs consistent with AGD. For the Atlantic salmon 100% of the fish got infected with visible gross gill score and more severe pathology than found in the ballan wrasse. The presence of amoeba was confirmed in apposition to hyperplastic and hypertrophic gill epithelial tissue for both species although with significant difference in inflammatory response. An increase in mucosal activity was observed for the ballan wrasse, but mucous cell density estimation showed little significant differences that can't be ruled out as random variance. The observations in this study support the fact that non-salmonid species like ballan wrasse are more resistant to infection with *P. perurans* than Atlantic salmon. The slower progression of AGD development in cleaner fish compared to salmonids, underlines the possibility that cleaner fish like ballan wrasse might be a reservoir and vector for the disease in Norwegian aquaculture.

1. INTRODUCTION

1. 1 Salmonid aquaculture from a Norwegian perspective

Norway is at the very forefront of innovation and production in aquaculture, and one of the leading nations on farming salmonids (Ababouch *et al.,* 2016). Norway produced 1 281 872 tonnes of Atlantic salmon (*Salmo salar,* Linnaeus 1758*),* and 68 015 tonnes of rainbow trout (*Oncorhynchus mykiss*, Walbaum 1972) (preliminary figures published at SSB (Statistics Norway) for the period 2017-2018). Although there has been a significant increase in production and export of Norwegian seafood, it now stands on the verge of its maximum potential with current regulations. With the expansion of fish farms along the coast, challenges related to disease, parasites, bacteria and viruses are a constant threat to the industry. The prime antagonist of aquaculture farming in Norway is the ectoparasite *Lepeoptheirus salmonis* (Krøyer 1837) (Johnson and Albright, 1991). Over the course of the last decade, there has been a significant drop in effective treatments with the ectoparasites resistance to medicinal and chemical treatments (Hannisdal *et al.*, 2020). Thermal and biological delousing methods are therefore more in use than ever before (Wiik-Nielsen *et al.*, 2019). With the increasing difficulty of managing infestation of salmon lice in the aquaculture industry, the implementation of the traffic light system (Produksjonsområdeforskriften, 2017) was initiated as a means of last resort to reduce infection pressure in the Norwegian fjords. The system divides the Norwegian coastline into production areas with the pressure of Salmon lice on aquaculture pens as the regulating factor. However, the ectoparasite continues to trouble the Norwegian aquaculture and the issue has not been solved with the use of thermal and mechanical treatments. In recent studies increased mortality have been linked to the use of such methods (Overton *et al.*, 2018) and The Norwegian Food Safety Authority stated the use of thermal delicing will be disbanded within a two-year period. This indicates that non-handling methods such as the use of cleaner fish might be more important in the future. Atlantic salmon aquaculture in Norway also struggles with Piscine myocarditis virus (Cardiomyopathy syndrome), Salmonid alphavirus (Pancreas disease) and *Neoparamoeba perurans* (Amoebic gill disease) (Wiik-Nielsen *et al.*, 2019).

1. 2 Cleaner fish as a biological asset

The salmon lice have always represented a challenge for the industry and the battle against the parasitic copepod has lasted since the late 70's. From 1992 the use of various therapeutic chemicals to combat the salmon lice infestation gradually increased up until 2015 where absolute resistance occurred in the salmon lice against most of the available therapeutic chemicals (Hannisdal *et al.,* 2020). From 2015 till 2017 the prescriptions for medicinal treatments came to an abrupt halt, with an "overnight change" and a 61% reduction of prescribed anti-lice medicine from 2016 (Jansen *et al.,* 2018; Wiik-Nielsen *et al.*, 2019). At the same time non-medicinal treatments arose as an alternative to the now ineffective therapeutic chemicals. The non-medicinal treatments include use of thermic (delousing with heated water), mechanic (delousing with hydro jets or brushes) or freshwater (well boats with freshwater in the tanks) to treat infection with the salmon lice (Wiik-Nielsen *et al.*, 2019). In 2017 thermic delousing accounted for 74% of the non-medicinal treatments alone (Jansen *et al.*, 2018). Although the non-medicinal treatments have been found to increase stress levels and, cause trauma which can impair the immune system and cause infections they are still the main tool for delousing in aquaculture to this day (Wiik-Nielsen *et al.*, 2019). The use of cleaner fish as a preventive biological delousing strategy is viewed as a gentle and less invasive way to supress the density of the salmon lice in large scale farming.

Both farmed lumpfish and wrasse species have been proven to graze upon infected fish, contributing to a reduction in the copepodite infection pressure in aquaculture pens (Imsland *et al.*, 2018; Overton *et al.*, 2020). This trait makes them a valuable resource for the Norwegian aquaculture and sought after means to combat salmon lice infection.

In 2019, a total of 42 million lumpfish (*Cyclopterus lumpus,* Linnaeus 1758) and 695 thousand ballan wrasse (*Labrus bergylta,* Ascanius 1767) where farmed with the intention of using them as a tool for reducing the salmon lice infestation (Firskeridirektoratet, 2019; Wiik-Nielsen *et al.*, 2019). Unfortunately, the high mortality and reduced effect of farmed lumpfish during the summer months has led to an industry revolving around the capture of wild wrasse. In 2019 the supplement of 17.5 million wild caught wrasse brought the total use of cleaner fish to an astounding 60.5 million in the Norwegian aquaculture industry. This industry moves large amount of wrasse over vast distances putting great pressure on the wild stock and can function as potential vectors for disease (Mortensen *et al.*, 2016; Grefsrud *et al.*, 2019; Sandlund *et al.*, 2020). In 2013 ballan wrasse were identified as a potential host for AGD in a rearing facility at the Institute of Marine Research (IMR) -station in Austevoll (Karlsbakk *et al.*, 2013). Later

Dahle, (2015) found that ballan wrasse infected with AGD had the potential to transfer amoeba to Atlantic salmon and function as a vector for disease. The same potential were later described for lumpfish by Haugland *et al.* (2017).

1. 3 The causative agent of the disease

Amoebic gill disease (AGD) in farmed salmonids was first described in Tasmania, around the mid 1980's (Munday, 1986; Oldham, Rodger and Nowak, 2016). Since then, the amoeba has steadily moved up north and was confirmed in Norway in 2006 (Wiik-Nielsen *et al.*, 2019). AGD has been responsible for great mortality with significant losses in Ireland, Scotland and Norway and is now considered a serious disease in Salmonids. The causative agent for AGD were previously not known and there has been many different *Neoparamoeba* spp. suspected to be associated with the disease (*N. permaquidensis, N. branchiaphila*) (Dyková and Lom, 2004; Wiik-Nielsen *et al.*, 2016). Later, *Neoparamoeba perurans* was described by Young *et al.*, (2007) as an amoeba which is directly associated with AGD lesions through in situ hybridization. It is therefore, believed to be the causal agent of AGD. *N. perurans* was successfully cultured in vitro in 2012 and through challenge trials shown to be the causative agent of AGD in so fulfilling Koch's postulate (Crosbie *et al.*, 2012).

1. 3 – 1 *Paramoeba perurans*

Neoparamoeba perurans and *Paramoeba perurans* are synonymous names (Feehan *et al.*, 2013; Hellebø, Stene *et al.*, 2017) and for this study *Paramoeba perurans* is used with the abbreviation *P. perurans.* Little is known about the biology of the *P. perurans,* beyond that they are free-living facultative ectoparasites. *P. perurans* can either adhere itself to a surface in what is known as the locomotive form or appear in a free-floating state. Its shape varies greatly with its state and while free floating the amoeba is measured to be approximately 20-30μm in diameter, with distinct extended pseudopodia's (Karlsbakk *et al.*, 2013). In locomotive state the amoeba has been measured between 41-56 μm in diameter, with mamilliform pseudopodia (Dyková and Lom, 2004; Young *et al.*, 2007; Wiik-Nielsen *et al.*, 2016).

P. perurans has only been associated with the gills and there has been no evidence of invasion of the gill epithelium or other tissues suggesting that the amoebae is entirely ectoparasitic (Powell *et al.*, 2008). Pathology is limited to the gills, where AGD of infected fish have characteristic white or greyish slimy patches and/or lesions covering the gill arches and filaments. Lesions tend to be focal and concentrated with smaller spots on the lamellae early on and evolves to big white patches as the disease progress in severity.

Amoebae can easily be detected and confirmed through a simple mucous smear under a light microscope and histology gives the most accurate detection (Wiik-Nielsen *et al.*, 2019). Key risk factors for an upcoming AGD-outbreak are linked to high salinity, with high water temperature. As the amoeba has a relatively low host specificity, it can be found in several, species, however, susceptibility does not mean that infected species develop AGD, as there are great variations in resistance against *P. perurans* (Karlsbakk *et al.*, 2013; Oldham, Rodger and Nowak, 2016; Nowak and Archibald, 2018; English *et al.*, 2019; Dahle *et al.*, 2020).

1. 4 Aim of the study

Although AGD is a known disease in both Atlantic salmon and ballan wrasse (Karlsbakk et al. 2013) little is known about transmission vectors, susceptibility and resistance in ballan wrasse. Initially, the main aim of this study was to look at dose response in ballan wrasse through an infection challenge with various concentrations of *P. perurans*. When the ballan wrasse in the study did not display clinical or morphological changes compatible with AGD the aim shifted to look at differences between a previous infected ballan wrasse group and the non-infected group from this study. Through proteomics, genome sequencing and histology the goal was to discover why the fish from this study had such an increased resistance towards the amoeba while the same challenge experiment yielded infected fish in the previous challenge. Due to covid-19 the lab work at IMR and the Veterinary institute was halted and both proteomics and genomics are not included in this master thesis but will be included in future works. Through histology, immunostaining and development of a digital mucous cell density estimation this study seeks to map out variances that can explain why the ballan wrasse had such high resistance towards the *P. perurans.*

1. 5 COVID - 19

COVID-19 interfered with the final stages of lab work for this thesis. Therefore, some of the work that was planned, was not completed. The university of Bergen has recognised that some of the results may not be as complete as one would like, but that the master students should complete their thesis in a manner which allows them to progress as normally as possible.

2. Material & Methods

2. 1 Origin of *Paramoeba perurans* culture

The polyclonal amoeba culture used in this study originated from an isolate of the C2-clone (H02/13P*p*C2). The C2-clone originates from farmed Atlantic salmon diagnosed with AGD sampled at Sotra on the west-coast of Norway in 2013 by ILAB, Bergen (Haugland *et al.*, 2017). Culturing and maintaining the amoebae in this study was preformed using flat bottomed culture flasks with a malt-yeast broth (MYB: 0.01% malt extract, 0.01% yeast extract and filtered sterile seawater at 15°C) with the cultures being subdivided every 14-20 days (Wennberg and Powell, 2015). Prior to inoculating the tanks with *P. perurans* the cultures were counted in a counting chamber with a stereo optical microscope (Grigoryev, 2013; Høstmark, 2016; Rosenlund, 2017).

2. 2 Husbandry of ballan wrasse

Ballan wrasse for this study were hatched at IMR - station in Austevoll, Norway. The wrasse were then transported by road to the wet lab facilities at IMR in Bergen and placed in 16, 250 L tanks with ambient water intake 128 m below the surface and maintained with a flow of 400 L/h with 80 fish per tank with an mean weight between 5-22 g. The average water temperature was maintained at 14°C (± 0,10 SD) with a salinity of 34,5 g/L and an oxygen saturation of 90,91‰ (± 7,77 SD). The ballan wrasse were fed a commercial diet (Otohime C1 grow-out diet, 580 – 840 µm granules) which was in turn mixed with a supplement of minced cooked prawns in a 1:2 ratio (33% minced cooked prawns and 67% Otohime C1) (Powell and Wennberg, 2016).

2. 2. 1 Husbandry of Atlantic salmon

The Atlantic salmon used as a sentinel species for this study were an all-male heterozygote isogen line from the IMR - station in Matre. Bred by crossing a double haploid female (homozygote across the whole genome) with a double haploid male. The same environmental parameters as described for the ballan wrasse were maintained for the Atlantic salmon.

2. 3 Study design

Ballan wrasse, with no known clinical history associated with *P. perurans*, were divided into groups of 80 fish in a total of 16 tanks two weeks prior of infection to climatize. A total of five challenge doses (cd) were used in the trial referred to as cells per litre (cells/L). One cell is equivalent to one amoeba per litre (L) which would give approximately 250 amoebae in a tank

with infection dose 1 cell/L. In trial one two parallel lines of tanks (X and Y) were set up in two separate wet labs **(figure 1)**. Line X consisted of six tanks, two control, two 10 cells/L and two 1000 cell/L and was isolated in wet lab 4. Line Y consisted of ten tanks, two control, two 1 cell/L, two 10 cells/L, two 100 cells/L and two 1000 cells/L in wet lab 5. Line X was left alone for the duration of trial one except for normal tending to the tanks and feeding as shown in **(figure 1).** Line Y was sampled weekly with five fish from each tank. The infection challenge of the ballan wrasse was carried out by stopping the waterflow to each tank and reducing the volume from 250 L to 100 L and adding the appropriate value of amoeba culture for one hour. Amoebae were administered by adding a 30 mL of medium spiked with the appropriate amount of amoeba for each infection dose calculated by the formula from Grigoryev, (2013). The 1 cell/L tanks were inoculated by diluting the 3 mL of culture to 30 mL with seawater from the tank, and subsequently total of 300 µl was added to the tank. 30 mL of amoeba-free medium were added to the control tanks to give all tanks the same treatment. One hour after inoculating the tanks with amoeba culture, the water flow and water level were restored to normal levels and the ballan wrasse were fed.

Trial two consisted of a total of nine tanks, two control tanks continued from trial one, and six 1000 cells/L tanks with ballan wrasse and one 1000 cells/L with n = 11 salmon as a sentinel species to confirm the virulence of the C2-clone. The tanks with 1000 cells/L were re-infected following the same protocol as in trial one **(figure 1)**.

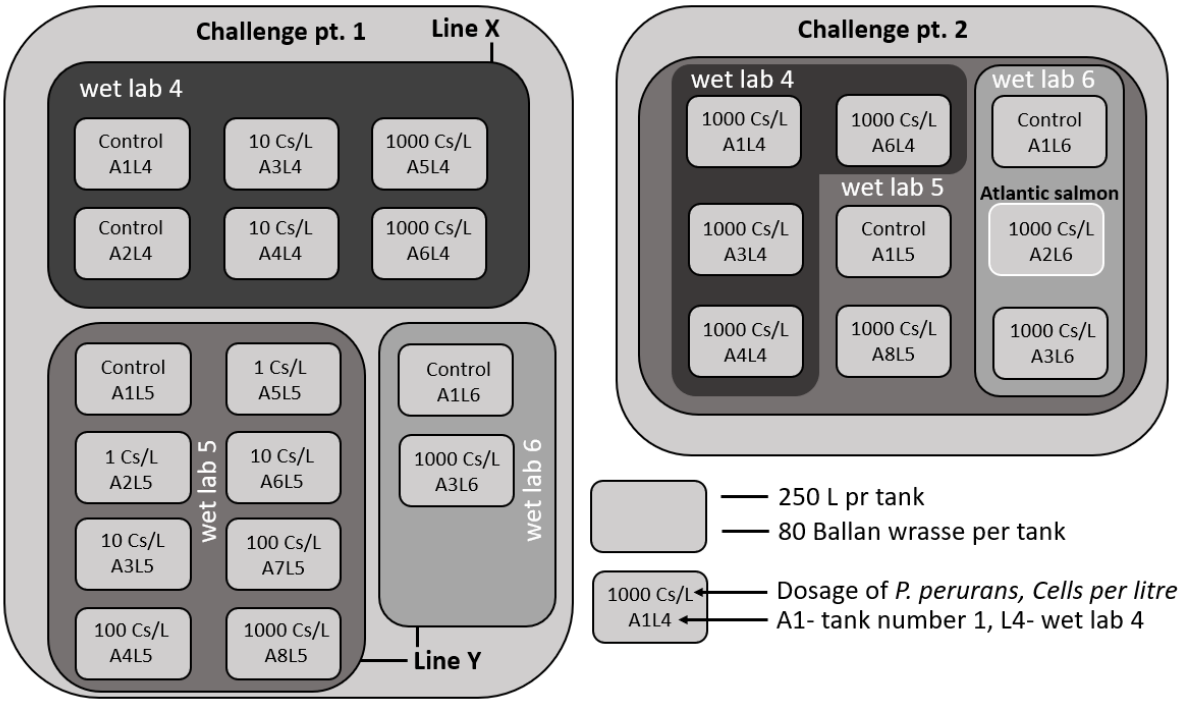


Figure 1 – Tank design for challenge pt.1 and challenge pt.2 of the study. Part 1 of the challenge included both line X and line Y. The isolated line X consisting of 6 tanks, two controls, two 10 Cells/L and two 1000 Cells/L and where located in wet lab 4. Wet lab 5 contained 1 control tank, two 1 Cell/L, two 10 Cells/L, two 100 Cells/L and one 1000 Cells/L and was a part of line Y. Wet lab 6 housed one control tank and one 1000 Cells/L tank and were also included in line Y. Line Y were sampled weekly with 5 fish from each tank. Part 2 of the challenge tanks were re-infected with the high dose of 1000 Cells/L across all tanks. The tanks that were put through to the next round consisted mostly of the isolated tanks from wet lab 4 two tanks from wet lab 5 and two from wet lab 6. In addition to the two existing tanks from wet lab 6 another tank was introduced and filled with 11 Atlantic salmon indicated with a white ring in challenge pt. 2. These 11 Atlantic salmon were used as a sentinel species to control the virulence of the P. perurans *isolate (C2-clone) that was used in the study.*

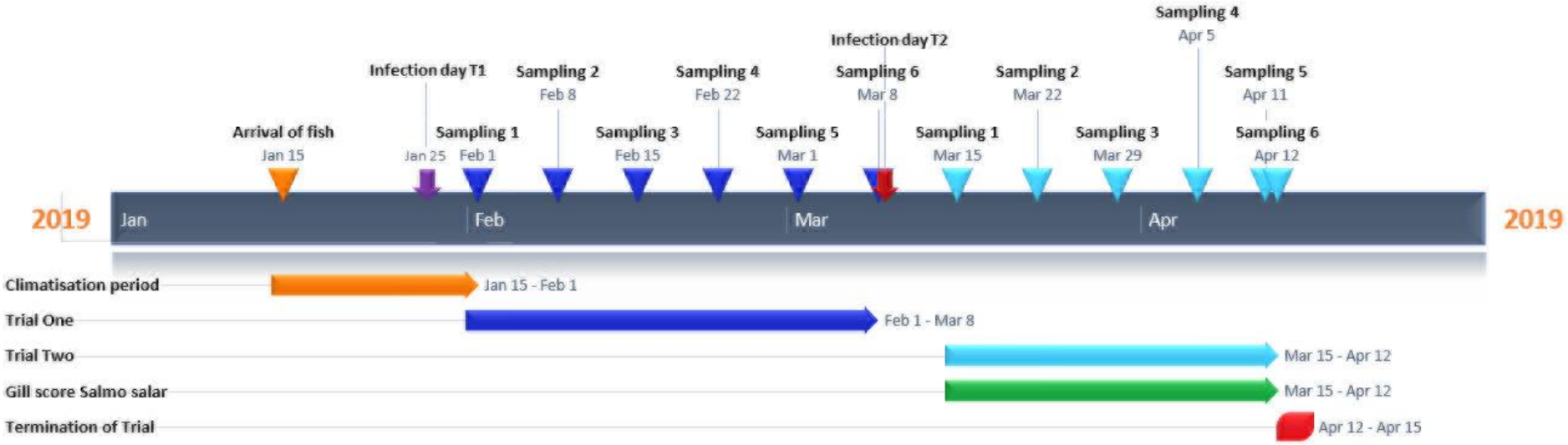


Figure 2 – Study timeline showing the different trial stages, infection dates, sampling points, trial phases and duration.

2. 4 Sampling

Sampling was conducted weekly from one-week post-challenge (WPC) (01.02.19) until 11 WPC (12.04.19). Samples used in the study were from 1, 3 and 5 WPC in trial one (1 – 6 WPC) and 1, 3 and 5 WPC in trial two (7 – 11 WPC). The ballan wrasse were starved the day before sampling as to minimize feed particles and other contaminants impairing the gills complicating scoring. Five fish were caught by net from each tank in line y and administering an overdose of anaesthesia (MS-222, Metacain 100 mg/L, Sigma Aldrich, Norway). The fish were weighed before being placed on their ventral side and then on the lateral side (randomly left or right) and then covered with two sterile pieces (each of 2.5 x 7.0 cm) of white medical wipes (Kimberly-Clark, Irving, TX, USA) to absorb mucous for 10 s, following the method of Fæste *et al.*, (2020). The wipes were then gently removed with forceps and placed into the upper compartments of an 2.5 mL modified Eppendorf tube, with an internal pipette tube clipped (acting as a filter) to prevent the paper from being spun down while centrifuging, while allowing flow of mucous to the bottom of the tube. The mucous samples were stored on ice and mucus fluid was extracted from the medical wipes by centrifugation through the modified 2.5 mL Eppendorf tubes with 500 g for 10 min at 4 ℃ (Fæste *et al.*, 2020), the samples were later used for metagenomic and proteomic analysis at the Veterinary institute. The fish were then measured and examined for external lesions. Gross gill score following the method described by Taylor *et al.*, (2009) were used as an indicative for amoebic gill disease in sampled fish. The ballan wrasse were starved the day before sampling as to minimize feed particles and other contaminants impairing the gills complicating scoring. The tail of the fish was cut off using a scalpel to reduce the amount of contaminating blood when the operculum and gill arches were removed with sterile scissors. The left-hand side gills were removed firstly and placed in a petri dish with a few drops of water from the tank to keep them wet. Two petri dishes were used to separate the left and right-side gills. Gill arches were scored on both sides giving a total of 16 scored surfaces per fish. Petri dishes and forceps were disinfected between tanks. The third gill arch on the right side of each sampled fish were placed into RNAlater for PCR-analysis. Gill arch 2 and 3 on the left side were placed in a casting cassette and fixed in neutral phosphate buffered formalin (VWR) solution (Fish 1 (F1) - F250) or fixated in Davidsons seawater fix (F251 - F549 and F600 - F611).

2. 5 Gill scoring

Gills were scored after Taylor *et al.*, (2009), examining all 16 gill surfaces for both species with a stereo optical microscope for the ballan wrasse. The Atlantic salmon were examined by eyesight gently opening the operculum using the blunt handle of a pair of forceps or finger on the Atlantic salmon as shown in (**figure 5**). Progression of gill scores in this study are referred to as an increase in severity (IS) indicating progression of AGD within the host.

2. 6 Dehydration / Paraffin infiltration and Sectioning

2. 6.-1 Preparation of fixed tissue for histology

Formalin or Davidson's fixed casting cassettes containing sampled gill tissue were transferred from the fixative to the histokinette and followed the protocol referred to in table 3 (appendix 1). This preliminary step was executed prior to embedding the tissue with paraffin see table 4 (appendix 1). Sectioning was done by a Micros HM354S Thermo Scientific waterslide microtome. Each block was left in the freezer for 10-15 min prior to sectioning. The blocks were sectioned to 3 µm and cut from the trailing edge towards the leading edge to minimize tearing while cutting through the gills. Gills from each fish were sectioned at least 6 times (maximum of 10) and then mounted on commercial Poly-L-lysine coated slides (Sigma Aldrich). Each slide was marked accordingly with sample date project number, fish id and section number (I-X).

2. 7 Histological staining

The poly-L-lysine slides (3µm) were deparaffinized following a standard protocol (table 3; appendix 1) and rehydrated in a descending alcohol series.

2. 7 – 1 H&E staining

H&E staining followed the steps listed in (table 4; appendix 1).

2. 7 – 2 HES staining

HES staining followed the steps listen in (table 5; appendix 1).

2. 7 – 3 Combined Alcian blue- PAS technique (Mowry, 1956)

AB-PAS staining followed the steps listed in (table 6; appendix 1).

2. 7 – 4 Proliferating cell nuclear antigen (PCNA) staining

Slides with AGD-like lesions and evident *P. perurans* where stained with PCNA-solution to access lesion characterisation, cell turnover and regeneration in and around inflamed tissue. The molecular lab at IMR had to develop a custom protocol in order to get a clear positive signal from the staining.

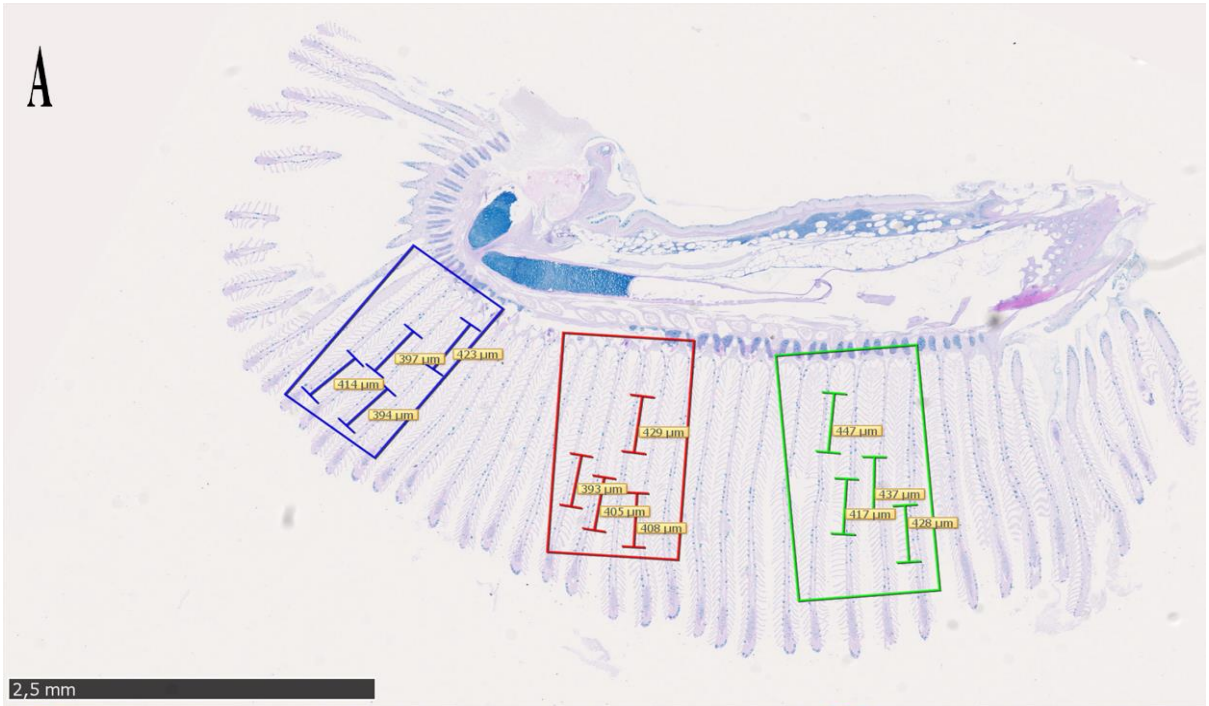
The paraffin sections to be immunostained were deparaffinized and hydrated. After the hydration they were microwaved with a citrate buffer for five min x 2 (repeated twice) on maximum power (800W). The sections were cooled for 45 min in a fume hood before they were transferred to a TRIS-buffer for three minutes. A PAP-pen (Sigma Aldrich) were used to draw a thin film like hydrophobic barrier around the sections to avoid dehydration of tissue. The sections were transferred to a dark moisture chamber (Sigma Aldrich) placed in a fume hood. The sections were incubated for 20 min in a 5% BSA (bovine serum albumin) in a TRIS-buffer following with deactivation of reagents. Sections were incubated with a monoclonal primary antibody anti-PCNA (Antibody used for this method were, Santa Crux biotechnology PCNA (PC 10) Sc-56, monoclonal mouse) diluted in 1:10 ratio in 2.5 % BSA in Tris-buffer for 60 min. The antibody was blocked and washed for five min in TRIS-buffer, on a lab-vibrating table. Sections were then incubated with Dako Envision ^{TM} + Dual link System -HRP (K4063) for 30 min. The reagents were blocked and washed in a TRIS-buffer and then placed on a lab-vibrating table for five min. Followed by incubating with a vector peroxidase substrate kit AEC (SK-4200), for 30 min. The sections were then rinsed in running cold tap-water for five min. The sections were counterstained with haematoxylin for 10-15 seconds. 1-2 drops of watery Aquatex (VVR) on a suitable cover slip (Thermo scientific). The cover slip was flipped over the section and weight placed on top to squeeze out air bubbles. When the slide mounting media were done curing, sections were ready for screening. The sections were kept in the dark to preserve staining.

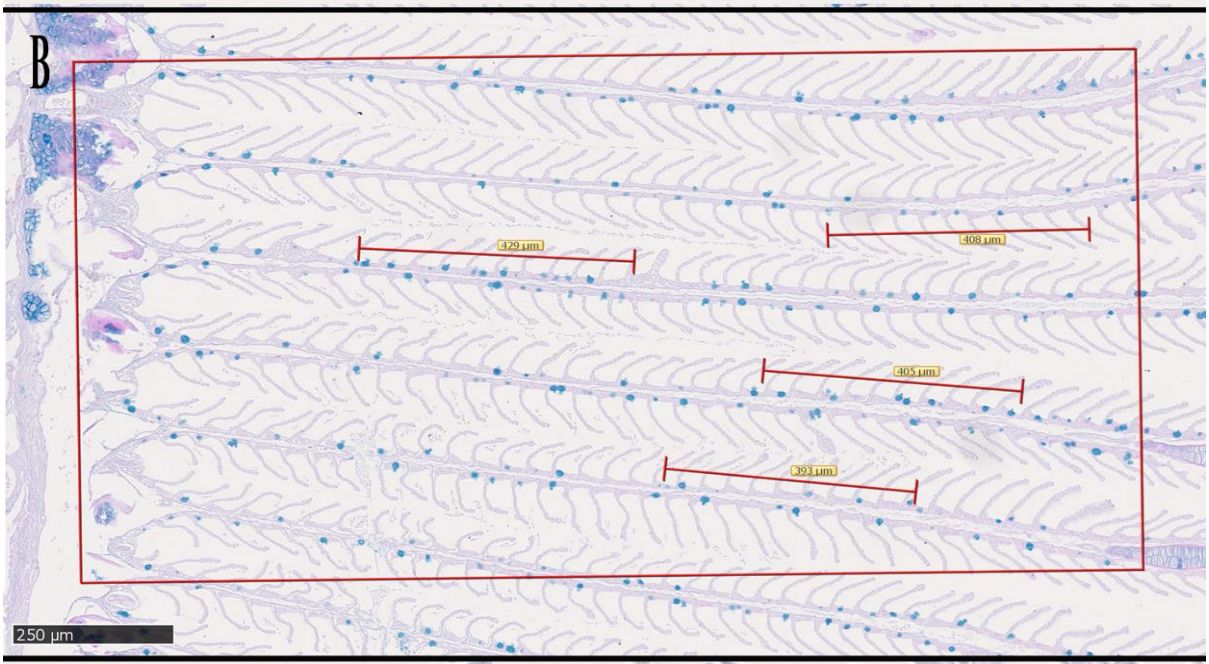
The anti-PCNA antibody stained positive (red brown) with AEC-substrate in proliferative cell nucleus hence it is of the utmost importance to avoid overstaining with haematoxylin.

2. 8 Mucosal cell count

Slides (3 µm) from ballan wrasse gills stained with AB-PAS were scanned by a Nanozoomer s60 (Hamamatsu). Each slide was manually corrected for false focus points, and then processed for a 9-layer depth digital scan. Each digital slide was processed in Nanozoom digital pathology viewer (NDP-viewer). The counting of mucous cells adapted the method described in (Roberts and Powell, 2003) which was adapted from Speare et al. (1997). The method differs in the

counting of four well-oriented filaments as supposed to five from a minimum of two (maximum three) areas of well-oriented filaments with 10 interlamellar units (ILU's). These areas are colour coded (green, red and blue) within the NDP-viewer interface (**figure 3**). A minimum of two squares, green and red on a random location where the section had four well oriented filaments, had to be fulfilled for the slide to be approved. The squares should be located from either the ventral, middle or dorsal region but preferably all of them. Within the squares 10 ILU's were selected on four well oriented filaments and connected by fusion of the gill filaments to the interbranchial septum. Where the counting took place on the filaments was decided by a random number generator (RNG) based on the total number of lamellae in each filament. Squares were placed by the base of the filaments with the cartilage extending through mid-section of the gill arch. A square was defined as valid if it had between 16-45 ILU's. Validation of the squares requires enough ILU's to get a representative expression and stay within the mid-section of the gill arch. The placement of squares should not extend to far out towards the edge of the gill surface as. The orientation of the gill arch followed that of (Hytterød *et al.*, 2018) with the lamellae closest to the cartilage valued as one with and increasing value towards the edge of the filament. The RNG value states the starting point of the 10 ILU's in orientation from the leading edge towards the trailing edge. If the value would exceed its parameter (square), the 10 ILU's would be estimated from trailing edge towards the leading edge. Estimation and counting of mucous cells were done over four filaments on both sides of the 10 ILU's. The values from minimum two squares were expressed as the gills total mean value representing a "general mucosal cell presence" in each individual fish.


A
397 µm
423 µm
414 µm
394 µm
429 µm
393 µm
405 µm
408 µm
447 µm
437 µm
417 µm
428 µm
2,5 mm


B
429 µm
408 µm
405 µm
393 µm
250 µm


C
50 µm

Figure 3 - NDP-viewer interface used for assessing density of mucous cells on digital scans of histological slides stained with AB-PAS. ***A:*** *Overview of a gill stained with AB-PAS in the digital processing software. Three colour coded squares (green, red, blue) with four well oriented filaments are selected for estimation of mucous cell density. A random number generator (RNG) decides the placement of the four rulers within the square each ruler indicating 10 ILU's. The sum of mucous cells present within the four rulers make up a mean value for each square, and the sum of the squares again makes up a total mean value of cell mucous density per gill arch. 1,75x enlargement.* ***B:*** *Closeup of a red colour coded square with well oriented filaments and four rulers marking the randomly selected 10 ILU's from the RNG program. The square is placed so that it's covering the cartilage at the left side flowing over into free filaments at the right side stopping before it reaches the end of the free filaments. Mucous cells are counted on both sides of the filament to get a total value of mucous cells per 10 ILU's. Mucous cells are clearly visible in the ILU's with a light blue colour as indicated with black arrowhead in picture C. 8,43x enlargement.* ***C:*** *Mucous cells are clearly stained with a distinct light blue colour; mucous cells are indicated with black bold arrowheads. Only mucous cells that are clearly in the same plane with a distinct round border are counted for the mucous cell density estimation. 54x enlargement.*

2. 9 Statistical analysis

R-Studio 1.3.1056 and Microsoft Excel 2018 (version 1908), were used to plot and calculate average values, standard deviations, standard error and correlation. Two-Way analysis of variance (ANOVA) was used to compare both challenges one and two, in respect to weight, length, cd, samplings and mucosal cell counts. A TUKEY test used as a post hoc analysis ran multiple comparisons to compare group means within the datasets. Outliers which could potentially severely affect normality and homogeneity were identified with checking the homogeneity of variance assumption in R-Studio. A Levene-test was used to further check homogeneity of variances, which showed no statistically significant differences in variance across groups. A normality plot was used to verify that the residuals were normally distributed, and a Shapiro-Wilk test was in turn used to confirm normality.

2. 10 PCR-Analysis

Samples of gill tissue were analysed by Pharmaq Analytiq using a standard commercial qPCR test for *P. perurans.* The entire third gill arch on the right side were used for PCR-analysis, to verify presence of amoeba in the challenge tanks.

3. Results

3. 1 – 1 Growth

The ballan wrasse ranged from 3 to 41 grams and 6.5 to 14.3 cm across all sampled fish. With a mean value for weight at 15.1 grams (± 6.6 g) and length 9.7 cm (± 1.4 cm) (**figure 4**). Through modulation in R-studio, boxplots for the representative parts of challenge one and two with respects to weight and length were estimated over time (**figure 4**). This initial representation of the dataset showed what appeared to be an increase over time for both weight and length in both challenges (**figure 4**). A combined linear regression comparing weight and

length across both challenges also showed a trend towards gradual increase in weight following an increase of length (**figure 4**). Sample size was equal to ten fish from each cd and a total of 50 fish per sampling point in challenge one. For challenge two ten fish were sampled from the control group and 30 fish was sampled for cd 1000 with a total of 40 fish per sampling point. To investigate if either length or weight were dependant on cd or sampling a two-way ANOVA was run for both challenges. For challenge one, cd had no significance on increase in weight ($F_4 = 1.206, Pvalue = 0.308$), however sampling had a statistically significant effect ($F_5 = 4.390, Pvalue < 0.001$). Between sampling five and two (TUKEY, *Pvalue* = 0.001) and sampling six and two (TUKEY, *Pvalue* = 0.006) there was a statistically significant effect over time for increase in weight. For length there was no dependency on cd ($F_4 = 1.223, Pvalue = 0.296$),, but sampling showed a significant effect between length and sampling ($F_5 = 4.640, Pvalue < 0.001$). Increase in length over time was not dependent on cd ($F_4 = 1.223, Pvalue = 0.296$), however sampling showed a significant effect on length ($F_5 = 4.640, Pvalue < 0.001$). This effect occurred between sampling four and two (TUKEY, *Pvalue* = 0.016), sampling five and two (TUKEY, *Pvalue* = 0.003) and sampling 6 and two (TUKEY, *Pvalue* = 0.012). For challenge two, cd did not show any significance on increase in weight either ($F_1 = 2.203, Pvalue$ 0.131) while sampling continued to have a statistically significant effect ($F_5 = 5.300, Pvalue < 0.001$). Tukey test showed statistically significant differences between sampling twelve and seven (TUKEY, *Pvalue* = 0.002), twelve and eight (TUKEY, *Pvalue* = 0.002), twelve and nine (TUKEY, *Pvalue* = 0.004) and twelve and ten (0.008). The same pattern repeated itself for length as cd had no statistical significance on length increase ($F_1 = 1.718, Pvalue$ 0.191), while sampling had a statistically significant effect on length ($F_5 = 5.736, Pvalue < 0.001$). Tukey test showed a statistically significant effect on length between samplings listed in table eleven and seven (TUKEY, *Pvalue* = 0.012), twelve and seven (TUKEY, *Pvalue* = 0.004), eleven and eight (TUKEY, *Pvalue* = 0.025), twelve and eight (TUKEY, *Pvalue* = 0.008), eleven and nine (TUKEY, *Pvalue* = 0.014), twelve and nine (TUKEY, *Pvalue* = 0.004), twelve and ten (TUKEY, *Pvalue* = 0.037).

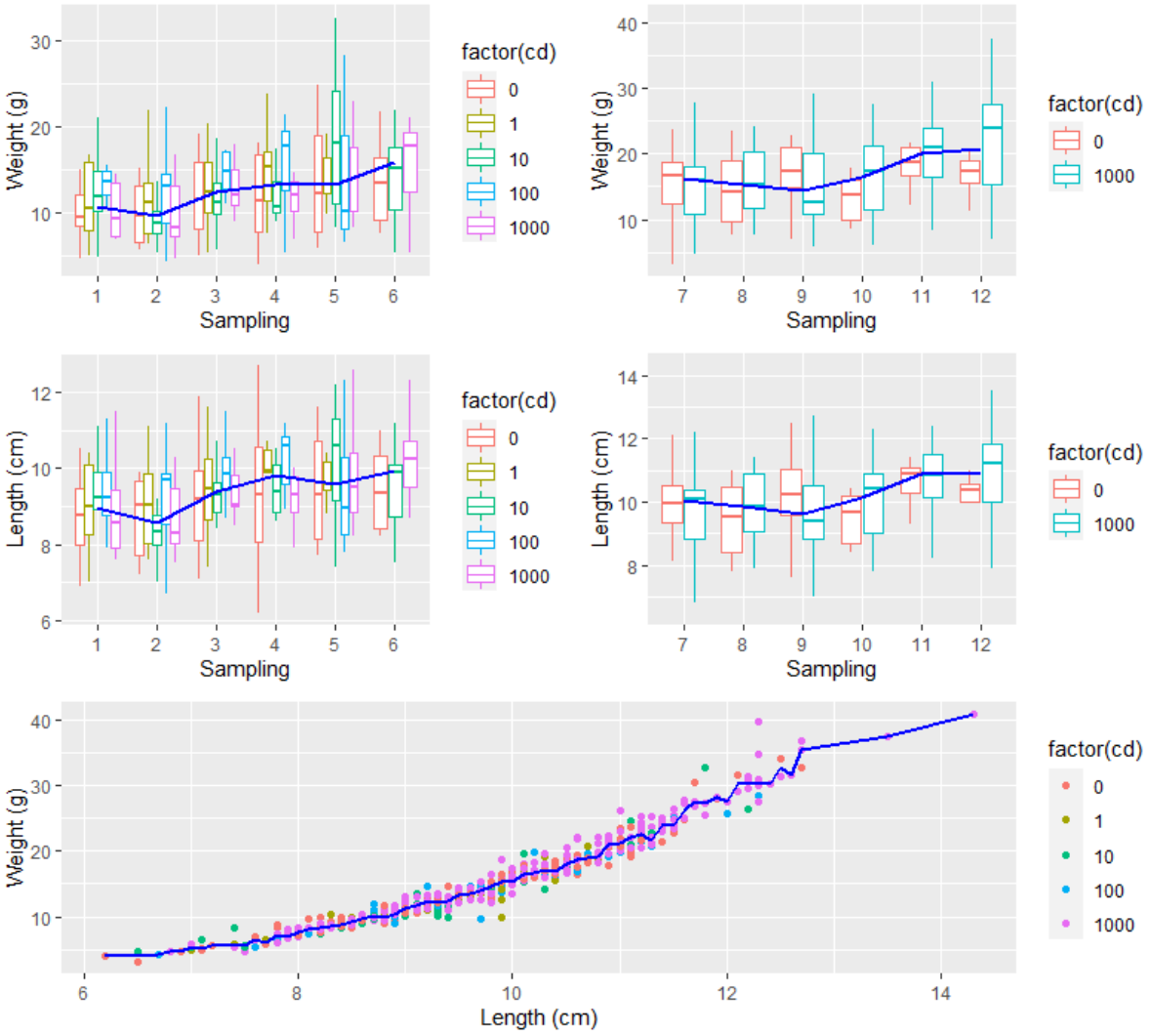


Figure 4 – Models from R-studio: Geom_boxplot in the top four small squares showing variations in weight (g) and length (cm) in challenge one (sampling 1-6) and challenge two (7-12). Boxplot one (orientation from left to right) from the top left and two from the top right show variations in weight (g) between the different challenge doses (cd), across samplings in challenge one and two. The third boxplot shows variations in length (cm) between cd in challenge one and the fourth boxplot also shows variations in length (cm) for challenge two. The geom_point plotted in R-studio shows distribution of weight (g) and length (cm) combined for both challenges. Different challenge doses (cd) represented by ((cd 0, orange), (cd 1, yellow), (cd 10, green), (cd 100, blue), (cd 1000, purple)). Sample size for challenge one was n=10 per cd per sampling point. Sample size for challenge two was n=10 for cd 0 and n=30 for cd 1000 per sampling point.

3. 1 – 2 Gill score and observations

Throughout, the study none of the gills from the ballan wrasse showed any signs of raised white mucoid spots and plaques on the gill filaments associated with classical AGD. Observations during sampling showed an increase in mucosal excretion from both gills, operculum and body in the ballan wrasse starting 1 WPC with a peak at 3 WPC and with a following decline towards 5 WPC. This pattern repeated itself for both challenge part one and challenge part two. Atlantic salmon showed an expected progression with clinical signs consistent with AGD (**figure 5**). The gill score for Atlantic salmon showed a gradual increase in severity of the gross gill score. 1 WPC none of the eleven salmon had any clinical signs consistent with AGD. 2 WPC the

number of fish with a gill score of one had increased from zero to eight out of eleven fish (72% increase in severity (IS)). 3 WPC, four out of eleven Atlantic salmon had progressed to a gill score of two (36% (IS)), 4 WPC, three out of eleven fish had progressed to a gill score of three (27% (IS)) and by 5 WPC, five out of ten fish had progressed to a gill score of three (50% (IS)) (**figure 5 and 6**). Histologically lesions consistent with AGD were compared between species (**figure 7 and figure 8**). Classic AGD lesions were observed for Atlantic salmon, with signs of proliferation and reduction in gill surface area (**figure 7**). The lesion shows a heightened presence of mucosal cells and increased activity in mucosal excretion and migration. More focal lesions from ballan wrasse (**figure 8**) showed a distinct difference in number and activity of mucous cells compared to the Atlantic salmon (**figure 7**). Although histological examination showed branchial irritation and morphological changes in both species, Atlantic salmon showed a more severe reaction to *P. perurans* than ballan wrasse (**figure 7 and figure 8**).

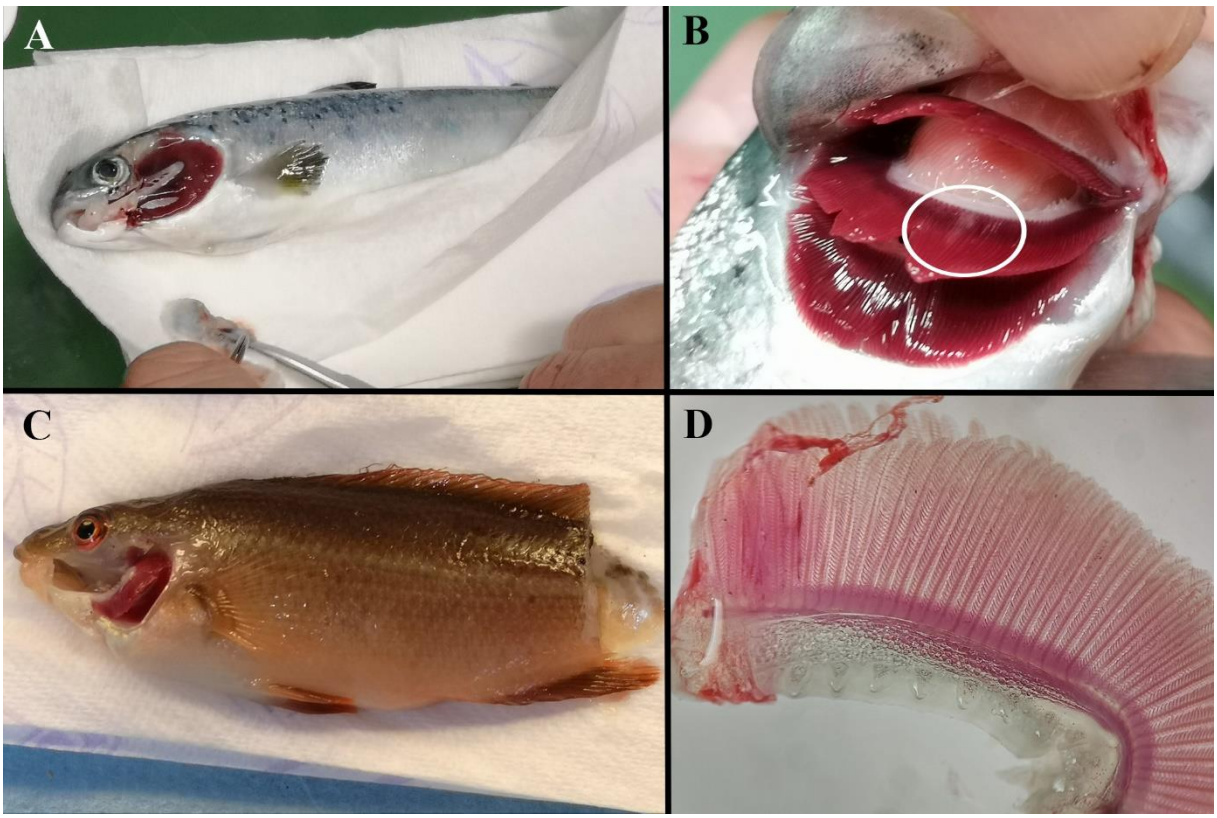


Figure 5 - Gill scoring of AGD and sampling of gill arches for histology and gill tissue for PCR-analysis. ***A:*** *Collection of gill tissue from* Atlantic salmon with removed operculum *for histology and PCR-analysis.* ***B:*** *Clearly visible white slimy translucent patch characteristic for AGD, indicated with a white ring with a total gill score of 1.* ***C:*** *Ballan wrasse with removed operculum and removed tailfin to reduce the amount of blood contaminating the gill arches when cutting these out for scoring.* ***D:*** *Typical gill arch from a ballan wrasse placed in a petri dish under a stereo optical loupe showing the ventral region along with the middle region of the free gill filaments along with the cartilage.*

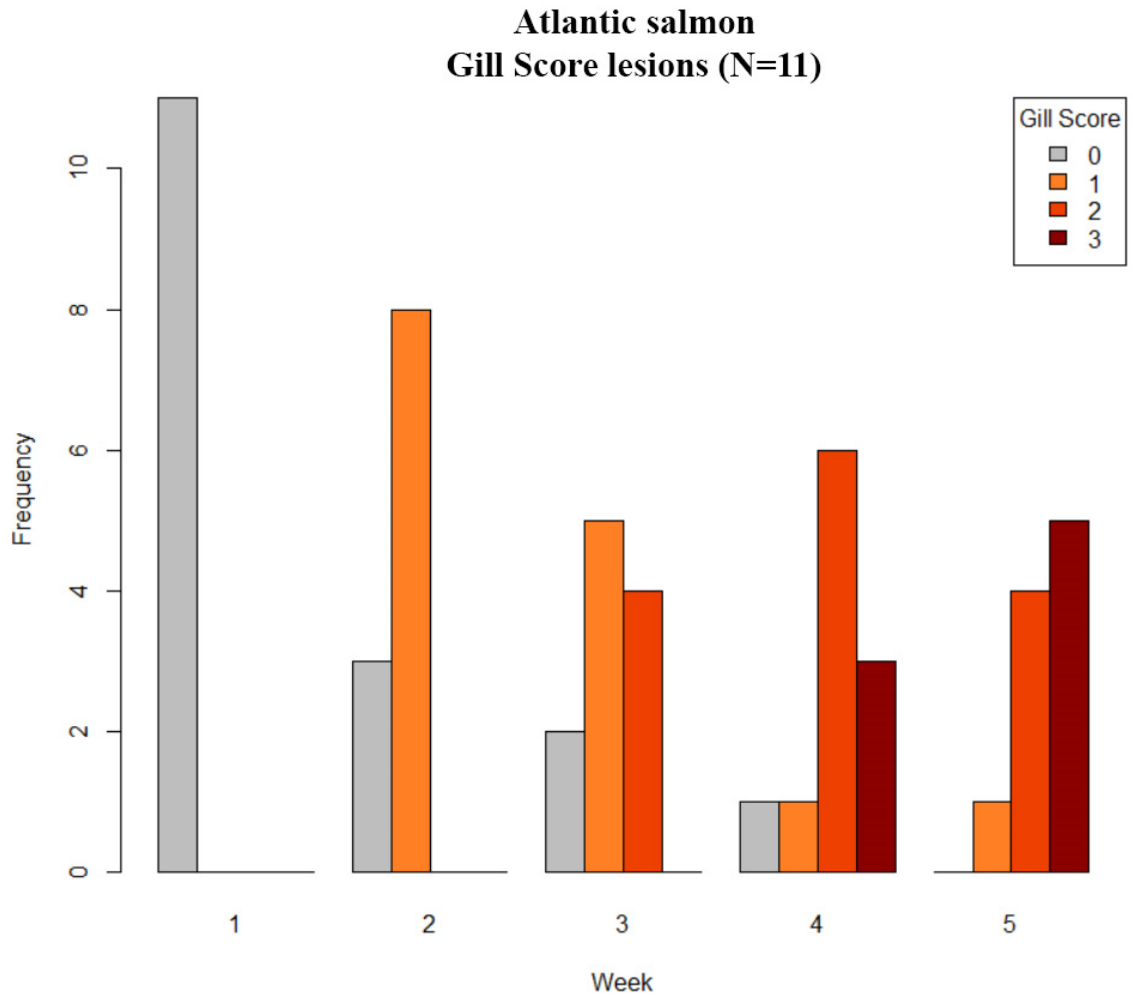


Figure 6 – Frequency of P. perurans *present in the gill tissue of Atlantic salmon. A total of 11 Atlantic salmon were used as a sentinel species to test virulence of the C2-clone in challenge pt. 2. Colour coded columns (light grey - burnt orange) represent different gill scores from 0-3. The gills were scored weekly with the method described by* Taylor, Muller, et al. (2009) *with 0- being an uninfected and normal healthy gill, 1- defined as very light, 2- light, 3 -moderate, 4- advanced, 5 Heavy. No gill scores exceeded 3 throughout the 5 WPC. The Y-axis indicates frequency of occurrence from the respective gill scores and the X-axis represents WPC.*

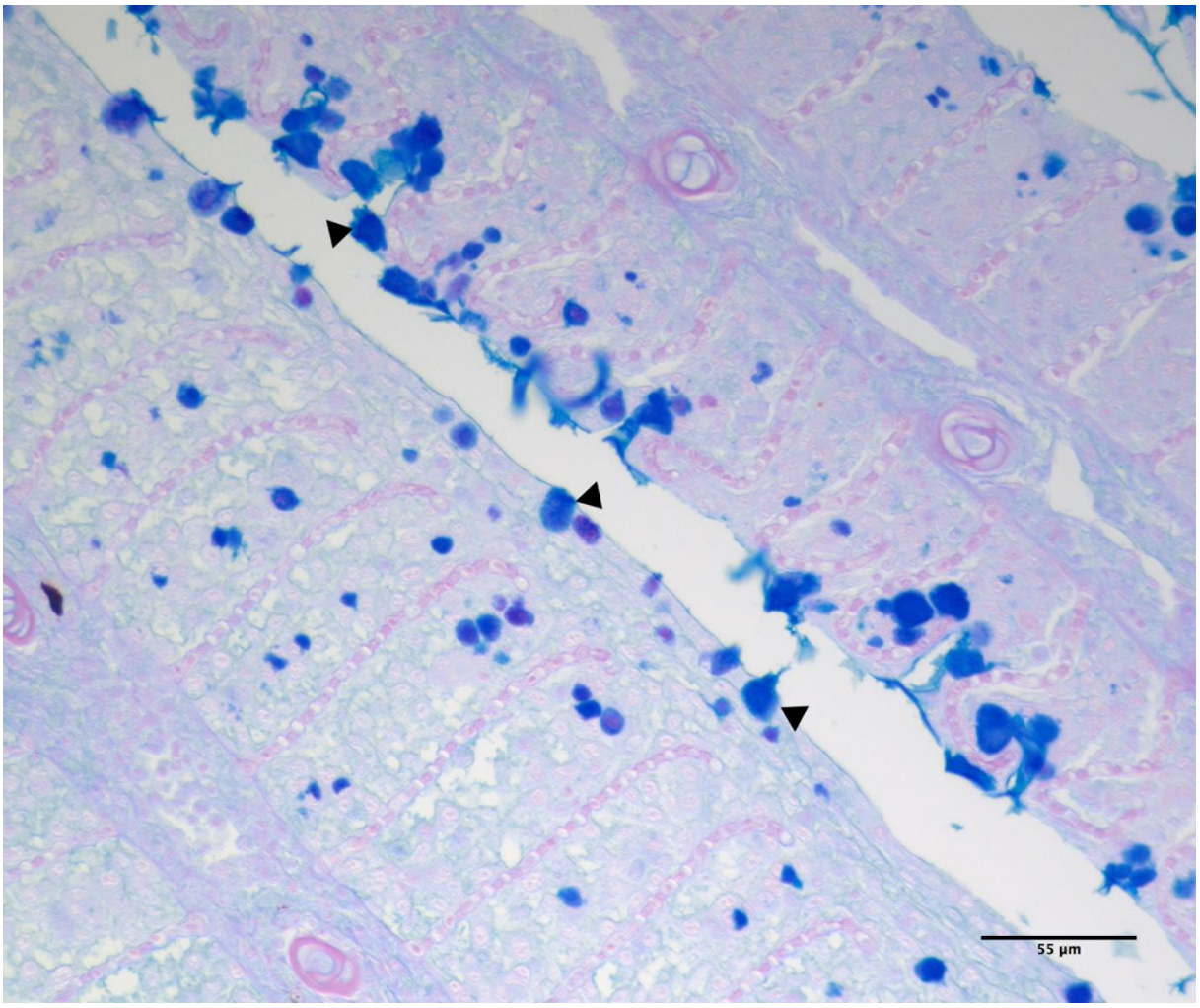


Figure 7 - AB-PAS staining of Atlantic salmon *(fish id 607) showing a classic AGD lesion with an abnormal amount of respiratory epithelial cells (proliferation) causing adhesion and reduction in gill surface area. Some of the secondary lamellae have rounded tips and cavernae formed in the interlamellar units, these morphological changes are consistent with AGD. Secondary lamellae are stained lightly pink with visible pillar cells. Black bold arrowheads indicate some mucosal cells, clearly differentiated from other cells stained with a clear royal blue colour. There is an increase in mucosal activity, migration and excretion along the borders of the clinical AGD lesion. 200x enlargement, scalebar 55 µm.*

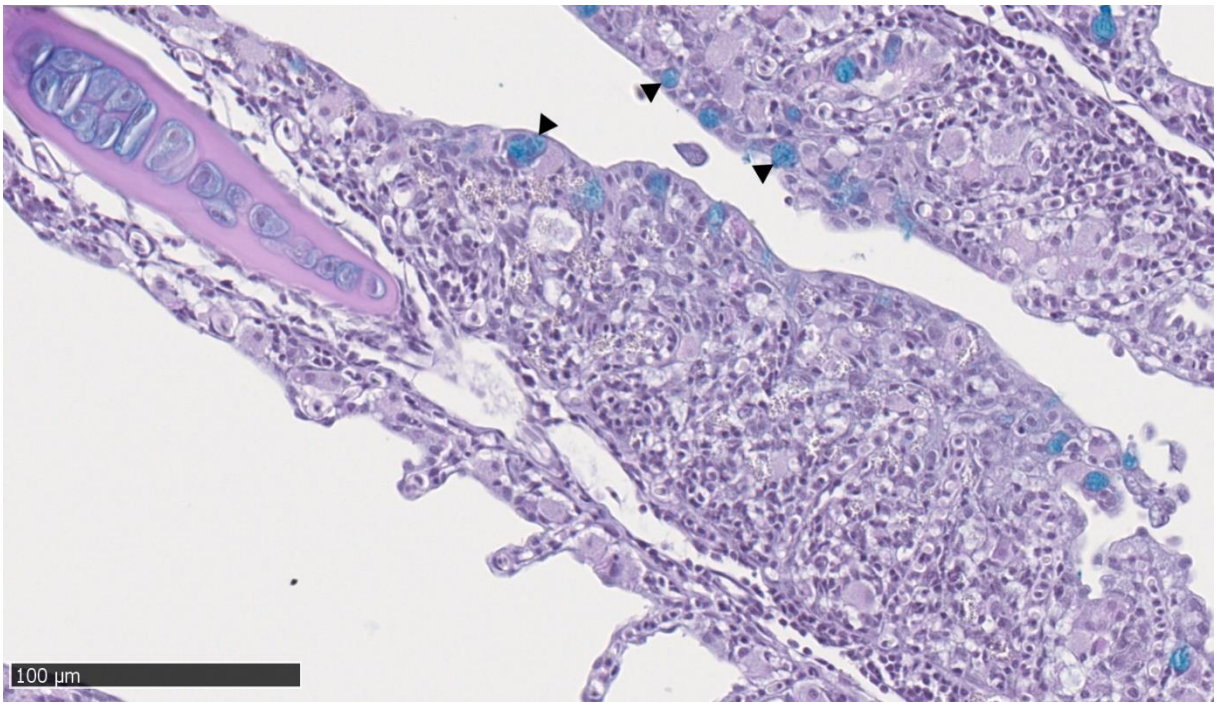


Figure 8 – AB-PAS staining with haematoxylin staining nuclei, from ballan wrasse (*fish id 41) showing a focal AGD lesion. Black bold arrowheads indicate some mucosal cells stained with a clear turquoise colour. There is a heightened presence of mucous cells along the border of the lesion. Secondary lamellae are hard to make out from the background noise the haematoxylin stain adds to the slide, but there is clear signs of hyperplasia and hypertrophic changes as in* ***figure 7****. 33x enlargement in NDP-viewer, scalebar 100 µm.*

3. 1 – 3 Histology in Atlantic salmon and ballan wrasse

Throughout the study there were pathological differences observed histologically between species. Out of the 549 ballan wrasse only 6 had clinical signs consistent with AGD or *P. perurans* present in the gill tissue, in contrast all 11 Atlantic salmon included in the study had morphological changes consistent with AGD. Various degree of branchial irritation and increased mucous excretion across three gill filaments from an Atlantic salmon with confirmed presence of *P. perurans* (**figure 9**). The amoebae were in close apposition to the gill epithelial surface causing the secondary lamellae to shorten due to rounding and fuse, creating interlamellar vesicles or cavernae (**figure 9 - B**). Squamation of chloride cells was also evident in (**figure 9** – **C**). Characteristic for the Atlantic salmon in non-lesion areas were squamation and sloughing of chloride cells from the base of the secondary lamellae (**figure 10**) indicating necrosis of epithelial gill tissue. AGD lesions in Atlantic salmon also appeared to be more severe, affecting multiple gill filaments (**figure 11**) in contrast to more focal lesions as observed in ballan wrasse (**figure 12**).

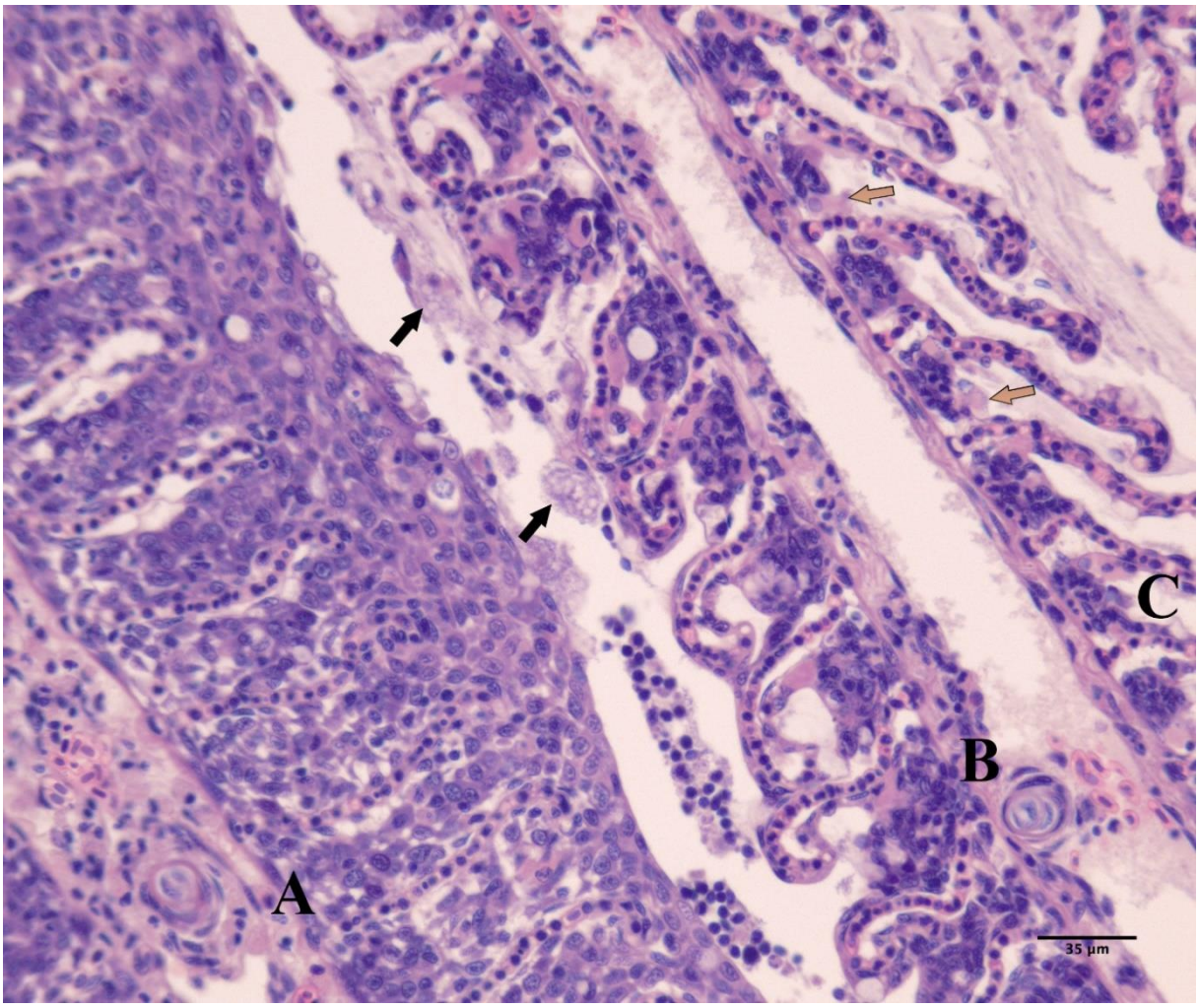


Figure 9 – HE-staining in Atlantic salmon (fish id 602) showing variations in pathology associated with AGD, 200x enlargement, scalebar 35 µm. ***A:*** *Showing a classic AGD lesion with smooth edges due to proliferation of gill tissue, caused by the presence of amoebae. The primary lamellae show the central venous sinus with erythrocytes to the left of the capital letter* ***A****. Secondary lamellae are stained lightly pink with clear structures like pillar cells stained dark purple.* ***B:*** *Part of a primary filament going through various stages of branchial irritation, the secondary lamellae are rounding causing a shortening of length and some lamellae have fused together forming interlamellar vesicles or cavernae. Two* P. perurans *are adhered to the gill surface vegetating on epithelial tissue, indicated with bold black arrows.* ***C:*** *Showing branchial irritation, with an initial response to the presence of amoebae with sloughing of chloride cells, indicated with light brown bold arrows, and heavy excretion of mucous in between filaments.*

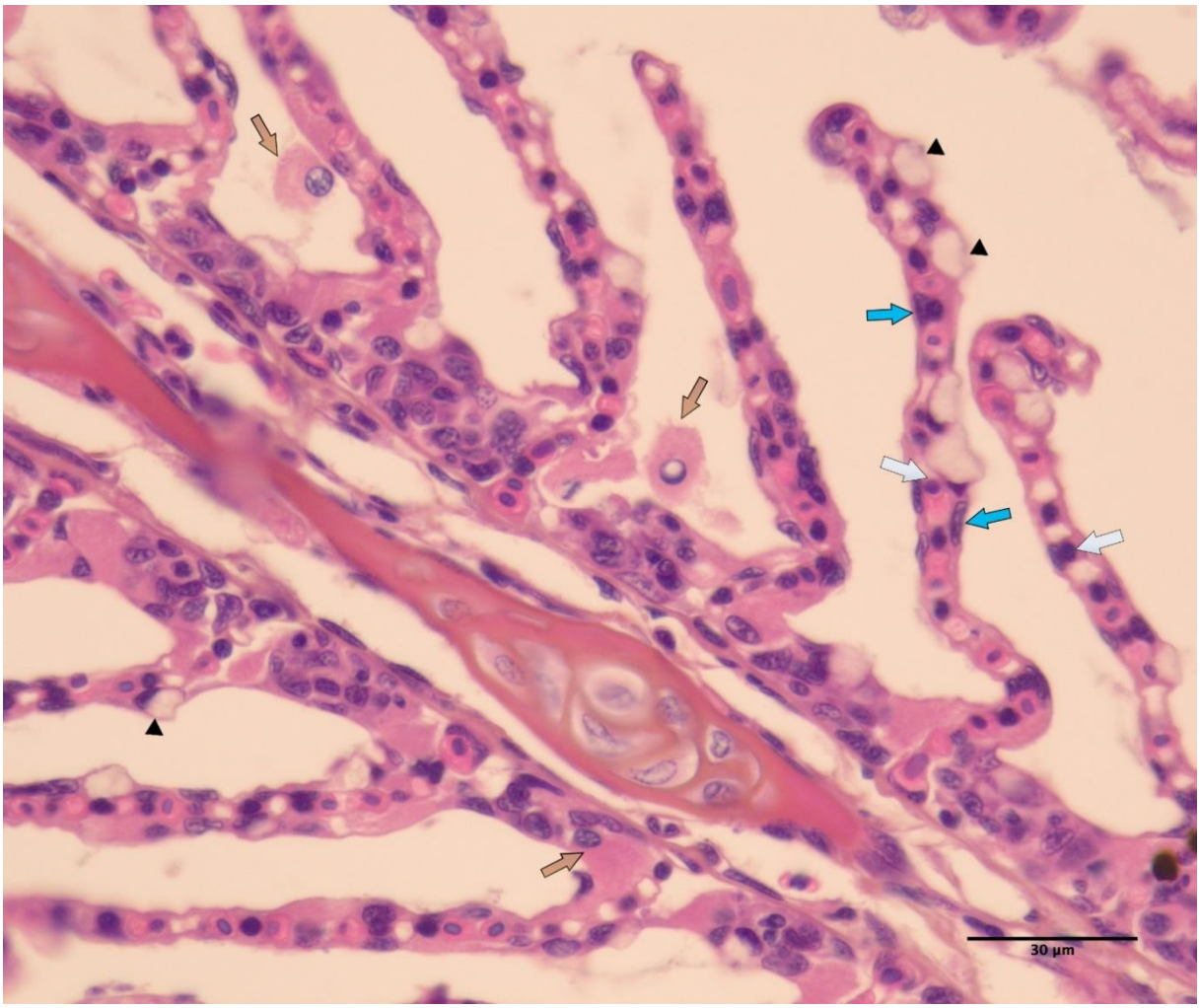


Figure 10 – HES-staining in Atlantic salmon (fish id 602) showing morphological changes in gill tissue related to infection with P. perurans*, 200x enlargement, scalebar 30 µm. Primary lamellae with central cartilage, central venous sinus and visible erythrocytes. Secondary lamellae are easily visible coloured pink with some pillar cells indicated by white bold arrows and some epithelial cells indicated with turquoise bold arrows. Sloughing of chloride cells, indicated with light brown bold arrows, are visible between lamellae indicating branchial irritation and necrosis of epithelial gill tissue. Some mucous cells are indicated with black bold arrowheads.*

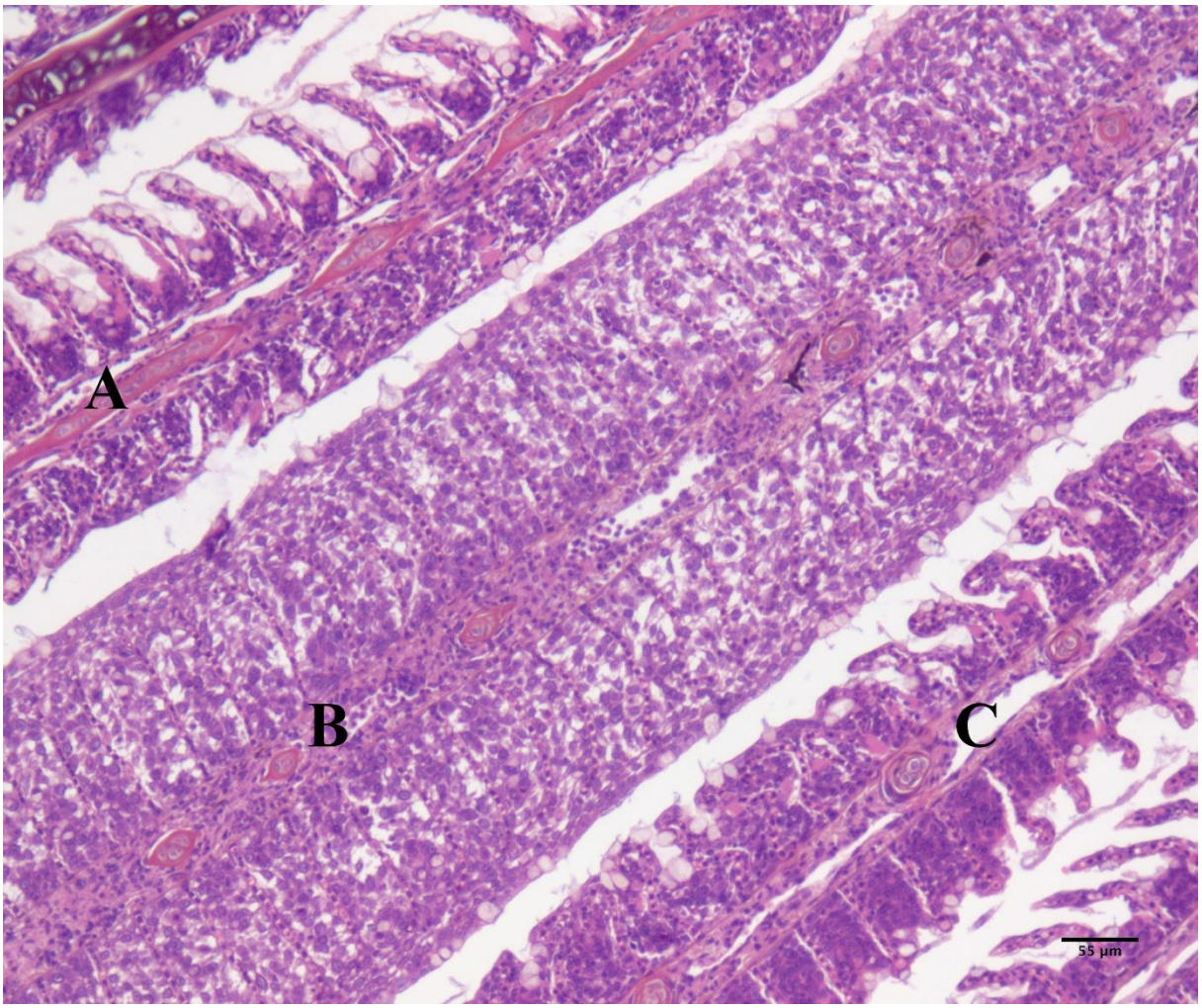


*Figure 11 - HES-staining in Atlantic salmon (fish id 607) showing multiple gill filaments with morphological changes in gill tissue related to infection with P. perurans, 100x enlargement, scalebar 55 µm. These gill filaments show visible centre cartilage as well as erythrocytes in the central venous sinus. The centre filament **B** shows secondary lamellae stained lightly pink with visible pillar cells stained purple and hyperplasia of respiratory epithelium forming a smooth edge on the filaments. At the borders of the edge there is a heightened presence of mucosal cells discharging mucus. The nearby filaments display the same morphological changes in the epithelial gill tissue, though with various degree of branchial irritation **A, C**.*

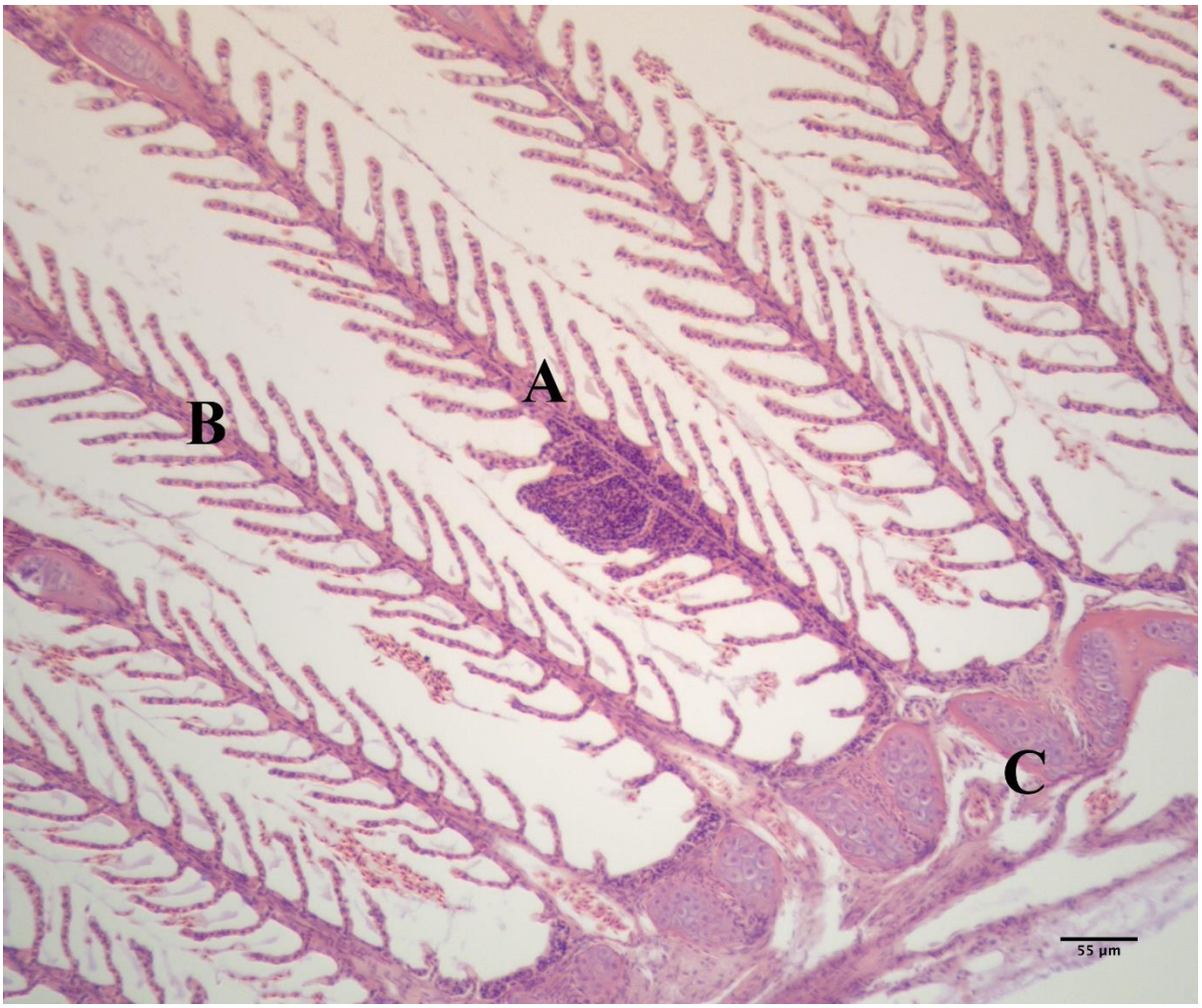


*Figure 12 - HE-staining in ballan wrasse (fish id 373) showing a section of the gill arch with gill filaments, 100x enlargement, scalebar 55 µm. **A**: Showing a focal lesion on a gill filament with an increase in respiratory epithelial cells (hypertrophy) and some rounding and shortening of the lamellae. **B**: Filament with secondary lamellae at equidistant length showing unaffected gills with normal orientation. **C**: Section of cartilage from the gill arch showing the anchor point of the primary filaments fused to the interbranchial septum and erythrocytes involved in the circulatory gas exchange system through capillaries and the central venous sinus.*

3. 1 – 4 Slides positive for amoebae

Upon histological examination six wrasse showed the presence of amoebae associated with hyperplastic lesions in the gills and five of the eleven Atlantic salmon had amoebae present near hyperplastic lesions (**table 1**). Amoebae were found across multiple histological stains often in proximity to lesions with inflamed gill tissue. The histological observations from ballan wrasse showed normal unaffected gills as the general observation, with a few exceptions (**table 1, figure 13 and figure 14)**. AB-PAS were used to estimate mucosal presence and excretory activity of mucus within clinical AGD lesions and in areas with epithelial changes and or inflammatory responses. In **figure 13** the same lesion from a ballan wrasse with clinical signs consistent for AGD were sectioned and stained with both AB-PAS and HE to illustrate how the same pathology was expressed across stains (**figure 13**). In the ballan wrasse most of the

sampled fish (98.9%) showed no signs or morphological changes in response to the presence of amoebae in tanks as seen in **figure 14 – B**. However, the infected fish (1.1%) displayed similar pathology as observed in infected Atlantic salmon with lesions forming around amoebae, although more defined (**figure 14-A, -C**). Variations in pathology between HES and HE stains related to inflammatory response related to infection with *P. perurans* in Atlantic salmon (**figure 15**). The amoebae were clearly visible with a bubbly appearance, vacuoles and a purple stained nucleus, either close apposition on the gill epithelial tissue (**figure 15 - A**) or incapsulated in interlamellar vesicles (**figure 15 - B**). Along the borders of the lesion, an increase in mucosal activity and excretion of mucus from discharging mucous cells was observed (**figure 15**). Fish 400 from 3WPC (challenge two) had very little inflammatory response to the presence of *P. perurans* (**figure 16 - B, - C**). With more similarities towards the unaffected fish with normal gill tissue (**figure 16 – A**).

Amoebae were generally discovered in higher numbers and density located at the base of the gill filaments near the cartilage, this was characteristic for both species. However, for ballan wrasse observed lesions were more focal spanning over fever ILU's and less severe than those in the Atlantic salmon. Characteristic pathology associated with AGD in Atlantic salmon, were rounding and shortening of the secondary lamellae, interlamellar vesicles containing amoebae, proliferation of gill tissue, squamation and stratification of gill epithelial tissue and increased in activity in mucous excretion (**figure 15 and figure 17**).

The frequency of observed amoebae in Atlantic salmon had a higher density in the surrounding tissue of lesions than in ballan wrasse. The amoebas and lesions were mostly located at the base or medial of the filaments close to the cartilage, a finding which was characteristic for both species. In Atlantic salmon 602 a *P. perurans* was observed with pseudopodia probing the epithelial tissue causing a massive inflammatory response which caused a heightened presence of mucous cells and mucosal activity (**figure 17**). There were also necrotic cells within the ILU were the vegetating amoebae had adhered itself to the gill epithelium and rounding of the secondary lamellae (**figure 17**).

Table 1 : Positive histological sections with lesions corresponding to Amoebic gill disease (AGD). Sections with P. perurans *present in the gill tissue marked with *. Trial part is indicated with phase 1 for trial stage one and phase 2 for trial stage two. Tank ID are highlighted, and special stains specified with abbreviations.*

Tank ID	Infection Dose	ID	Species	Stain	Sampling	Phase
A8L5	1000 Cells/L	**41 ***	*Labrus bergylta*	**HE/AB-PAS**	Week 1	**1**
A4L5	100 Cells/L	**133***	*Labrus bergylta*	**HE/PCNA**	Week 3	**1**
A8L5	1000 Cells/L	**144***	*Labrus bergylta*	**PCNA**	Week 3	**1**
A6L4	1000 Cells/L	**396**	*Labrus bergylta*	**HE**	Week 3	**2**
A6L4	1000 Cells/L	**400 ***	*Labrus bergylta*	**HE**	Week 3	**2**
A8L5	1000 Cells/L	**373**	*Labrus bergylta*	**HE, HES**	Week 3	**2**
A2L6	1000 Cells/L	**602 ***	*Salmo salar*	**HE/AB-PAS**	Week 5	**2**
A2L6	1000 Cells/L	**605***	*Salmo salar*	**PCNA**	Week 5	**2**
A2L6	1000 Cells/L	**606***	*Salmo salar*	**PCNA**	Week 5	**2**
A2L6	1000 Cells/L	**607***	*Salmo salar*	**PCNA**	Week 5	**2**
A2L6	1000 Cells/L	**610***	*Salmo salar*	**PCNA**	Week 5	**2**

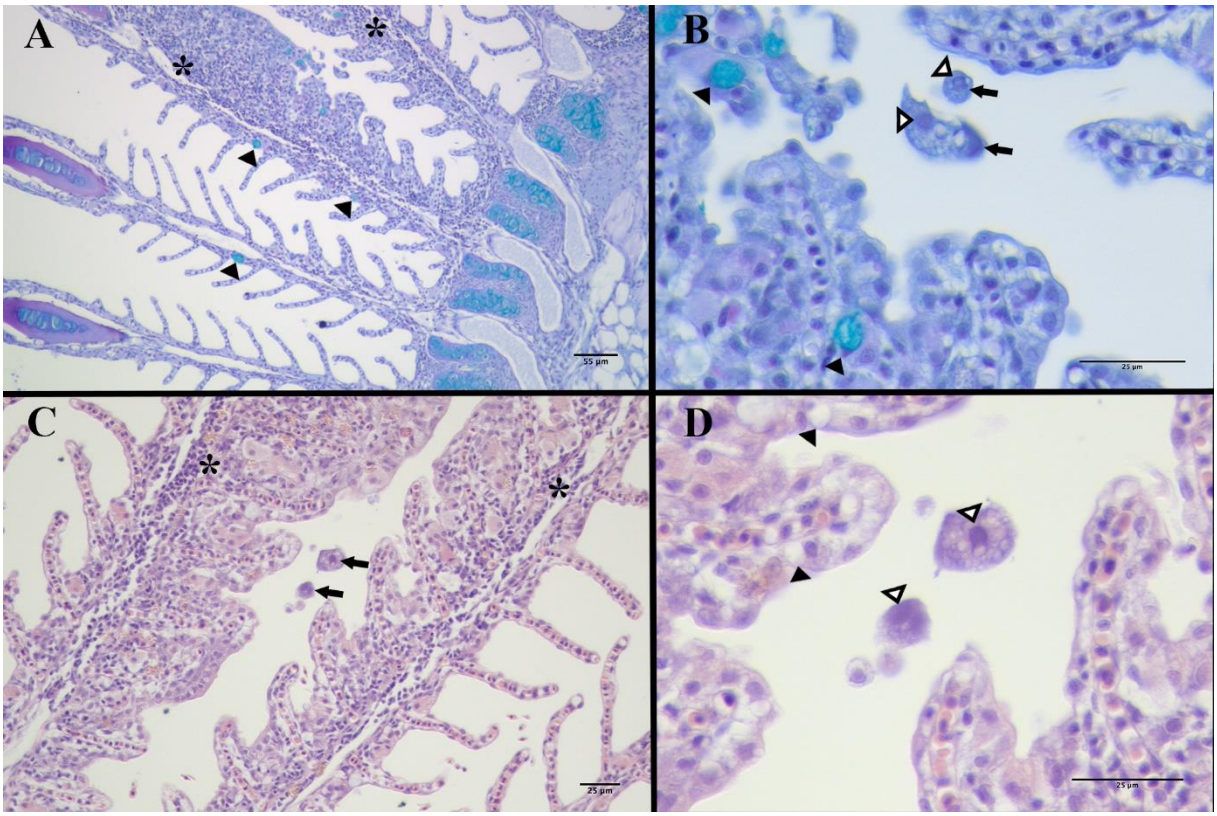


Figure 13 – Comparison of gill filaments from ballan wrasse *(Fish id 41) showing variations in AB-PAS and HE-staining.* ***A:*** *AB-PAS staining, overview of a typical AGD-lesion with hyperplasia and hypertrophic changes. Enlargement 100x, scalebar 55 µm* ***B:*** *AB-PAS staining, close-up of* P. perurans *1-week post infection. 630x enlargement, scalebar 55 µm* ***C:*** *HE-staining overview of the same fish on another section, lesions indicated with Asterix (*) and bold black arrows showing two* P. perurans *between the two lesions. Enlargement 200x, scalebar 25 µm* ***D:*** *HE-staining, closeup of amoeba between AGD-like lesions, nucleus indicated with black and white arrowheads and mucous cells indicated with black arrowheads. Enlargement 630x, scalebar 25 µm.*

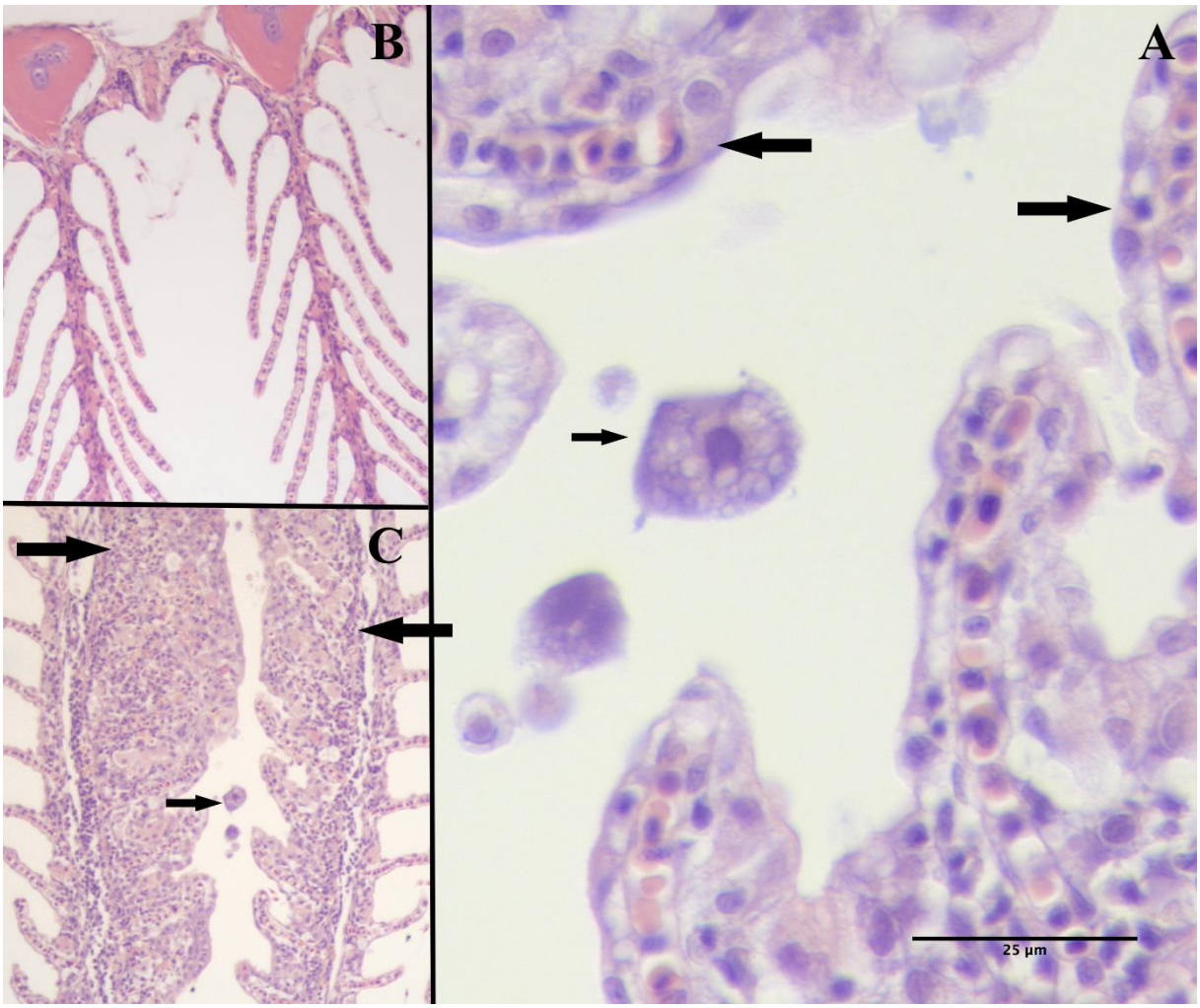


Figure 14 - Differences in gill morphology from ballan wrasse*; **A:** Fish id 41; HE-stained, showing* P. perurans *(small bold arrow) with a clear nuclei, located in between two filaments with typical lesions associated with AGD. The big bold arrows indicate the AGD lesions smooth edges. Below the indicated* P. perurans *is another partial amoeba where the nuclei are barely visible, this amoeba is also in picture C. **B:** Fish id 387; HE-stained, showing normal pathology with neat order of primary lamellae and equidistant secondary lamellae with normal composition of pillar cells, epithelial cells, mucous cells and chloride cells which can be easily differentiated at 100x enlargement. **C:** Fish id 41; HE-stained, Overview of AGD lesion indicated by (bold arrows) and* P. perurans *between fused gill arches at 630x enlargement, scalebar 25 µm.*

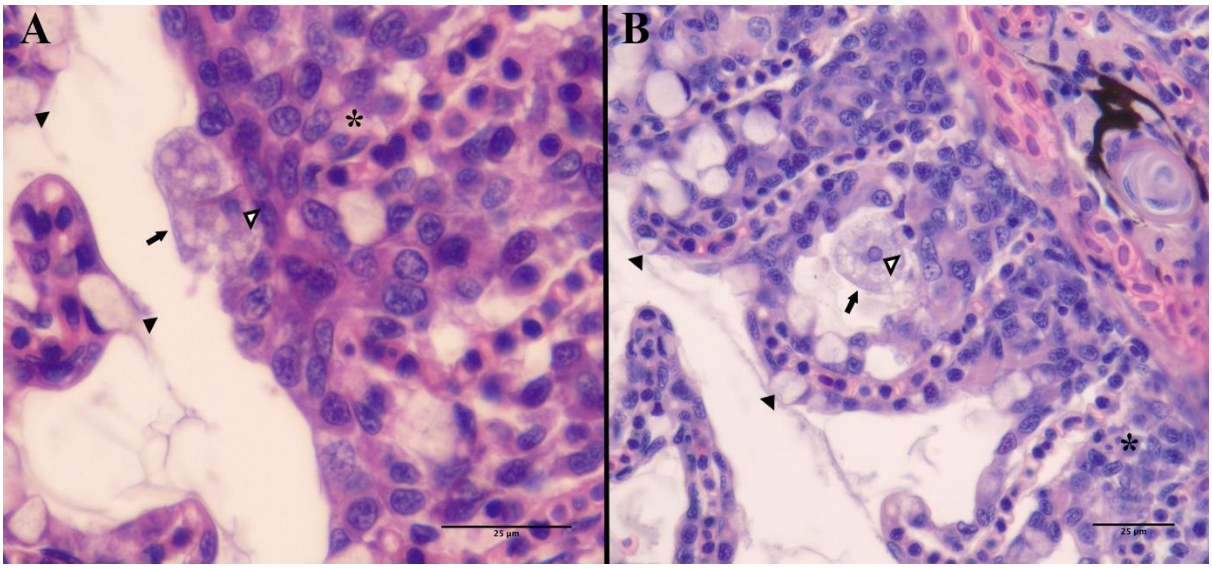


Figure 15 – Differences in staining comparing HES in picture (A) with HE in picture (B) gill sections sampled from Atlantic salmon *fish id 602.* ***A:*** *HES-staining showing* P. perurans *indicated with black bold arrow. Typical AGD lesion is marked with Asterix. Nuclei of the* P. perurans *indicated with white and black arrowhead, mucous cells indicated with black bold arrowhead. 630x enlargement, scalebar 25 µm.* ***B:*** *HE-staining showing* P. perurans (*indicated with a black bold arrow) incapsulated in an interlamellar vesicle. Vacuoles are visible within the amoeba and the nuclei is indicated with a white and black bold arrowhead. Excreting mucous cells are indicated with a bold black arrowhead and the lesion starting point indicated with an Asterix. 400x enlargement, scalebar 25 µm.*

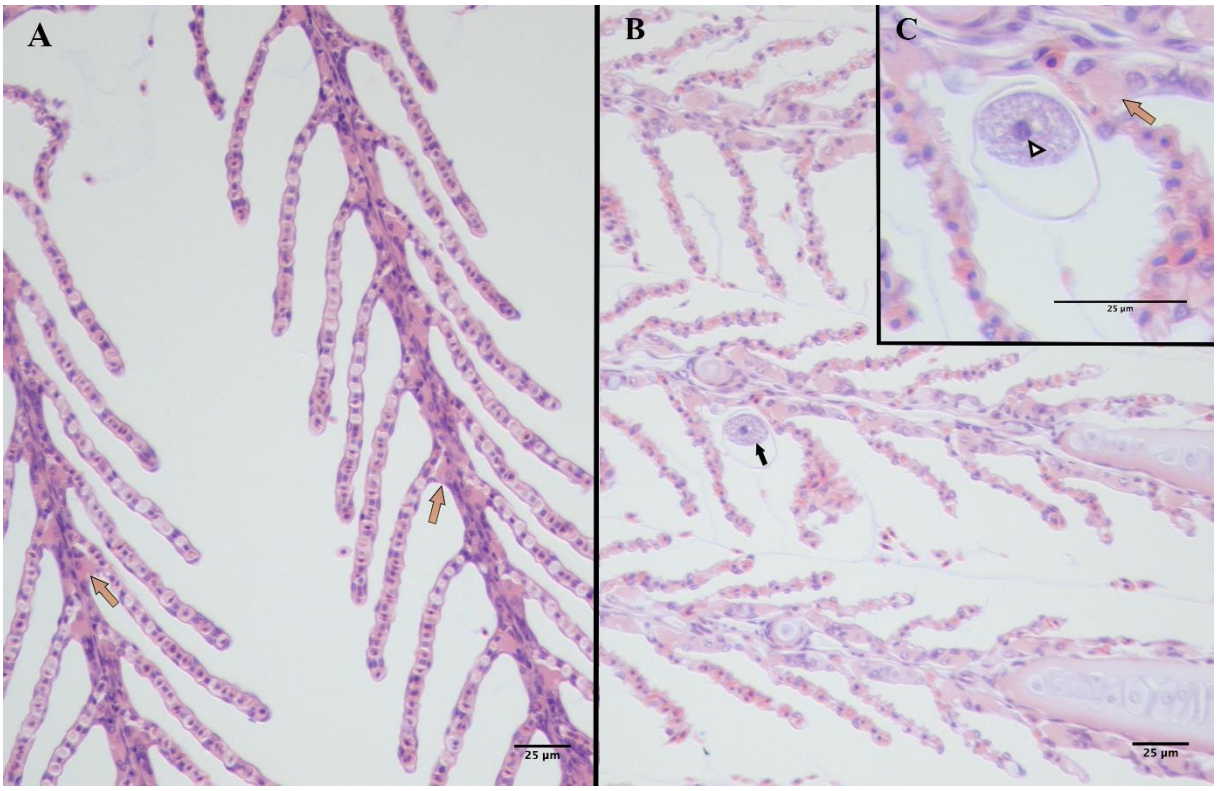


Figure 16 – Variations in pathology related to presence of P. perurans *causing AGD;* ***A:*** *HE-staining from* ballan wrasse *(fish id 387) (1000Cells/L) showing normal gill filaments with no apparent lesions or morphological changes associated with clinical signs of AGD. Some chloride cells indicated at the base of the secondary lamellae with light brown arrows. Pillar cells are clearly stained dark purple and located in the centre of the secondary filaments as well as epithelial cells on the edges of the lamellae. Enlargement 200x, scalebar 25 µm.* ***B:*** *HE-staining from fish id 400 (1000Cells/L) showing a* P. perurans *located in an interlamellar unit causing epithelial changes within on the secondary lamellae. The presence of* P. perurans *is affecting the secondary lamellae showing signs of hypertrophy like rounding and fusion of the lamellae forming an interlamellar vesicle.*

Enlargement 200x, scalebar 25 µm ***C:*** *Closeup from the amoeba in picture B, nuclei indicated with a black and white bold arrowhead and a chloride cell right next to it indicated with a light brown arrow. The faint barrier around the* P. perurans *is due to a bubble forming while mounting the plate of the section with xylene. Enlargement 630x, scalebar 25 µm.*

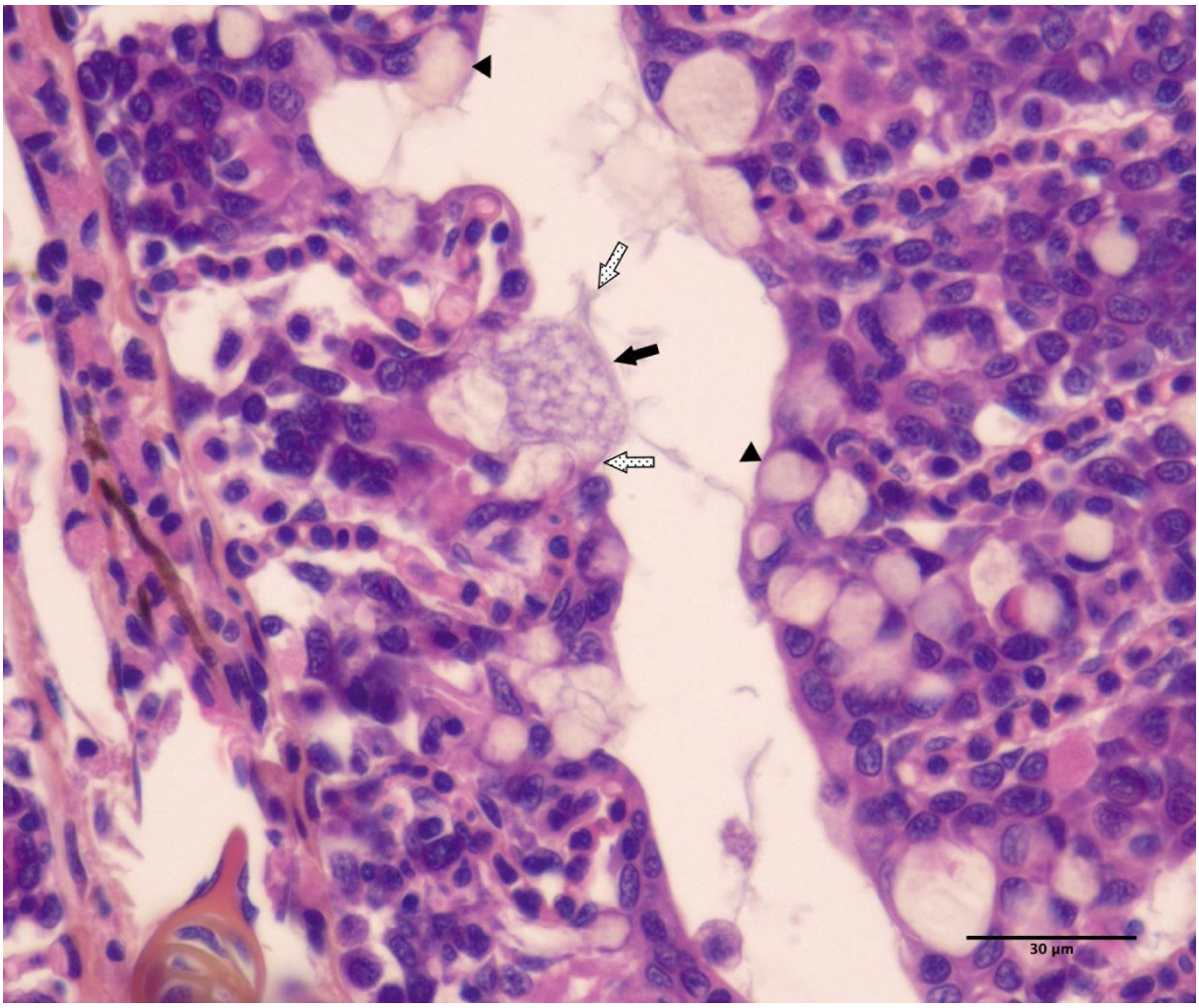


Figure 17 – HES-staining in Atlantic salmon *(fish id 602) showing* P. perurans *indicated with a bold black arrow located on the surface of a clinical lesion consistent with AGD, 400x enlargement, scalebar 30 µm. Pseudopodia (indicated with white and black dotted arrows) are in contact with the gill tissue causing an inflammatory response with multifocal hyperplasia and hypertrophic changes in the gill epithelium. High recruitment and migration of mucus cells along the edge of a classic smooth AGD lesion (some indicated with black bold arrows) excreting mucous.*

3. 1 – 5 Proliferating cell nuclear antigen (PCNA)

Proliferating cell nuclear antigen (PCNA) staining was carried out on sections from both species *S. salar* and *L. bergylta*. PCNA positive cells were stained faint mauve differentiating them from the PCNA negative cells with a purple-dark blue hue (**figure 18. A - F**). AGD causes a chronic gill inflammation over time, which were observed in both species as an abnormal increase of respiratory epithelial cells with fusing of the secondary lamellae and reduced respiratory surface (**figure 18 – A, 18 – D** and **18 – F**). Gill filaments with variance in branchial irritation related to AGD, displaying the same morphological differences with proliferation and

the presence of PCNA positive cells within lesions (**figure 18 – A**). PCNA positive cells were observed both in normal secondary lamellae with no evidence of inflammation and in more focal lesions (**figure 18 – B**). Examining differences between location of PCNA positive cells, in general there were higher density for positive cells within lesions than in normal tissue in Atlantic salmon (**figure 18 – C**). For ballan wrasse the same variance in PCNA positive cells were displayed, however with a slightly reduced number of positives within lesions (**figure 18 – D**). However, the secondary filaments in ballan wrasse showed a heightened presence of PCNA positive cells in the normal unaffected tissue than what was observed in Atlantic salmon (**figure 18 – E**). Although the lesions were similar in both Atlantic salmon and ballan wrasse with pathology consistent with AGD like hypertrophy and hyperplasia, they displayed various levels of cell turnover (**figure 18 – A and figure 18 – F**). Fixation dependant staining differences in pathology and expression of PCNA positive cells were observed between formalin and Davidsons sea water fixative (**figure 19**). Through examination of PCNA stained sections an eosinophilic granular cell (EGC) were found to be stained with a dark mauve colour and observed amidst erythrocytes in the central venous sinus (**figure 20**). Similar granular structures consistent with EGCs were also discovered in the base of some secondary lamellae in ballan wrasse, they were, however, not detected in any lesions (**figure 21**).

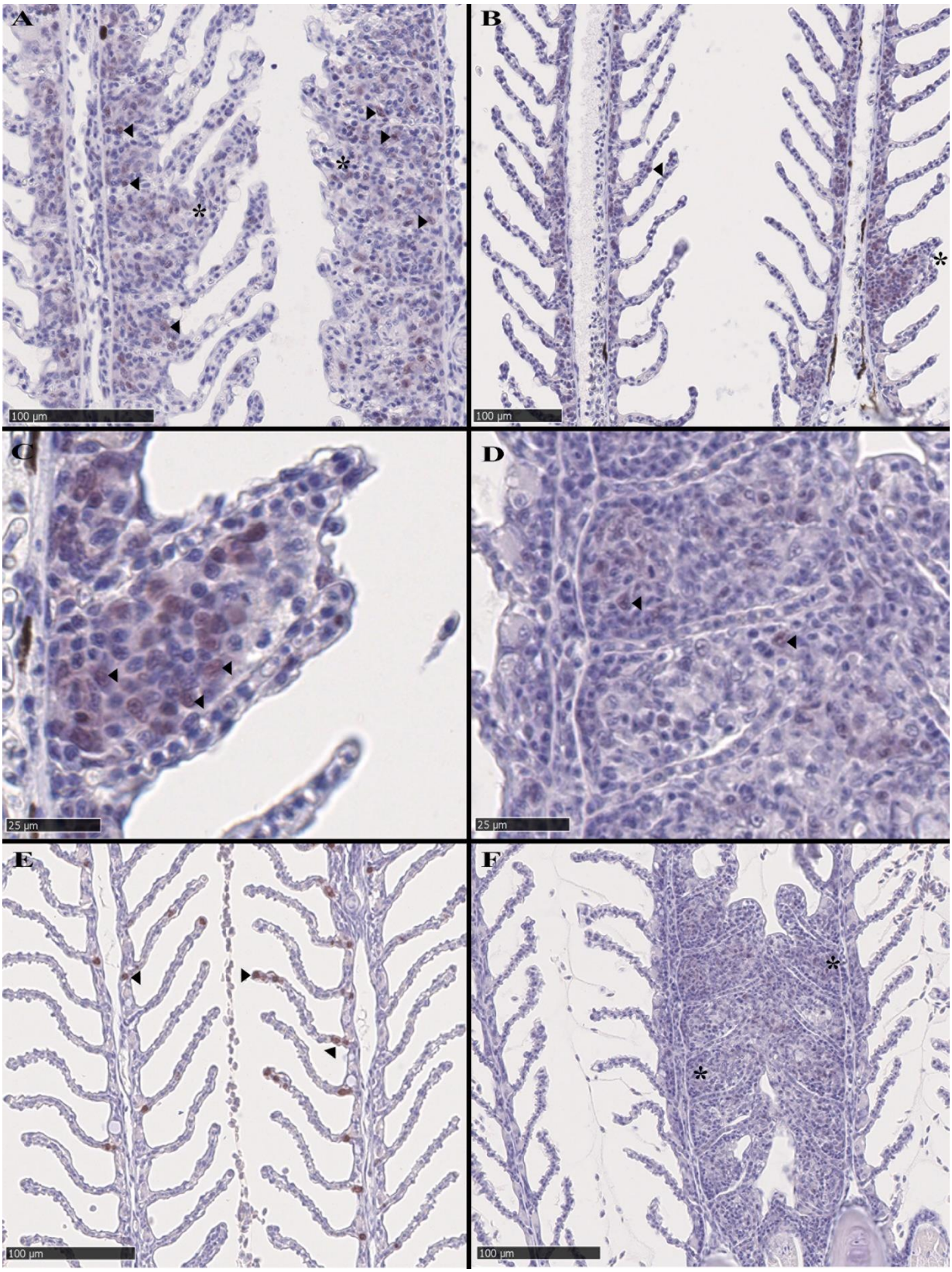


Figure 18 - Proliferating cell nuclear antigen (PCNA) immunostaining on Atlantic salmon *(A-C) and* ballan wrasse (*D-F) showing variations in saturation and absorbance of staining related to cellular "turnover".* ***A****: PCNA-staining on a gill section from* Atlantic salmon *(fish id 606); Positive cells indicated with a black bold arrowhead, note not all positive cells are marked. Lesion is marked with Asterix (*).-Scalebar 100 µm, NDP-viewer 30,4x enlargement, infection dose (1000 Cells/L).* ***B****: PCNA-staining on a gill section from* Atlantic salmon *(fish id 609); Normal gill filament to the left and filament with a small lesion covering 2 ILU's on the right filament. Scalebar 100 µm, NDP-viewer 18,3x enlargement, infection dose (1000 Cells/L).* ***C****: Closeup from lesion in B showing hyperplasia and hypertrophic changes, positive cells indicated with black bold arrowhead,*

Scalebar 25 µm, NDP-viewer 80x enlargement, infection dose (1000 cells/L). ***D****: PCNA-staining on a gill section from* ballan wrasse *(fish id 396); Closeup from lesion in picture F, showing hyperplasia and hypertrophic changes associated with AGD. PCNA-Positive cells indicated with black bold arrowhead. Scalebar 25 µm, NDP-viewer 80x enlargement, infection dose (1000 Cells/L).* ***E****: PCNA-staining on a gill section from* ballan wrasse *(fish id 133); Showing filaments with normal histology and no evident hyperplasia or hypertrophic changes. Scalebar 100 µm, NDP-viewer 27,5x enlargement infection dose (100 Cells/L).* ***F****: PCNA-staining on a gill section from ballan wrasse (fish id 396); Normal filament located at the left corner of E and Lesion marked with Asterix (*) between two gill filaments. Area marked with Asterix showing coalescence between two filaments by the base of the gill arch with hyperplasia and hypertrophic changes in the gill tissue. Scalebar 100 µm, NDP-viewer 26,4x enlargement, infection dose (1000 Cells/L).*

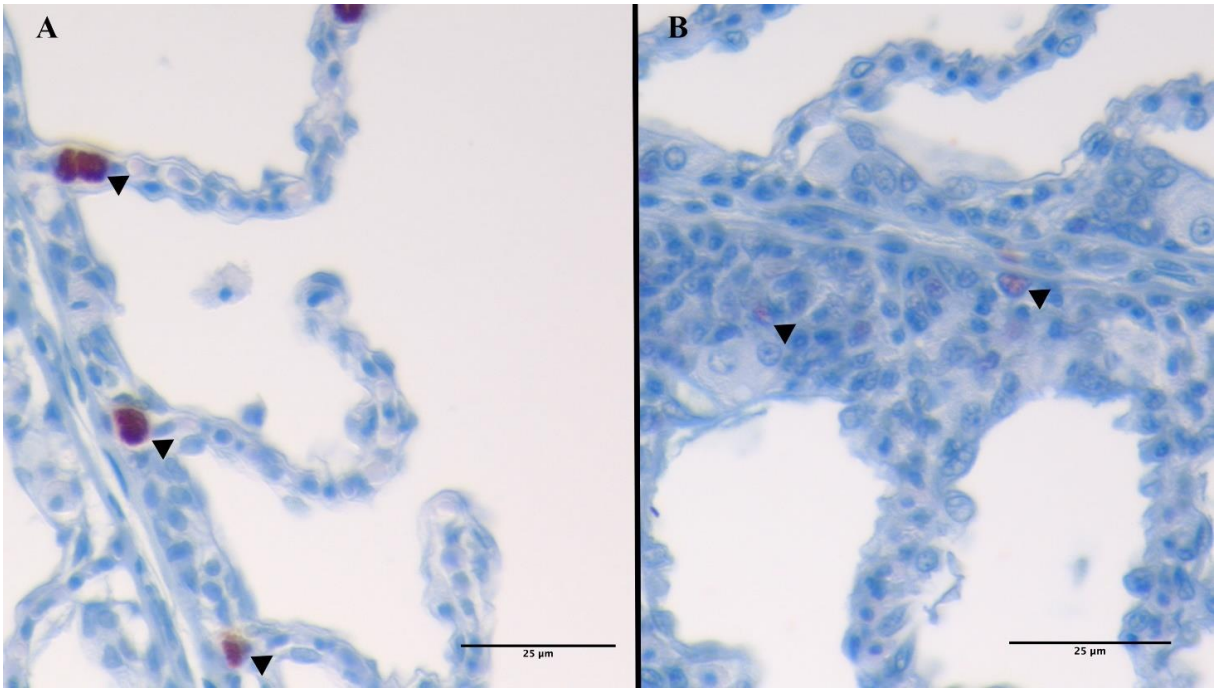


Figure 19 – Immunostaining of ballan wrasse, showing variations in PCNA staining affected by fixative. ***A:*** *Fish id 135; PCNA staining on gill tissue fixated with formalin, with clear bright mauve colouration on PCNA positive cells indicated with black bold arrowheads. The lamellae and filaments are stained lightly blue with less background noise than in picture B. 630x enlargement, scalebar 25 µm.* ***B:*** *Fish id 400; PCNA staining on gill tissue fixated with Davidsons seawater fixative, the PCNA positive cells indicated with black bold arrowheads are fainter with a weak mauve colouration quite distinct from the formalin fixated gill tissue in picture A. Davidsons sea water fixative effects the background colouration of the lamellae. 630x enlargement, scalebar 25 µm.*

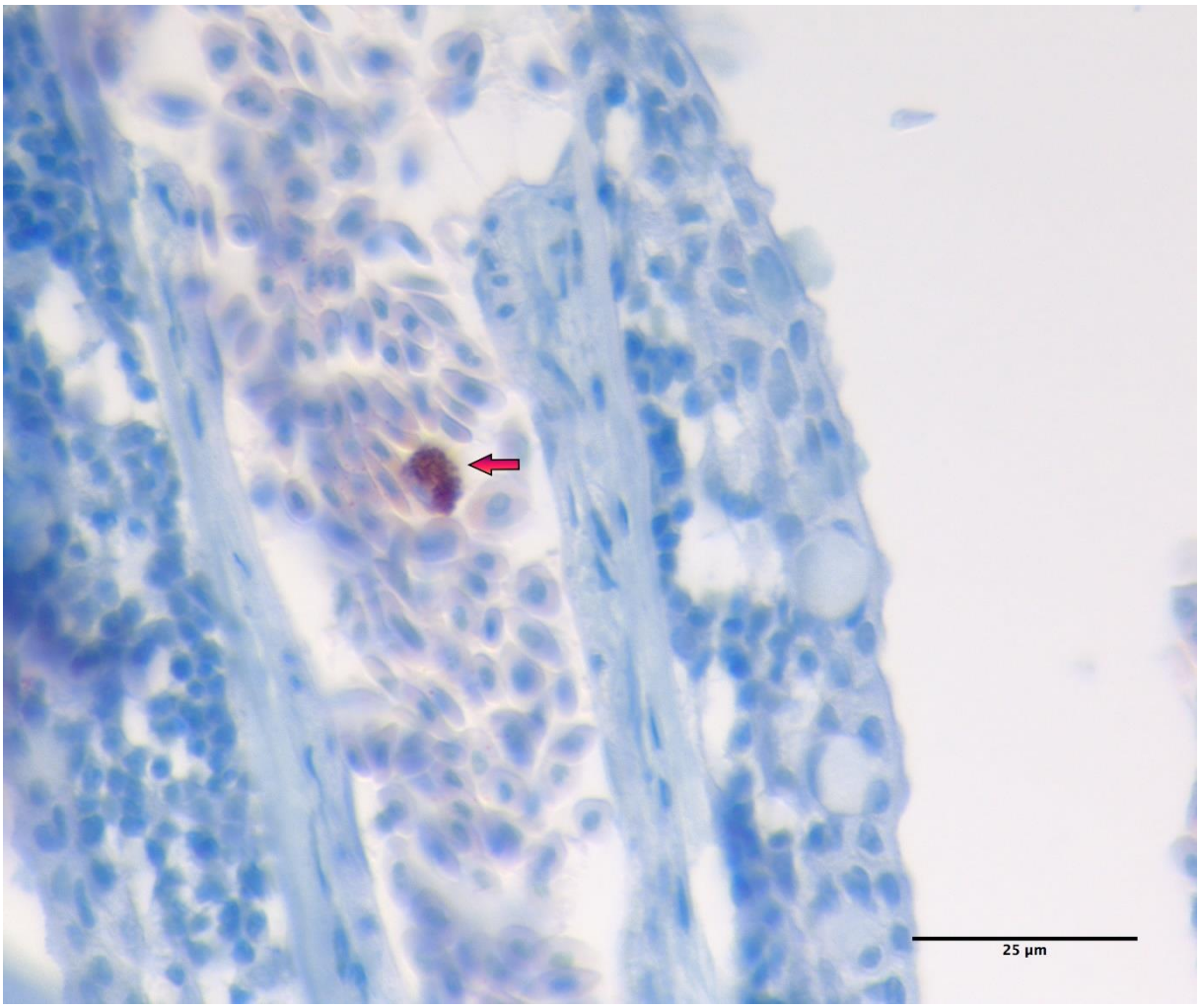


Figure 20 - PCNA-staining from ballan wrasse *(fish id 135) showing the cartilage and blood vessel in the primary lamellae, 630x enlargement, scalebar 25 µm. Erythrocytes are clearly visible with a light pink staining and a sky-blue core contained within a central venous sinus. An eosinophilic granular cell (EGC) is indicated with a bold red arrow, located in the bloodstream positively stained by the PCNA.*

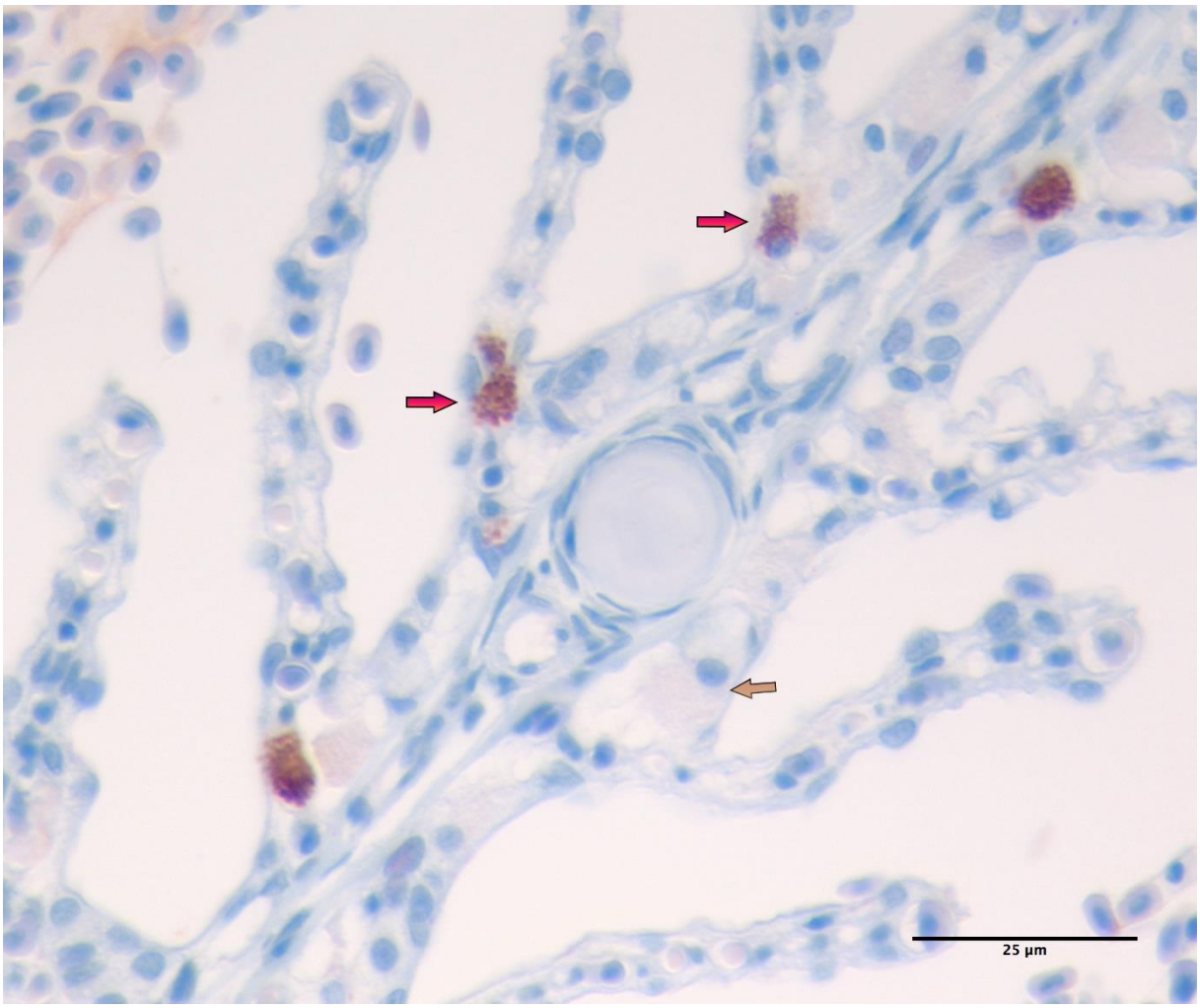


Figure 21 - PCNA-staining from ballan wrasse (fish id 35) showing multiple positive stained EGCs, 630x enlargement, scalebar 25 µm. Primary filament with cartilage and equidistant secondary lamellae, some positive EGCs indicated with bold red arrows, and a chloride cell indicated with a light brown bold arrow at the base of a secondary lamellae. Erythrocytes are also viewable in the top right corner with a slight pink cell membrane and a sky-blue nucleus.

3. 1 – 6 Mucous cell counts AB-PAS

Mucous cell counts were used to assess the mucosal activity and presence of mucous cells in gill tissue of the ballan wrasse across cd. In challenge one cd 0, cd 100, cd 1000 were used and cd 0 and cd 1000 were used for challenge two. In challenge one both challenge doses (cd 100 and cd 1000) had a higher density of mucous cells than in the control group (cd 0) (**figure 22**), however, the number of mucous cells did not appear to be dependent on either challenge dose ($F_2 = 0.372\,, Pvalue = 0.6916$) or WPC ($F_2 = 2.539\,, Pvalue = 0.0902$). The highest mucous cell density was registered at 3 WPC with a mean value of 13,6 mucous cells per 10 ILU's (**figure 22)**. The mucosal cell density trends towards a peak in 3 WPC with a following decline in 5 WPC for both challenge doses, cd 100 and cd 1000, but the control group (cd 0) has a steady decline from 1 WPC – 5 WPC (**figure 22**). There was also no significant

interaction between challenge dose and WPC (TUKEY, *Pvalue* = 0.471). In challenge two the control group (cd 0) showed the same trend as for the high doses in challenge one with a peak in 3 WPC and a following decline in 5 WPC which coincided with mucus excretion observed while sampling. The differences observed in mucosal cell counts were also statistically significant for cd ($F_1 = 8.569$, $Pvalue = 0.005$). However, the high dose (cd 1000) did not follow the same trend (**figure 23**) where the control group (cd 0) expressed a higher number of mucous cells than the high dose, showing a significant effect of cd (TUKEY, *Pvalue* = 0.005).

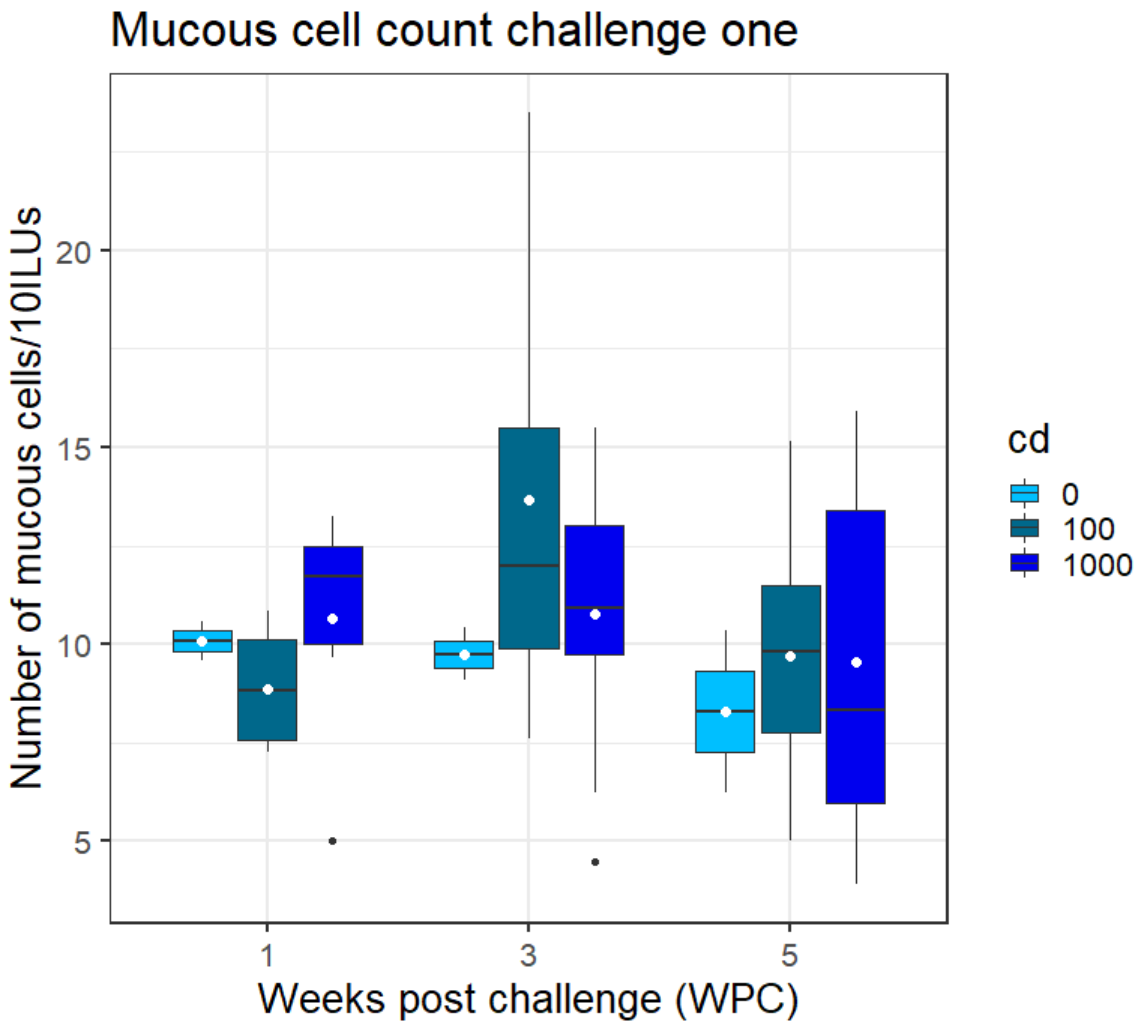


Figure 22 - Mucous cell count challenge one, colour coded boxplots (turquoise for the cd 0, blue green for cd 100 and royal blue for cd 1000) showing mean values (white dot) for the different challenge doses (cd 0, 100, 1000) included in challenge part one.

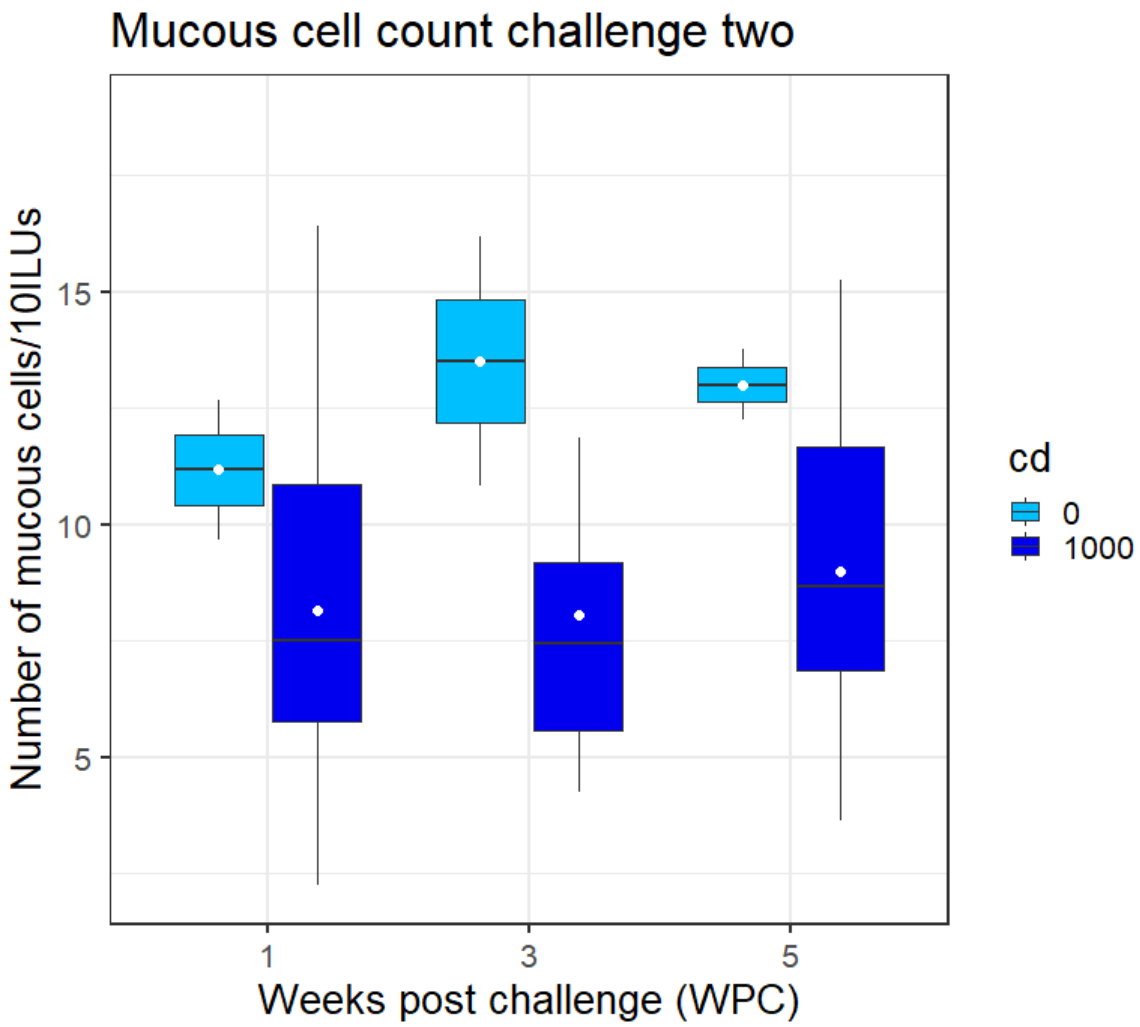


Figure 23 - Mucous cell count challenge two, colour coded boxplots (turquoise for the cd 0 and royal blue for cd 1000) showing mean values (white dot) for the different challenge doses (cd 0, 1000) included in challenge part two.

3. 1 – 7 Polymerase Chain Reaction (PCR) analysis

From the sampled gill tissue analysed for *P. perurans*, only one positive was found from 314 samples of ballan wrasse by qPCR. The positive sample showed a low detection from fish ID 320 a 1000 cells/L group. From the sampled gill tissue from Atlantic salmon 2 out of 5 samples were positive for *P. perurans*. The positive cells showed a moderate detection from fish ID 600 and low detection in fish id 603.

4. Discussion

The aim of the study was to investigate the susceptibility of ballan wrasse to *P. perurans* the aetiological agent of amoebic gill disease as proposed by Young *et al.*, (2008). This was done by exposing different groups of ballan wrasse to various concentrations of *P. perurans* to

identify a minimum threshold dose of amoeba required to contract AGD. Through challenge one there were no gross gill score or clinical signs indicating infection with *P. perurans* from any of the challenge doses in the wrasse. There was however, observed an increase in mucosal excretion with a peak in 3 WPC and with a following decline (**figure 22, 23**). This raised the question if the C2-clone had perhaps lost some of its ability to proliferate or adhere to the epithelial tissue of the gills which has been suggested to affect virulence (Collins *et al.*, 2017). Clonal cultures have also been observed to loose virulence over time as seen for Bridle *et al.* (2015) where the clonal isolate became completely avirulent. The C2-clone in this study was kept in continuous culture since 2013 surpassing the three years described in Bridle *et al.* (2015) by another 1095 days (over 70 passages).

To further investigate this and determine whether the ballan wrasse either had an unusually high tolerance for the clonal isolate or the amoeba had lost virulence, a sentinel species, Atlantic salmon was introduced in challenge two. The ballan wrasse displayed the same trend as in challenge one, with no gross gill score and an increase of mucosal activity with a following decline. However, the Atlantic salmon developed gross gill lesions consistent with previous challenges with AGD (Adams *et al*., 2004; Mitchell *et al*., 2011; Oldham *et al*., 2016; Dahle *et al.*, 2020) and progressed to a gross gill score of three at the end of the challenge (**figure 6**). This contested the fact that the clonal isolate had lost virulence while in culture and pointed towards a variance in susceptibility between species and innate response towards amoebae.

4. 1 Differences in susceptibility between species

Clinic varied greatly throughout the study between species (**figure 5**). For the ballan wrasse there were no visual signs suggesting that they were infected with AGD. Through eleven weeks of challenge only six out of 549 ballan wrasse displayed pathology consistent with AGD (**table 1**). However, for the sentinel species Atlantic salmon all showed clinical signs consistent with AGD with slightly different development of the disease (**figure 6**). Such variations in susceptibility had previously been described for Taylor *et al.* (2007) showing anecdotal evidence of considerable variation in AGD related to severity and survival rate in Atlantic salmon. Bridle *et al.*, (2005) showed evidence of a level of innate immunity towards AGD (Taylor *et al.* 2009). This supports the observations of inter individual progression of disease with clinical manifestation not appearing uniform even in a clonal strain of Atlantic salmon (**figure 6**).

Previous studies have shown different capability to generate clinic consistent with AGD between clonal isolates (Crosbie *et al.*, 2010; Dahle, 2015; Haugland *et al.*, 2017; Dahle *et al.*, 2020). Suggesting that virulence of clonal isolates may be affected in numerous ways, including increased proliferation, longer duration, better attachment or immunocompromising the hosts ability to clear amoebae by production of virulence factors interacting with the host (Collins *et al.*, 2017). *P. perurans* has a low host specificity affecting several marine teleost fish (Karlsbakk *et al.*, 2013; Oldham, Rodger and Nowak, 2016; Collins *et al.*, 2017; Haugland *et al.*, 2017; Hellebø, Stene and Aspehaug, 2017; Steigen *et al.*, 2018) with considerable variation in described clinic and severity between salmonids and other marine teleost's like ballan wrasse and lumpfish. Through a co-habitant infection study Dahle *et al.* (2020) observed that clonal isolates of amoebae derived from ballan wrasse caused more severe AGD in Atlantic salmon than the salmon isolates did in the wrasse. This may indicate that ballan wrasse is less susceptible and has a higher tolerance for AGD, as has been proven for lumpfish (Haugland *et al.*, 2017). The C2-clonal isolate has also been reported to not cause severe AGD in ballan wrasse (Andersen, ILAB, pers.com) although it generates clinic in Atlantic salmon which supports that the ballan wrasse is less susceptible to AGD than Atlantic salmon. This also underlines that even if a species is susceptible to *P. perurans* it does not necessarily mean that it will develop clinic consistent with AGD (Haugland *et al.*, 2017).

4. 2 Growth and metabolic cost of AGD

Throughout the study the ballan wrasse did not show any evidence of moribund fish or other lethality than the sampling itself. Observed behaviour suggested acclimatisation to the tanks and that they adapted well to the feeding regime. Over time a trend indicating growth, linked to an increase of weight and length was observed (**figure 4**). This was further emphasized through the sampling points (SP) showing a range from 3 to 41 grams in weight and 6.5 to 14.3 cm in length. The median weight for the study also changed from 12.0 grams (± 5.0 g) in SP-1 to 21.3 grams (± 7.7 g) in SP-12. The median length also displayed the same pattern with 9.0 cm (± 1.2 cm) in SP-1 and 10.7 cm (± 1.2 cm) in SP-12 (**figure 4**). This contrasts with the study conducted by (Kvinnsland, 2017) were ballan wrasse developed severe AGD, and growth data from both the positive and negative fish showed little change during the six weeks of challenge.

Infection with AGD in teleost fish have been linked to a significant metabolic cost, that has been associated with reduction in growth if left untreated (Lovy *et al.*, 2007; Jones, 2008;

Powell *et al.*, 2008). This could indicate that ballan wrasse with a severe infection of AGD would lead to a stagnation in growth as was observed in (Kvinnsland, 2017). However, the non-infected control groups displayed even less median progression for either weight or length suggesting other factors may have impacted growth (Kvinnsland, 2017). Stressors have previously been reported to cause a metabolic cost effectively compromising normal functions as growth, immuno-function, digestion and reproduction (Leclercq *et al*., 2014). Therefore, this stagnation in growth could be caused by other impeding stress factors. For the present study however, no such metabolic cost was evident as the growth of the ballan wrasse was shown to have statistically significant differences between SP, but no significant effect from cd. Indicating that the presence of *P. perurans* was not a factor significantly affecting the fish as is supported from the lack of clinic consistent with AGD for the majority of the ballan wrasse taking part in this study.

4. 3 Mucosal activity and excretion

For both challenges the amount of excreted mucus had an observed peak at 3 WPC. Initially the mucus covered mainly the gills and the operculum, but as the challenge progressed the mucus was found in excess amounts across the body surface, gills and the operculum. Infection with *Paramoeba perurans* has been shown to affect migration and activity in mucous cells (Roberts *et al.*, 2003; Adams *et al.*, 2004b; Adams *et al.*, 2004; Powell *et al.*, 2008; Mitchell *et al.*, 2011; Chalmers *et al.*, 2017; Cano *et al.*, 2019). Excessive production of mucus has also been linked to an increase in the number of branchial mucous cells (Roberts *et al*., 2003; Chalmers *et al.*, 2017). Pathologically discharging mucous cells were observed lining the edges of AGD lesions both in Atlantic salmon (**figure 7**) and ballan wrasse (**figure 8**). Indicating hyperplasia of mucous cells and an inflammatory response in the affected gill filaments.

A higher number of mucous cells present within lesions than in non-affected filaments has been reported before (Roberts *et al.*, 2003). This supports the pathological findings in this study where there was no observed increase in mucous cell numbers for the non-infected ballan wrasse, despite the excessive production of mucus. The mucous cell counts for challenge one mirrored the same trend of mucosal activity (**figure 22**). However, statistical analysis showed no significant interaction between cd or WPC. The same trend repeated itself in challenge two, but with one interesting change, being that the control group had a higher mean of mucous cells than the 1000 cells/L group (**figure 23**). This variance between cd was found to be statistically significant and a reduction in mucous cells numbers was observed in the 1000 cells/L group from challenge one (**figure 23**). Unchanged mucous cell populations were observed by Adams

et al., (2004a) suggesting that infection with AGD not necessarily causes an increase in mucous cell numbers. However, Roberts *et al*., (2008) found that an excess mucus production leads to an immediate decrease in mucous cell numbers, followed by an increased excretion and possibly alteration in mucus composition, which may have affected the amount of visible mucous cells present histologically causing the statistically significant interaction between cd in challenge two.

Five out of six ballan wrasse with pathology consistent with AGD were found 3 WPC (**table 1**). This coincides with the observed alteration of mucus viscosity and production. Thinning of mucus has been linked to a "self-cleaning" action as a response to the presence of amoeba in salmonids (Roberts *et al*., 2003, 2005; Butler *et al*., 2004; Powell *et al.*, 2008). Therefore, it is likely that the increased production of mucus is related to an unknown number of trophozoite attaching to the gill epithelium causing branchial irritation and initiating a response (Butler *et al* ., 2004).

4. 4 Histological conformation of *P. perurans*

The pathological findings of this study showed presence of amoebae for both seemingly unaffected ballan wrasse and the Atlantic salmon with more severe signs of amoebic infection. These findings suggest that Atlantic salmon had a stronger response to the presence of *P. perurans* (**figure 11**) than what was observed in the majority of the ballan wrasse (**figure 12**). This variation in expressed pathology is likely related to variance in susceptibility towards AGD as shown in Dahle *et al.*, (2020). The Atlantic salmon generally showed more severe pathology with proliferation of the gill epithelium spanning over several ILUs and effectively reducing the gill surface area (**figure 9, 15, 17**) which has been reported in several previous studies with AGD (Adams *et al*., 2004; Lovy *et al.*, 2007; Powell *et al.*, 2008; Taylor *et al.*, 2009; English *et al.*, 2019). Amoebae were also located in greater numbers along the edge of lesions, in interlamellar vesicles or adhered to the lesion surface (**figure 9, 15**) as described by (Adams *et al*., 2004b; Powell *et al.*, 2008). Adams *et al*., (2004a) also found evidence suggesting that existing proliferative epithelial tissue has an inhibitory effect on trophozoite attachment. Supported by the fact that lesion distribution and amoeba settlement favours the low water flow areas of the dorsal gill region (Adams and Nowak, 2001), is consistent with observations in this study. Whether the direct contact between amoeba and the gill epithelium as seen in **figure 17** or substances secreted by the amoeba initiates the hosts response is not yet known (Nowak *et al.*, 2014). However, *P. perurans* have been found to not secrete cell damaging enzymes (Nowak *et al.*, 2014). Chloride cells were also observed being sloughed off from the gill

filaments in (**figure 10**) indicating epithelial desquamation linked to AGD (Adams *et al*., 2004b).

For the positive infected ballan wrasse, lesions tended to be more focal spanning over fewer ILUs with less impact on surrounding gill filaments (**figure 12**) which is similar to what (Karlsbakk *et al.*, 2013; Dahle, 2015; Lepperød, 2017; Herman, 2017) observed in infected wrasse. However, some exceptions were found with more severe pathology closer to the response observed in Atlantic salmon (**figure 13, 14**). Morrison *et al*., (2004) described the fact that lesion development is in direct proportion to the infective parasite concentration and progression of the infection. Indicating that the more severe lesions and responses in ballan wrasse were due to a higher infective load of *P. perurans*. This is likely due to variations in inter individual susceptibility. One ballan wrasse also showed a primary attachment of a *P. perurans* at an early interaction stage, with extremely focal morphological changes within the hosts ILU (**figure 16**). This further emphasizes the differences in progression of the disease where Atlantic salmon has a much faster rate of development than other susceptible species such as wrasse and lumpfish (Haugland *et al.*, 2017; Kvinnsland, 2017; Dahle *et al.*, 2020).

Immunostaining with PCNA revealed multiple positive cells (**figure 18**) for both species. In general, the PCNA positive cells were observed near the primary filament or at the base of the secondary filament with an increased presence within lesions. PCNA positive cells are related to the replication and repair machinery of the cell (Paunesku *et al.*, 2001) and indicates cellular turnover associated with cell cycle (Ortego *et al.*, 1995; Powell *et al.*, 2014). This shows that there is an increased cellular activity regarding cellular-repair or cellular apoptosis within lesions (Powell *et al.*, 2014). The Atlantic salmon had more PCNA positive cells within areas of epithelial proliferation than what was observed for lesions in ballan wrasse. This however is likely a reflection of variance in severity of AGD-response between species. There was also a fixation related effect on staining as seen in **figure 19** between formalin and Davidsons effectively masking the PCNA positives, making them harder to spot. For the ballan wrasse positive stained granular cells resembling EGCs in the central venous sinus (**figure 20**) were observed as well. The same PCNA positive cells were also observed in the base of the secondary lamellae (**figure 21, figure 19**), which coincides with the findings of EGCs done in previous studies with Ballan wrasse (Karlsbakk *et al.*, 2013; Kvinnsland, 2017). EGCs have also been found present in the tip of the filaments or in the connective tissue with close proximity to blood vessels (Kvinnsland, 2017). The presence of these cells has also been related to tagging lesions and ulcerative dermal necrosis in Atlantic salmon (Reite and Evensen, 2006). In Kvinnsland,

(2017) filaments with lesions had an substantial increase of EGCs in the hyperplastic tissue in the inter lamellar space. However, for this study no such increase was observed.

4. 5 Limitations and challenges

Detection and diagnosis of amoebic infections are dependent on method and can be difficult, likely underestimating the occurrence of disease (Nowak *et al.*, 2014). The most common way of accessing gill health and evidence of AGD is the use of gross gill score adapted from Taylor *et al.*, (2009)(Downes *et al.*, 2017). However, gross gill score is an indicative measure of gill proliferation and does not directly access numbers of parasitic amoeba (Taylor *et al.*, 2009). Fresh gill smears and light microscopy are also used for diagnostical purposes. However, this is more complicated and requires a trained eye to differentiate cellular structures and amoebae. AGD, particularly at low gill score is often hard to detect, especially in non-salmonid species like ballan wrasse and lumpfish (Karlsbakk *et al.*, 2013; Dahle, 2015; Stagg *et al.*, 2015; Haugland *et al.*, 2017; Lepperød, 2017). This opens for the possibility that seemingly healthy individuals of cleaner fish species may be asymptomatic carriers of amoeba (Oldham *et al*., 2016; Hellebø *et al*., 2017; Steigen *et al.*, 2018; Dahle *et al.*, 2020) and function as a vector in a naïve population. This trait has also been observed in Atlantic salmon with no gross gill score, were histopathology confirmed that the fish were tolerant, but not necessarily resistant to the parasite (Taylor *et al.*, 2009).

The case definition for AGD is conducted by histopathology, observing amoebae and associated pathology (Clark and Nowak, 1999; Rodger, 2014; Downes *et al.*, 2017). While histopathology has the potential to detect both the presence of pathogen and the host response, it requires destructive sampling, potentially limiting its appliance for screening (Adams *et al*., 2004; Downes *et al.*, 2017). The identification of *P. perurans* as the aetiological agent of AGD by Young *et al.*, (2007) paved the way for molecular methods of detection. Many previous studies have shown this to be a valuable and highly sensitive tool observing a high degree of correlation between observed gross gill score and detection by PCR (Young *et al.*, 2008; Fringuelli *et al.*, 2012; Rodger, 2014; Downes *et al.*, 2015, 2017). For this study the detection and observed gill score did not correlate for either species, indicating that the PCR assay might not be as sensitive when used on low gill scores, posing a greater risk for AGD to be unrecognized especially in cleaner fish species like ballan wrasse and lumpfish where detecting disease can be difficult (Haugland *et al.*, 2017; Dahle *et al.*, 2020).

5. Concluding remarks

The observations in this study support the fact that non-salmonid species like ballan wrasse are more resistant to infection with *P. perurans* than Atlantic salmon. This highlights the possibility that ballan wrasse may act as asymptomatic carriers of amoeba, with seemingly "healthy" fish transferring disease to more susceptible species like Atlantic salmon. The frequent use of cleaner fish as a means to supress salmon lice infestation also represents a risk of introducing *P. perurans* to new locations (Hellebø, Stene and Aspehaug, 2017; Steigen *et al.*, 2018; Dahle *et al.*, 2020). Further emphasizing this Dahle *et al.*, (2020) found that isolates derived from ballan wrasse also led to more severe AGD in Atlantic salmon than the salmon isolates did in ballan wrasse. This combined with findings indicating slower progression of AGD in non-salmonids shows that ballan wrasse poses a great threat for amoebae transmission to naïve more susceptible populations if infection goes unrecognized.

6. Future works

Ballan wrasse has been used more frequently to supress salmon lice infestation in Norwegian aquaculture and was quite recently shown to be susceptible to AGD. Evidence suggest that ballan wrasse is less susceptible than Atlantic salmon, with the possibility of transmission between species. Further research is required to fully understand the role and potential ballan wrasse has as an agent for spreading disease. A continuing work on characterizing disease progression, host immune system and inflammatory response is necessary to fully understand interaction with parasitic amoeba. Understanding the interaction between amoebae and ballan wrasse could potentially help minimize risk of AGD-outbreaks in Norwegian aquaculture.

7. Appendix I

7. 1 Dehydration and Paraffin infiltration

Table 2 - Histokinette carousel, dehydration and paraffin infiltration steps.

Bath	**Solution**	**Duration**
1	buffer /4% phosphate buffered formalin	1h
2	50% ethanol	1h
3	70% ethanol	1h

4	80% ethanol	1h
5	96% ethanol	2h
6	ethanol 96%	2h
7	ethanol 100%	2h
8	ethanol 100%	2h
9	xylen, hist	2h
10	xylen, hist	2h
11	Paraffin/Histowax 56-58°C	2h
12	Paraffin/Histowax 56-58°C	2h

Table 3 - Paraffin casting steps

Steps	Work description
1	When the histokinette has completed the cycle, the samples are covered in liquid paraffin/Histowax (56-58°C) in bath 12.
2	The carousel is lifted, and the casting cassette is placed on the heating block of the Histowax embedding machine. Avoid spilling paraffin during the transfer. Close the histokinette.
3	A stainless casting mould is filled with liquid paraffin (Histowax 56-58°C) from the embedding machines dispenser.
4	Tissue is placed in the mould with desired cur surface down.
5	The stainless mould is then placed on a cooling element that needs to be stored in a freezer. With heated tweezer the tissue is carefully pressed towards the bottom of the stainless mould until the tissue attaches to the metal. Work quickly since the tissue will cling to the tweezer as soon as it starts to cool.
6	The marked part of the casting cassette from the infiltration process is placed over the mould with the tissue sample so that the marking is clearly visible. The mould is then filled with liquid paraffin. The mould should be filled all the way up with paraffin to avoid tearing during the sectioning. If it takes too long before the second paraffin layer is added delamination and breakpoints tend to occur, work quickly.
7	The cooling element with the stainless mould is then placed in a freezer (-20°C) for 10 to 20 minutes, then the paraffin block detaches easily from the mould.

7. 2 Solutions used in histological staining

Solutions used in the challenge were either supplied with the kit or made at the lab, following the protocols listed up in this appendix I under 6.3 staining protocols.

7. 2 – 1 HE

Table 4 - Standardised H&E staining protocol for histological sections.

Bath	Solution	Time
1	Distilled water	1 min
2	Shandon instant haematoxylin	3 min
3	Hydrochloric acid 0.1% aqueous	2 sec
4	Running tap water	3 - 5 min
5	Eosin Y-solution 0.5%, aqueous, working solution	3 min
6	Running tap water	30 sec
7	Ethanol 70%	1 min
8	Ethanol 70%	1 min
9	Ethanol 96%	1 min
10	Ethanol 96%	1 min
11	Ethanol 100%	1 min
12	Ethanol 100%	1 min
13	Xylene or Neo-Clear®	5 min
14	Xylene or Neo-Clear®	5 min

7. 2 – 2 HES

Table 5 – Standardised HES staining protocol for histological sections.

Bath	Solution	Time
1	Xylene – hist	10 min
2	Ethanol 100%	5 min
3	Ethanol 100%	5 min
4	Ethanol 96%	5 min
5	Ethanol 80%	5 min
6	Ethanol 50%	5 min
7	Running tap water	5 min
8	Filtrated haematoxylin	2,5 min
9	Running tap water	4 min
10	Erythrosine 1%, aqueous, pH=6,5	1,5 min
11	Running tap water	1 min
12	Ethanol 96%	1 min
13	Ethanol 100%	1 min
14	Alcoholic saffron	20 sec
15	Ethanol 100%	1 min
16	Ethanol 100%	1 min
17	Xylene – hist	5 min
18	Xylene – hist	5 min

7. 2 – 3 AB - PAS

Acid mucins and neutral mucins are clearly separated (**table 7**). Staining all acid mucins first PAS-positive, they will not react with the PAS reaction only PAS-neutral mucins, resulting in a good colour distinction between acid and neutral moieties (Stevens and Bancroft, 1977; Johannes and Klessen, 1984). Not all sections included step 8 and 9 as haematoxylin was used for core staining.

Table 6 – Standardised AB-PAS staining protocol steps for histological sections.

Steps	Method	Solution	Time
1	De-wax sections and bring to water		
2	Dip in	Alcian blue solution	5 min
3	Rinse in water, then	Distilled water	
4	Dip in	1 % aqueous periodic acid	2 min
5	Rinse well in	Distilled water	
6	Dip in	Schiff's reagent	8 min
7	Wash in	Running tap water	5 -10 min
8	Stain nuclei lightly in	Haematoxylin solution	1,5 min
9	Wash in	Running tap water	
10	Rinse in	Absolute alcohol	
11	Clear in xylene and mount	Xylene – hist	

Table 7 - Interpretation of staining results with AB-PAS

Results		Staining colour
Acid	Mucins	Blue
Neutral	Mucins	Magenta
	Nuclei	Pale Blue
Mixtures	With mixtures of the above colouration depends on the dominant entity and will range from blue - purple through to a violet or mauve colour.	

7. 3 Fixatives

7. 3 – 1 Davidsons Sea water fixative

Table 8 – Listing of chemicals used in preparation of Davidsons seawater fixative

Protocol yields 1000ml fixative	
Chemicals	**Volume/weight**
37% formaldehyde	200ml
Concentrated glycerol	100ml
96% ethanol	300ml
Filtrated seawater (from sample location)	300ml
Concentrated acetic acid	100ml

Acetic acid is added in the end, slowly, so that there is no heat exchange. Tissue samples is fixated in Davidsons for a minimum of 4 days (2). Change fixation fluid after two days. Samples that are being stored remains on fixative.

7. 3 – 2 Formalin

4 percent neutral buffered formaldehyde solution. The fixative solution is used with paraffin routine diagnostics and for immunohistochemistry. Fixation solution is not suited for EM and in situ hybridisation.

Table 9 - Neutral buffered formaldehyde solution recipe

Protocol yields 1000ml neutral buffered formaldehyde solution		
Chemicals	**Volume/Weight**	**Manufacturer**
37% formaldehyde (without precipitate)	100ml	Merck 1.04003.1000
Na2HPO4 x 2H2O	8.15g	Merck 1.06580.1000
NaH2PO4 x H2O	4.00g	Merck 1.06346.1000
Tapwater	900ml	

Phosphate salts are dissolved in lukewarm tap water before the formaldehyde solution is added. The acidity should be around pH 7.2. Fixation time for fish tissue is around 8-24 hours.

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